Methods in Molecular Biology™

Volume 92

PCR IN BIOANALYSIS

Edited by

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Humana Press

PCR in Bioanalysis

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Humana Press  **Totowa, New Jersey**

999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

This publication is printed on acid-free paper.
ANSI Z39.48-1984 (American Standards Institute)
Permanence of Paper for Printed Library Materials.

Cover illustration: Figure 3 from Chapter 4, "Prenatal and Presymptomatic Diagnosis of Marfan Syndrome Using Fluorescence PCR and an Automated Sequencer," by Mei Wang and Maurice Godfrey.

Cover design by Jill Nogrady.

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Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

Library of Congress Cataloging in Publication Data

Main entry under title:

Methods in molecular biology™.

PCR in bioanalysis/edited by Stephen J. Meltzer.
p. cm.—(Methods in molecular biology; 92)
Includes bibliographical references and index.
ISBN 0-89603-497-6 (alk. paper)
1. Polymerase chain reaction—Laboratory manuals. 2. Molecular diagnosis—Laboratory manuals. 3. DNA fingerprinting—Laboratory manuals. I. Meltzer, Stephen J. II. Series: Methods in molecular biology (Totowa, NJ); 92.
[DNLM: 1. Polymerase Chain Reaction—methods. 2. Microbiological Techniques. W1 ME9616J v. 92 1998/QH 450.3 P3475 1998]
RB43.8.P64P37 1998
616.07' 58—dc21
DNLM/DLC 97-37496
CIP

Preface

The invention of the polymerase chain reaction (PCR) eventually earned Kary B. Mullis half of the 1993 Nobel Prize for Chemistry ***(1–4)***. However, for several years, issues of quality control and reproducibility interfered with attempts at commercial or clinical application of PCR.

More recently, persistent work and numerous methodological innovations and refinements have resulted in the establishment of PCR as a routine, sensitive, and specific detection method in hospital and agricultural laboratories. This transformation of PCR from an experimental research technique to an established bioassay tool formed the impetus behind *PCR in Bioanalysis*.

PCR has proven particularly valuable in clinical microbiology and the diagnosis of infectious diseases in humans and animals. This large and diverse group of applications is reviewed in Chapter 1 by Gorm Lisby. Specific organisms now detectable by PCR include hepatitis C virus (protocols presented in Chapter 14), *Mycobacterium tuberculosis* (Chapter 18), *Chlamydia* and *Trichomonas* species (Chapter 20), *Toxoplasma gondii* (Chapter 17), *Legionella* species (Chapter 15), enterotoxigenic *Escherichia coli* (Chapters 9 and 10), HIV-1 subspecies (Chapter 8), bovine immunodeficiency-like virus (Chapter 6), rodent parvoviruses (Chapter 2), Ross River virus (Chapter 12), and porcine reproductive and respiratory syndrome virus (Chapter 7).

In addition, some methodologic breakthroughs are now routinely applied to clinical and industrial problems. In the analysis of human tissues, selective ultraviolet radiation fractionation (SURF) has become established as a means of selecting (or excluding) cells for subsequent PCR amplification (protocol presented in Chapter 3). In the evaluation of human tumors, new techniques for detection of amplified or deleted DNA sequences (Chapter 24) and circulating tumor cells (Chapter 19) have emerged. It is now routine to see PCR applied to the quantitative measurement of mRNA expression (Chapter 16), the examination of transgenic plants (Chapter 13) or mice (Chapter 22), the identification and cloning of differentially expressed genes (Chapter 21), and the measurement of cytokines or inducible nitric oxide synthase (Chapter 5). In vitro transcription/translation of PCR products is a successful screening technique to detect chain-terminating mutations (Chapter 11); this and other approaches are now commonly utilized to detect hereditary disease syndromes, such as familial adenomatous polyposis (Chapter 23) and Marfan's syndrome (Chapter 4).

In summary, these innovations and adaptations of existing technologies have launched a virtual bioanalytic revolution. Many more applications will probably follow in short order. We are now firmly entrenched in the PCR era, but we are still standing near that era's beginning.

References

1. Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230,** 1350–1354.
2. Saiki, R. K., Bugawan, T. L., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1986) Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes. *Nature* **324,** 163–166.
3. Mullis, K. B. and Faloona, F. A. (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.* **155,** 335–350.
4. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239,** 487–491.

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PCR in Bioanalysis

1

Application of Nucleic Acid Amplification in Clinical Microbiology

Gorm Lisby

1. Introduction

Since the discovery of the doublehelix structure of DNA ***(1)***, no single event has had the same impact on the field of molecular biology as the rediscovery by Kary Mullis in the early 1980s of the polymerase chain reaction (PCR) ***(2–4)***, which was first published in principle by Keld Kleppe in 1971 ***(5)***. This elegant technology with its apparent simple theory has revolutionized almost every aspect of classical molecular biology, and is at the present moment beginning to make a major impact upon many medical—especially diagnostic-specialities. The field of clinical microbiology has been among the first to embrace the polymerase chain reaction technology, and the expectations of the future impact of this technology are high. First and foremost, the diagnostic possibilities of this technology are stunning, but in this era of emerging implementation, it is crucial to focus not only on the possibilities, but also on the pitfalls of the technology. Failure to do so will increase the cost of implementation manifold, and will risk to disrepute the technology in the eyes of the clinicians.

2. PCR–Theory and Problems

2.1. "Classic" PCR

2.1.1. The Principle of Exponential Amplification

The hallmark of the polymerase chain reaction is an exponential amplification of a target DNA sequence. Each round of amplification is achieved by annealing specific oligonucleotides to each of the two complementary DNA

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

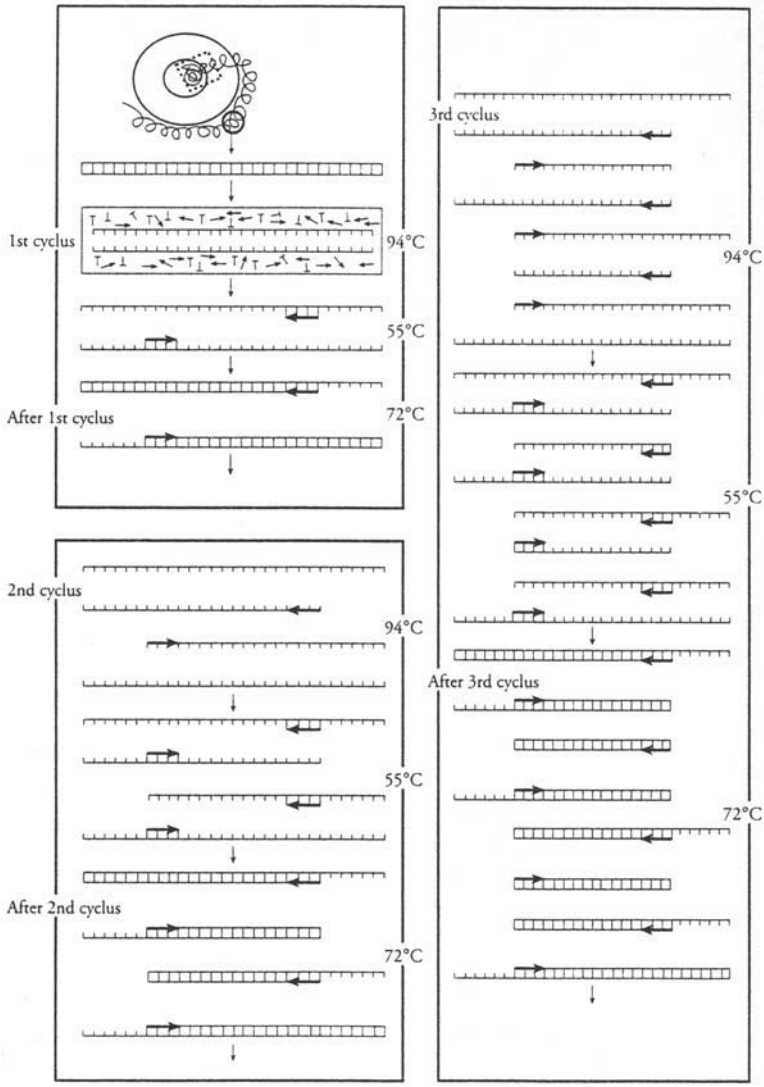


Fig. 1. The first three cycles of a standard PCR. The tentative annealing temperature of 55°C needs to be optimized for each PCR set-up.

strands after denaturation. Following annealing of the two oligonucleotides (primers), a thermostable DNA polymerase ***(6)*** will produce doublestranded DNA, thus in theory doubling the amount of specific DNA in each round (**Fig. 1**). After the third round of amplification, a specific product consisting of the target DNA fragment between the two primer annealing sites (and including

the two sites) will start to accumulate. When the usual 30–40 rounds of amplification are completed, up to several hundred million fold amplification of the specific target sequence can be achieved. The amplified products can be detected by numerous methods that vary in sensitivity, accuracy, and feasibility for routine application: From the classical agarose gel electrophoresis, Southern blot and Sanger dideoxy sequencing to probe capture and visualization in microtiterplates and direct realtime detection of the product in the PCR tube by fluorescens ***(7)***.

2.1.2. Primer Selection and Primer Annealing

Several aspects must be considered when a primerset for PCR is designed ***(8)***. Computer software programs have been constructed to deal with this problem ***(9–11)***, and these programs are based on the same considerations, as one has to take during a "manual" primer design:

The primers are typically between 15 and 30 bases long and do not have to be exactly the same size. However, it is crucial that the melting temperatures of the two primer/template duplexes are identical within 1–2°C. Since a billion-fold surplus of primers may exist in the beginning of a PCR when compared to the target sequence to be amplified, the optimal primer annealing temperature of a primer may be higher than the calculated melting temperature of the primer/template duplex (where 50% of the DNA molecules are doublestranded and 50% are singlestranded). Several formulas to calculate the annealing temperature exist ***(12–14)***, but eventually one has to establish the actual optimal annealing temperature by testing.

The location of the target sequence and thereby the size of the amplified product is not crucial for the sensitivity or the specificity of the analysis, and typically a fragment of 150–800 bases is amplified. Amplification of products sizing up to 42,000 basepairs has been reported ***(15)***. However, when fragments above 1000–2000 basepairs are amplified, problems with template reannealing can be encountered ***(15–17)***. The annealing step in a "long-range" PCR is thus a balance between keeping the templates denatured and facilitating primer annealing.

The composition of the two primer sequences must ensure specific annealing to the target sequence alone. The probability of this specificity can be made through a search in the computer databases (GeneBank or EMBL), but eventually this also has to be established empirically (absence of signals from DNA from other microorganisms than the target organism). It is of utmost importance, that the sequences at the 3' end of the two primers are not homologous, otherwise the two primers will self anneal with primer–dimer products and a possible false negative analysis as result. At the 5' end of a specific primer, a "tail" consisting of a recognition sequence for a restriction enzyme, a captur-

ing sequence or a radioactive or nonradioactive label can be added, normally without influencing the specificity of the primer annealing ***(18)***.

2.1.3. Choice of Enzyme

In recent years, almost every vendor of enzymes and molecular biology products offers a thermostable DNA polymerase. No independent analysis presents a complete overview of all available enzymes, so one has to consider the specific needs in a given analysis: affinity purified vs genetic engineered enzyme, proofreading activity versus no such activity and price-per-unit, which can be difficult to determine, since the actual activity per unit may vary between different enzymes. The final choice can be determined by a price/performance study, but one should also consider the fact that only enzymes with a license to perform PCR can be used legally in a laboratory performing PCR analysis.

2.1.4. Optimization of the Variables

The components of a PCR reaction need to be optimized each time a new PCR analysis is designed ***(19)***. Once the optimal annealing temperature is established, different concentrations of primers, enzyme, and $MgCl_2$ are combined, and the combination ensuring optimal sensitivity and specificity is chosen for future analysis. Whenever a variable in the analysis is changed, e.g., the DNA to be analyzed is extracted by another method, a new optimization may be needed.

2.2. Hot Start

When DNA is extracted from a sample, unavoidably some DNA will be in single-stranded form. If the components of the PCR analysis are mixed at room temperature, the primers may anneal unspecifically to the single-stranded DNA. Since the *Taq* polymerase possesses some activity at room temperature, unspecific DNA can be synthesized even before the sample is positioned in the thermal cycler. One way to avoid this is to withhold an essential component from the reaction (e.g., *Taq* polymerase or $MgCl_2$) until the temperature is at or above the optimal primer annealing temperature–the so called hot-start PCR ***(20)***. This can also be achieved by inhibition of the enzymatic activity by a monoclonal antibody that denatures at temperatures above the unspecific primer annealing level ***(21)*** or by using an inactive enzyme, one that is activated by incubation above 90°C for several minutes ***(22)***. Another method is to mix the PCR components at 0°C. At this temperature, DNA will not renature and the *Taq* polymerase has no activity. When the sample is placed directly in a preheated (94–96°C) thermal cycler, unspecific amplification is avoided—the so-called cold start PCR. If the carry-over prevention system (**Subheading 2.4.2.**) is used, a chemical hot-start is achieved, since any unspecific products

synthesized before the UNG is activated (just prior to initiation of the PCR profile) will be degraded by the UNG ***(23–25)***.

2.3. Quantitative Amplification

In the clinical setting, not only information regarding the presence or absence of a microorganism, but also information regarding the level of infection can be of great value. Since the PCR technology and the other nucleic acid amplification technologies (except bDNA signal amplification) comprises an exponential amplification followed by a linear phase, several built-in obstacles must be overcome in order to gain information about the initial target level. First, the final linear phase must be avoided by limiting the number of amplification cycles. Second, a known amount of an internal standard amplifiable by the same primerset as the target—but different from the target in sequence length and composition—must be included ***(26–28)***. However, since the amplification efficiency varies not only from cycle to cycle, but also between different targets ***(29)***, a semiquantitative rather than an absolute quantitative amplification seems to be the limit of the PCR technology (and LCR/3SR, *see* **Subheadings 5.1.** and **5.2.**) ***(30)***. Calculations of the tolerance limits of a quantitative HIV assay showed that an increase in HIV DNA copies of 60% or less, or a decrease in HIV DNA copies of 38% or less, could be explained by random and not by an actual increase or decrease in the number of HIV DNA copies ***(26)***. If an absolute quantitation is to be achieved, the bDNA signal amplification assay (**Subheading 5.3.**) can be implemented at the cost of a substantially lower sensitivity.

2.4. Sources of Error

2.4.1. False Negative Results

If the extraction procedure applied does not remove inhibitory factors present in the clinical material, even a high copy number of the target gene will not produce a positive signal. In theory, the PCR reaction can ensure a positive signal from just one copy of the target gene hidden in an infinite amount of unspecific DNA. In practical terms, however, 3–10 copies of the specific target gene sequence are needed to reproducibly give a positive signal, and more than 0.5–1 μg unspecific DNA will inhibit the analysis. If the primers are not specific, the primer annealing temperature is not optimized, or the concentration of the components of the reaction is not optimized, a false negative result can occur because of inefficient or unspecific amplification. Products only consisting of primer sequences can arise if the two primers have complementary sequences, but can also be seen if the primer and/or enzyme concentration is too high—even if the primers are not complementary. These primer–dimer artifacts will dramatically reduce the efficiency of the specific amplification and will likely result in a false negative result.

2.4.2. False Positive Results

If the primers are homologous to other sequences than the target gene or if products from previous similar PCR analysis are contaminating the reaction, a false positive signal will be the result. Primers crossreacting with other sequences can be a problem when conserved sequences (e.g., the bacterial ribosomal RNA gene) are amplified. The problem can be avoided by a homology search in GeneBank or EMBL combined with a screening test using DNA from a number of related as well as, unrelated microorganisms. Contamination has in the past been considered the major problem of the PCR technology ***(31,32)***, but this problem can be minimized by rigorous personnel training, designing the PCR laboratory according to the specific needs of this technology (*see* **Subheading 4.1.**) and application of the carryover prevention system already included in commercial PCR kits. This system substitutes uracil for thymine in the PCR, and if the following PCR analyses are initiated with an incubation with a uracil-degrading enzyme such as uracil-*N*-glycosylase, contaminating–but not wild-type–DNA will be degraded ***(23–25)***. Implementation of this technology in the PCR analysis has reduced the problem of contamination in most routine PCR laboratories.

3. Detection of Microorganisms

3.1. Relevant Microorganisms

At the present time, PCR cannot be considered as a substitution but rather a supplement for the classic routine bacteriology. The PCR is clearly inferior in terms of sensitivity to classic methods such as blood culture when fast-growing bacteria such as staphylococci are present. Moreover, although antibiotic resistance can be identified by PCR ***(33–38)***, the sequence still has to be known, whereas the classical disk methods will reveal the susceptibility and resistance no matter what genetic sequence (chromosomal or plasmid) the underlying mechanism is based upon. Even though PCR has been applied to detect a great number of bacteria (**Table 1**, **refs. *39–132***), only the detection of slowly or poorly growing bacteria (e.g., *Legionella* spp., *Mycobacterium* spp., or *Borrelia* spp.) are relevant in the clinical setting. In contrast, all pathogenic viruses and especially all pathogenic fungi would be candidates to detection by PCR or related technologies, because of the problems with speed and/or sensitivity of the current diagnostic methods.

3.2. Identification of Microorganisms

3.2.1. Identification by Ribosomal RNA PCR

The classical detection of microorganisms by PCR is based on the amplification of a sequence specific for the microorganism in question. If a broad

Table 1
Examples of Microorganisms Detected by PCR (refs. *39–132*)

Mycobacterium tuberculosis	Rhino virus
Mycobacterium paratuberculosis	Coxsackie virus
Mycobacterium leprae	Polio virus 1-3
Mycobacterium species	Echovirus
Legionella pneumophila	Enterovirus 68/70
Legionella species	Adeno virus type 40/41
Borrelia burgdorferii	Rota virus
Listeria monocytogenes	Rabies virus
Listeria species	Parvo virus B19
Haemophilus influenzae	Dengue virus
Bordetella pertussis	St. Louis encephalitis virus
Neisseria meningitidis	Japanese encephalitis virus
Treponema pallidum	Yellow fever virus
Helicobacter pylori	Lassa virus
Vibrio vulnificus	Hanta virus
Aeromonas hydrophilia	JC/BK virus
Yersinia pestis	
Yersinia pseudotuberculosis	*Rickettsia rickettsii*
Clostridium difficile	*Rickettsia typhi*
Escherichia coli	*Rickettsia prowazekii*
Shigella flexneri	*Rickettsia tsutsugamushi*
Shigella dysenteriae	*Rickettsia conorii*
Shigella boydii	*Rickettsia canada*
Shigeila sonnei	*Toxoplasma gondii*
Mycoplasma pneumoniae	*Taenia saginata*
Mycoplasma genitalium	*Schistosoma mansoni*
Mycoplasma fermentas	*Echinococcus multilocularis*
Chlamydia trachomatis	*Pneumocystis carinii*
Chlamydia psittaci	*Plasmodium falciparum*
Chlamydia pneumoniae	*Plasmodium vivax*
Whipple's disease bacillus (Trophyryma whipelii)	*Leishmania*
	Trypanosoma cruzi
HIV 1/2	*Trypanosoma brucei*
HTLV I/II	*Trypanosoma congolense*
Endogenous retrovirus	*Entamoeba histolytica*
Cytomegalovirus	*Naegleria fowleri*
Herpes simplex 1/2	*Giardia lamblia*
HHV 6/7/8	*Babesia microti*
Varicella-Zoster virus	
Epstein-Barr virus	*Candida albicans*
Hepatitis virus A/B/C/D/E/F/G/H	*Candidae species*
Human papilloma virus	*Cryptococcus species*
Rubella virus	*Trichosporon beigelii*
Morbilli virus	
Parotitis virus	
Influenza virus A	

range of bacterial pathogens is to be detected in a clinical sample, conserved genetic sequences must be sought. The bacterial 16S ribosomal gene contains variable as well as conserved regions ***(133)***, and is well suited for this strategy. By 16S RNA PCR, it is not only possible to detect all known bacteria (at kingdom level, ***[134]***), identification can also be performed at genus or species level (e.g., *mycobacterium* spp., *Mycobacterium tuberculosis* ***[135,136]***). Moreover, since some conserved sequences are present in all bacteria, it is now possible to detect unculturable bacteria. By application of this approach, the cause of Whipple's disease ***(137)*** as well as bacillary angiomatosis ***(138)*** has been identified. It is likely that more diseases of unknown etiology in the future will be correlated to the presence of unculturable bacteria by the application of 16S RNA PCR. Furthermore, since the typing and identification of bacteria at the present time are based upon phenotypical characterization (shape, staining, and biochemical behavior), typing at the genetypic level (e.g., by 16S RNA PCR) would most likely result in altered perception of the relation between at least some bacteria.

3.2.2. Identification by Random Amplification of Polymorphic DNA (RAPD)

Classical detection of microorganisms by PCR as well as amplification of bacterial 16S RNA sequences relies upon specific primer annealing. However, if one or two oligonucleotides of arbitrarily chosen sequence with no known homology to the target genes were used as primers during unspecific primer annealing conditions in a PCR assay, arrays of DNA fragments would be the result ***(139–141)***. Under carefully titrated conditions of the PCR, empirical identification of primers generating an informative number of DNA fragments can be made. By analyzing the pattern of DNA fragments, bacterial isolates can be differentiated, not only on genus level, but also on species and subspecies level ***(142–147)***. This method could prove to be an efficient tool for monitoring the epidemiology of infections such as hospital infections ***(148)***.

3.3. Sample Preparation

Once the variables of a PCR analysis have been optimized, the actual clinical performance is determined by the efficiency of the extraction method applied to the clinical material as well as the handling of the clinical material. Different clinical materials contain different levels of factors capable of inhibiting the PCR—some acting by direct inhibition of the enzyme, some by binding to other components of the PCR (e.g., the $MgCl_2$).

The optimal extraction method for any clinical material is a method that extracts and concentrates even a single target molecule into a volume that can be analyzed in a single PCR. Because of the loss of material during the extrac-

tion and the large amount of unspecific DNA if the specific target sequence is very scarce, the detection limit of any routine PCR will be more than 10 copies of target DNA per microgram total DNA if no specific concentration (e.g., capture by a specific probe) is performed. Thus, without concentration, more than one copy of the target gene must be present per 150,000 human cells in order to reproducibly give a positive signal. Various tissues are known to contain inhibitory substances, and various chemicals (such as heparin, heme, acidic polysaccharides, EDTA, SDS, and guanidinium HCl) are also known to inhibit the PCR ***(149–151)***.

In routine diagnostics, however, the optimal extraction procedure depends upon a cost/benefit analysis and is not necessarily the procedure with the greatest yield. Basically, one can choose between removing all other components from the sample rather than the nucleic acid using a classical lysis/extraction method ***(152)*** or to remove the target from the sample by a capture method ***(153,154)***. The classical lysis/extraction method (proteinase K digestion–phenol/chloroform extraction–ethanol precipitation) has been modified numerous times, and application in routine analysis requires this method to be simplified and to avoid the use of phenol/chloroform. The most commonly analyzed tissues in clinical microbiology can be ranked according to increasing problems with inhibition of the PCR: endocervical swaps–plasma/serum–cerebrospinal fluid–urine–whole blood–sputum–feces ***(155–162)***.

The simple and easy sample preparation method (and also the final detection method) is often the most obvious difference (apart from the cost) between a commercial kit and a corresponding "in-house" PCR analysis.

4. Routine Applications and Quality Control

4.1. Laboratory Design and Personnel Training

The powerful exponential amplification achieved by the nucleic acid amplification technology also results in a potential risk of false positive signals because of contamination. Since up to 10^{12} copies of a specific target sequence can be generated in a single PCR, even minimal amounts of aerosol can contain thousands of DNA copies. The essential factor in avoiding cross contamination is to physically separate the pre-PCR and the post-PCR work areas—ideally in two separate buildings. In a routine clinical laboratory this is not practical, but the "golden standard" (level 3) for a PCR laboratory performing in-house PCR (or in-house variants of the LCR and/or the 3SR technology–but not the bDNA technology, *see* **Subheadings 5.1.–5.3.**) should be considered: four separate rooms (**Fig. 2**) with unidirectional workflow (from laboratory 1 through 4) and unidirectional airflow if individual airflow cannot be installed ***(163)***. Each room should be separated from any of the other rooms by at least two doors, and, if possible, a positive air pressure in laboratories 1,

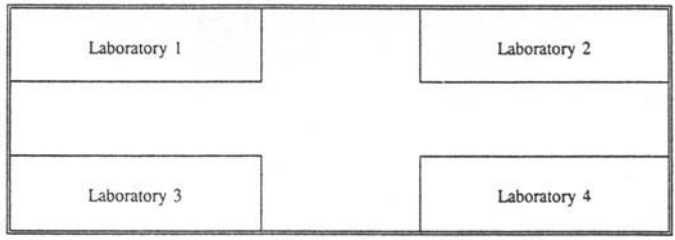


Fig. 2. Design of a PCR laboratory (level 3).

2, and 3 should be obtained. Laboratories 1, 2, and 3 should have a laminar airflow bench. In laboratory 1, no DNA is permitted. This laboratory is used for production of mastermixes and setup of the individual PCR analysis except addition of sample DNA. Laboratory 2 is used for extraction of clinical samples and in laboratory 3, the nucleic acids extracted from the clinical samples are added to the premade PCR mixes. In laboratory 4, the thermal cyclers are placed, and postamplification procedures such as detection can be performed in this laboratory.

Level 2: If the carry-over prevention system is included in the in-house analysis, laboratory 3 can be omitted. Extraction of the clinical material and addition of the extracted material to the premade PCR mixes are performed in laboratory 2—preferably in two laminar airflow benches. This laboratory design is also recommended if the LCR and/or 3SR technology including a carry-over prevention procedure are performed.

Level 1: If only commercial PCR, LCR, or 3SR kits are used, patient sample extraction and analysis setup (pre-amplification procedures) can be performed in two laminar airflow benches in laboratory 1. The amplification and postamplification procedures are performed in laboratory 4.

Since the bDNA technology (*see* **Subheading 5.3.**) does not involve amplification of the target sequence, there are no specific recommendations for the laboratory design.

Besides the recommendations regarding laboratory design, some general guidelines should also be observed: the use of dedicated pipeting devices in each laboratory, the use of gloves during all laboratory procedures, the use of filtertips in the preamplification areas and the use of containers with Clorox or a related product for minimizing potential aerosol problems during disposal of pipet tips containing DNA. Furthermore, the use of aliqouted reagents and the use of a low-copy-number positive control (no more than 100 copies) are recommended.

Because of the potential problems with the nucleic acid amplification technologies, especially if an in-house analysis is performed, it is essential to ensure that there is a high level of motivation, education, and information with the

personnel performing these analyses. This concern should override the principle of rotation applied in some routine laboratories, at least until better standardized and more robust analyses are implemented.

4.2. Quality Control

All routine analysis—no matter what technology applied—must be submitted to quality control. Because of the nature of nucleic amplification technology, problems are likely to arise, and the requirement for quality control is especially demanding during these procedures. The quality control program should consist of internal quality control as well as participation in an external quality control program.

The internal quality control program should be designed to test the individual procedures in the analysis ***(164)*** and should consist of the use of weak positive controls (to test the sensitivity), the use of negative controls without DNA (to test for contamination) as well as negative controls with irrelevant DNA (to test the specificity). The absence of inhibitors in negative patient samples can be verified by amplification of a housekeeping gene such as β-globin, and temperature variation between individual wells in the thermal cycler should be verified by a temperature probe with regular intervals.

Participation in an external quality control program is an overall evaluation of the performance of the laboratory and should be mandatory. Published external quality control studies have confirmed the suspected variation between individual laboratories. In the first multicenter study, five laboratories reported 1.8% false positive results using in-house methods when analyzing 200 samples for the presence of HIV-1 ***(139)***. In a later study, 31 laboratories were asked to analyze a blinded serum panel for the presence of hepatitis C virus using their own in-house analysis. Only nine laboratories identified all clinical samples correctly, and only five of the nine could correctly identify two dilution series ***(140)***. Later studies have confirmed these problems, and even when commercially available kits are evaluated, interlaboratory variation can be observed ***(141,142)***.

4.3. Commercial vs In-House PCR Analysis

PCR technology started out as a "home-brewed" technology in numerous laboratories throughout the world. Because of the fact that PCR technology initially was used for different research applications in different laboratories, and because of the initial overriding problem of contamination, the problem of standardizing the technology was not brought into focus until recently. The use of commercially available kits not only results in easier and faster pre- and postamplification procedures when compared to most in-house analysis, but also in higher agreement between individual laboratories when performing the same analysis. This agreement is, however, not 100% certain, and is probably

still at the lower end of what is acceptable for a routine diagnostic procedure. Financially, the lower reagent cost of in-house analyses are somewhat balanced by the mandatory license fee payable when performing clinical PCR.

5. Alternative Nucleic Acid Amplification Methods

Probably because of the vast commercial interest in diagnostic procedures, and as a result of the comprehensive patent protection of the PCR technology, several alternative nucleic acid amplification methods have been constructed. Three of the most promising technologies are described here, the first using a variation of the PCR technology, the second using RNA as a template and a different enzymatic approach, and the third using the template for signal amplification.

5.1. Ligase Chain Reaction (LCR)

This technology has many similarities with the PCR technology ***(169)***. LCR amplifies very short fragments (corresponding to the size of two primers) by annealing two primers to each of two DNA strands (**Fig. 3,** adapted from **ref. *169***). The primers anneal two and two directly opposite, and if a DNA ligase is present, the four annealed primers will be ligated two and two. Following denaturation, the ligated primers will act as a template for the annealing of the two opposite primers once the temperature reaches the level for specific annealing. If a thermostable DNA ligase is used, the denaturation-annealing-ligation process can be automated just like the PCR ***(169,170)***. The potential problem of this technology in addition to the risk of contamination is clearly the lack of conformation, since only primer sequences are amplified. To minimize this problem, the commercial variant of LCR combines ligase and DNA polymerase activity in a "gap-filling" reaction ***(171)***. If a gap of one or two different nucleotides exists between the two perfectly annealed primers, only the two relevant nucleotides are included in the reaction mix, and only primers annealing with the correct gap will be filled, and thus ligated. The present commercial "gap" variant of LCR has been applied to the detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* with excellent results regarding sensitivity as well as specificity ***(172–174)***.

5.2. Self-Sustained Sequence Replication (3SR)

This RNA amplification technology ***(175–177)*** is also described as nucleic acid-based amplification (NASBA ***[178]***) and transcription amplification system (TAS ***[179]***). The technology combines three different enzyme activities at the same temperature (42–50°C), and thus renders a temperature cycling device superfluous. First, the RNA template is transcribed to cDNA by reverse transcriptase initiated by a downstream primer with the recognition sequence for the T7 RNA polymerase at its 5' end (**Fig. 4** adapted from **ref. *177***). The tem-

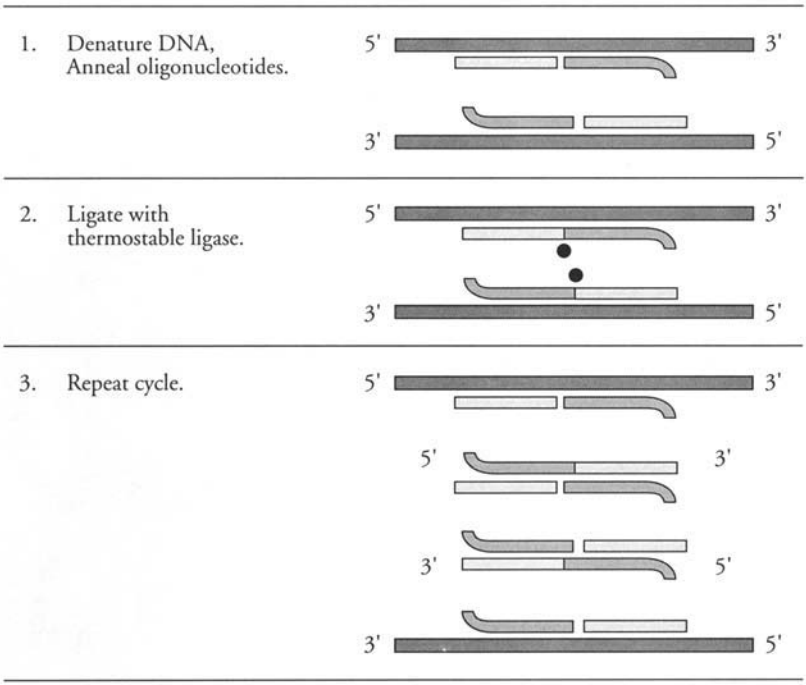


Fig. 3. The principle of the ligase chain reaction (LCR). Note the accumulation of primer–primer products without a target-specific sequence interspersed.

plate RNA is destroyed by RNase H activity as the cDNA is synthesized. The upstream primer will then anneal to the cDNA, and doublestranded DNA will be synthesized. The T7 RNA polymerase will then produce multiple antisense RNA transcripts, and the downstream primer will initiate the synthesis of cDNA from these transcripts. Following synthesis of double-stranded DNA, a new round of amplification can be initiated. This technology can amplify a RNA signal more than 10^8-fold in just 30 min ***(180)***. At the present time, there are still potential specificity problems, as not all enzymes exist in heatstable variants and the process must be kept at 42–50°C. 3SR is an RNA amplification technology, and one of the major advantages in its use in clinical microbiology is the capacity to discriminate between dead and viable microorganisms. So far, the use of 3SR has been concentrated around the detection of human immunodeficiency virus (HIV) type 1 and *Mycobacterium tuberculosis* ***(181–184)***.

5.3. Branched DNA Signal Amplification (bDNA)

A different approach than amplifying the target itself would be to amplify a signal generated by the target. This is achieved by the branched DNA signal

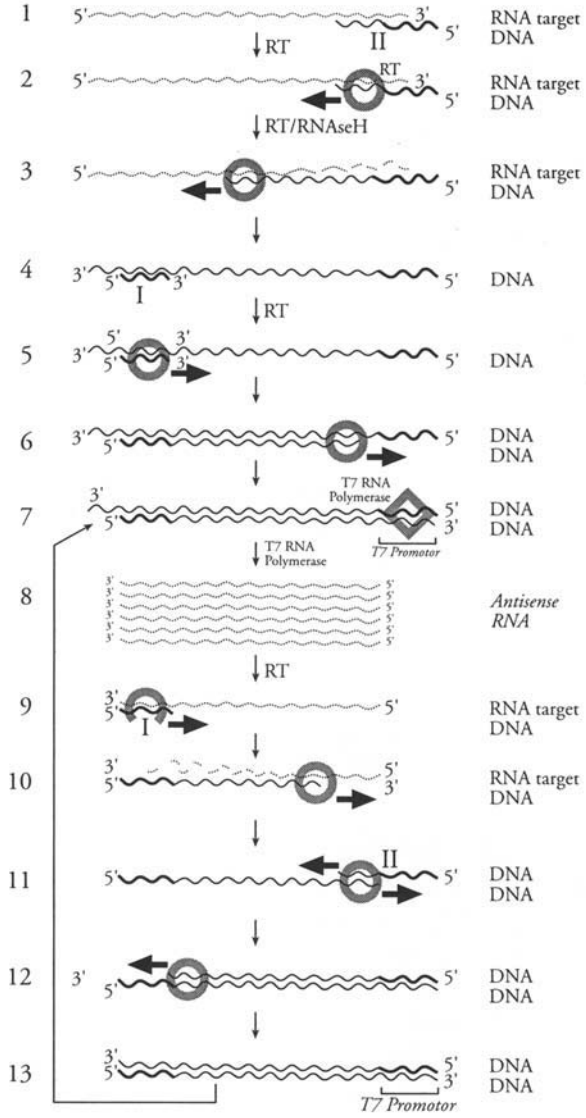


Fig. 4. The principle of the 3SR technology. Note the isothermal, multiple enzymatic activity amplifying an RNA target in a single buffer system.

amplification (bDNA) assay ***(185,186)***. When the target nucleic acid is immobilized on a solid surface (e.g., a microtiterplate), specific probes will connect "amplifier" molecules to the target nucleic acid. These amplifier molecules will hybridize to enzyme-labeled probes, and a chemiluminiscense substrate will emit light (**Fig. 5**). As the bDNA assay uses signal- and not target-

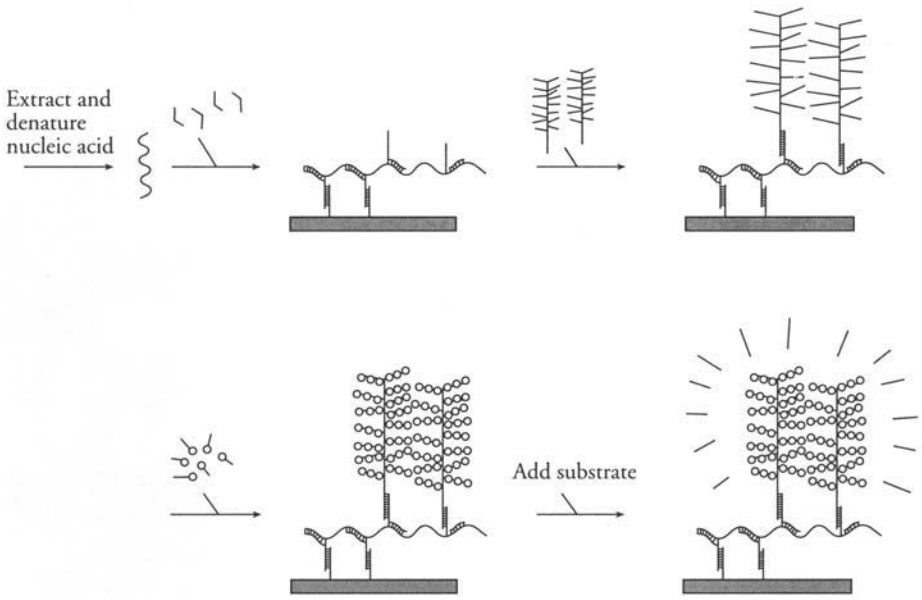


Fig. 5. The principle of the branched DNA signal amplification assay. Note the signal–and not target–amplification, making accurate quantitation possible.

amplification, the risk of contamination is minimal. Furthermore, because no exponential amplification of the target sequence takes place, a genuine quantification can be achieved compared to the semiquantification achievable by the PCR, LCR, or 3SR technologies. The sensitivity, however, is clearly lower compared with the other technologies, and at the present time approx 500 copies per milliliter of the target sequence can be detected. As with the other technologies, it is possible (per definition) to obtain a 100% specific analysis–depending upon the design of the capturing probes. Presently, the bDNA technology has been applied to the detection and quantification of HIV-1-RNA ***(187,188)*** hepatitis C virus (HCV)-RNA ***(189–192)***, hepatitis B virus (HBV)-DNA ***(193,194)***, and cytomegalovirus (CMV)-DNA ***(195)***.

5.4. Choice of Technology

When the optimal technologies for nucleic acid amplification in a specific laboratory–routine, research, or a combination–are chosen, several variables (often different between different laboratories) should be taken into consideration. The available technologies can be weighted and scored according to the specific needs of the individual laboratory, and an example of weighting and scoring in a routine laboratory is shown in **Table 2**. The example given here is not valid for all routine laboratories performing nucleic acid amplification for

Table 2
Example of a Scoring Sheet for a Routine Laboratory

Factor (weight)	PCR	LCR	3SR	bDNA
High sensitivity (1)	+	+	+	+/–
High specificity (1)	+	+	+	+
Genuine quantification (1/2)	–	–	–	+
No contamination risk (1)	+/–	–	+/–	+
Live microorganisms (1/4)	+	–	+	–
Easy to perform (1)	+	+	+	+
Commercial availability today (1)	+	+/–	+/–	+/–
Total score	4.75	3.5	4.25	4.5

diagnostic purposes, as individual design and needs will have to be taken into consideration. However, the general scoring and weighting principle are applicable to any laboratory.

6. Discussion

Since first described, the PCR technology has been applied in many fields, especially in detection of various microorganisms. Three problems have until now prevented the expected major breakthrough in routine clinical microbiology: contamination, standardization, and cost. Having moved toward minimizing the "child disease" problem of contamination, the problem of standardizing the nucleic amplification technology between different laboratories is the Achilles heal of the technology at the present moment. This problem is clearly unsatisfactory in a clinical setting, and in the very near future, license to perform clinical PCR and other nucleic amplification analysis—at least in the United States and the European Union—will probably be based on satisfactory performance in an impartial external quality control program.

The first commercially available PCR kits were prized relatively high. However, there is no reason to believe that the PCR technology will be the sole actor on the routine diagnostic scene. Several other technologies offer similar or comparable qualities, and the choice or combination of technology in a given routine laboratory depends upon an individual assessment in each laboratory. With increasing demand and competition, the cost of the analysis will inevitably be reduced in the near future.

In conclusion, there is no doubt that the nucleic acid amplification technologies will improve the routine detection of viruses, fungi, and slow-growing bacteria. As our knowledge of antibiotic resistance genes and mechanisms expands, these technologies will be able to supplement the classical phenotypical resistance detection methods. One way to minimize the present unsatisfac-

tory interlaboratory variation, even when applying commercially available kits, could be to expand the automated procedures. If sample preparation and nucleic acid extraction are included in the automated process, less interlaboratory variation would most likely be the result, thereby facilitating the acceptance of these technologies in the clinical community.

References

1. Watson, J. D. and Crick, F. H. C (1953) Molecular structure of nucleic acids: a structure for deoxyribose nucleic acid. *Nature* **171,** 737,738.
2. Mullis, K. B. (1990) The unusual origin of the polymerase chain reaction. *Sci. Am.* **262,** 36–43.
3. Saiki, R. K., Scharf, S., Faloona, F., and Mullis, K. B. (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230,** 1350–1354.
4. Saiki, R. K., Gelfand, D. H., Stoffel, S., and Scharf, S. J. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239,** 487–491.
5. Kleppe, K., Uhtsuka, E., Kleppe, R., Molineux, I., and Khorana, H. G. (1971) Study on polynucleotides: repair replication of short syntetic DNAs as catalyzed by DNA polymerases. *J. Mol. Biol.* **56,** 341–361.
6. Chien, A., Edgar, D. B., and Trela, J. M. (1976) Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus. J. Bacteriol.* **127,** 1550–1557.
7. Higuchi, R., Fockler, C., Dollinger, G., and Watson, R. (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology* **11,** 1026–1030.
8. Rychlik, W. (1995) Selection of primers for polymerase chain reaction. *Mol. Biotechnol.* **3,** 129–134.
9. Lucas, K., Busch, M., Mossinger, S., and Thompson, J. A. (1991) An improved microcomputerprogram for finding gene- or gene family-specific oligonucleotides suitable as primers for polymerase chain reactions or probes. *Comput. Appl. Biosci.* **7,** 525–529.
10. Osborne, B. I. (1992) HyperPCR: a Macintosh HyperCard program for the determination of optimal PCR annealing temperature. *Comput. Appl. Biosci.* **8,** 83.
11. Rychlik, W. and Rhoades, R. E. (1989) A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and *in vitro* amplification of DNA. *Nucleic Acid Res.* **17,** 8543–8551.
12. Rychlik, W., Spencer, W. J., and Rhoads, R. E. (1990) Optimization of the annealing temperature for DNA amplification in vitro. *Nucleic Acid. Res.* **18,** 6409–6412.
13. Wu, D. Y., Ugozzoli, L., Pal, B. K., Qian, J., and Wallace, R. B. (1991) The effect of temperature and oligonucleotide primer length on the specificity and efficiency of amplification by the polymerase chain reaction. *DNA Cell. Biol.* **10,** 233–238.
14. Meinkoth, J. and Wahl, G. (1984) Hybridization of nucleic acids immobilized on solid supports. *Anal. Biochem.* **138,** 267–284.

15. Cheng, S., Fockler, C., Barnes, W. M., and Higuchi, R. (1994) Effective amplification of long targets from cloned inserts and human genomic DNA. *Proc. Natl. Acad. Sci. USA* **91,** 5695–5699.
16. Maga, E. A. and Richardson, T. (1990) Amplification of a 9.0-kb fragment using PCR. *BioTechniques* **8,** 185,186.
17. Barnes, W. M. (1994) PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. *Proc. Natl. Acad. Sci. USA* **91,** 2216–2220.
18. Kaufman, D. L. and Evans, G. A. (1990) Restriction endonuclease cleavage at the termini of PCR products. *BioTechniques* **9,** 304–306.
19. Innis, M. A. and Gelfand, D. H. (1990) Optimization of PCR's, in *PCR Protocols, A Guide to Methods and Applications* (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds.), Academic, San Diego, CA, pp. 3–12.
20. Carter, I. W. and Cloonan, M. J. (1995) Comparison between three PCR methods for detection of human cytomegalovirus DNA. *Pathology* **27,** 161–164.
21. Sharkey, D. J., Scalice, E. R., Christy, K. G., Jr., Atwood, S. M., and Daiss, J. L. (1994) Antibodies as thermolabile switches: high temperature triggering for the polymerase chain reaction. *Biotechnology* **12,** 506–509.
22. Birch, D. E., Kolmodin, L., Laird, W. J., McKinney, N., Wong, J., Young, K. Y., Zangenberg, G. A., and Zoccoli, M. A. (1996) Simplified hot start PCR. *Nature* **381,** 445,446.
23. Longo, M. C., Berninger, M. S., and Hartley, J. L. (1990) Use of urasil DNA glucosylase to control carry-over contamination in polymerase chain reactions. *Gene* **93,** 125–128.
24. Lindahl, T., Ljungquist, S., Siegert, W., Nyberg, B., and Sperens, B. (1977) DNA N-glycosidases. *J. Biol. Chem.* **252,** 3286–3294.
25. Varshney, U., Hutcheon, T., and van de Sande, J. H. (1988) Sequence analysis expression and conservation of Escherichia coli uracil DNA glycosylase and its gene (ung). *J. Biol. Chem.* **263,** 7776–7784.
26. Ferre, F. (1992) Quantitative or semi-quantitative PCR: reality versus myth. *PCR Meth. App.* **2,** 1–9.
27. Gilliland, G., Perrin, S., Blanchard, K. A., and Bunn, H. F. (1990) Analysis of cytokine mRNA and DNA: detection and quantitation by competetive polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **87,** 2725–2729.
28. Murphy, L. D., Herzog, C. E., Rudick, J. B., Fojo, A. T., and Bates, S. E. (1990) Use of the polymerase chain reaction in the quantitation of *mdr*-1 gene expression. *Biochemistry* **29,** 10,351–10,356.
29. Conolly, A. R., Cleland, L. G., and Kirkham, B. W. (1995) Mathematical considerations of competetive polymerase chain reaction. *J. Immunol. Methods* **187,** 201–211.
30. Raeymaekers, L. (1993) Quantitative PCR: theoretical considerations with practical implications. *Anal. Biochem.* **214,** 582–585.
31. Kwok, S. and Higuchi, R. (1989) Avoiding false positives with PCR. *Lancet* **339,** 237,238.
32. Persing, D. H. (1990) Polymerase chain reaction: trenches to benches. *J. Clin. Microbiol.* **29,** 1281–1285.

33. Tokue, Y., Shoji, S., Satoh, K., Watanabe, A., and Motomiya, M. (1991) Detection of methicillin-resistant *Staphylococcus aureus* (MRSA) using polymerase chain Reaction Amplification. *Tohoku J. Exp. Med.* **163,** 31–37.
34. Murakami, K., Minamide, W., Wada, K., Nakamura, E., Teraoka, H., and Watanabe, S. (1991) Identification of methicillin-resistant strains of *Staphylococci* by polymerase chain reaction. *J. Clin. Microbiol.* **29,** 2240–2244.
35. Zhang, Q. Y., Jones, D. M., Nieto, J. A. S., Trallero, E. P., and Spratt, B. G. (1990) Genetic diversity of penicillin-binding protein 2 genes of penicillin-resistant strains of *Neisseria meningitidis* revealed by fingerprinting of amplified DNA. *Antimicrob. Agents Chemother.* **34,** 1523–1528.
36. Arthur, M., Molinas, C., Mabilat, C., and Courvalin, P. (1990) Detection of erythromycin resistance by the polymerase chain reaction using primers in conserved regions of *erm* rRNA methylase genes. *Antimicrob. Agents Chemother.* **34,** 2024–2026.
37. Vliegenhart, J. S., Ketelaar-Van Gaalen, P. A. G., and van de Klundert, J. A. M. (1990) Identification of three genes coding for aminoglycoside-modifying enzymes by means of the polymerase chain reaction. *J. Antimicrob. Chemother.* **25,** 759–765.
38. Levesque, C., Piche, L., Larose, C., and Roy, P. H. (1995) PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrob. Agents Chemother.* **39,** 185–191.
39. Brisson-Noel, A., Lecossier, D., Nassif, X., Gicquel, B., LevyFrebault, V., and Hance, A. J. (1989) Rapid diagnosis of tuberculosis by amplification of mycobacterial DNA in clinical samples. *Lancet* **ii,** 1069–1071.
40. Vary, P. H., Andersen, P. R., Green, E., Hermon-Taylor, J., and McFadden, J. J. (1990) Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. *J. Clin. Microbiol.* **28,** 933–937.
41. Hartskeerl, R. A., De Wit, M. Y. L., and Klatser, P. R. (1989) Polymerase chain reaction for the detection of *Mycobacterium leprae. J. Gen. Microbiol.* **135,** 2357–2364.
42. Starnback, M. N., Falkow, S., and Tompkins, L. S. (1989) Species-specific detection of *Legionella pneumophila* in water by DNA amplification and hybridization. *J. Clin. Microbiol.* **27,** 1257–1261.
43. Mahbubani, M. H., Bej, A. K., Miller, R., Haff, L., DiCesare, J., and Atlas, R. M. (1990) Detection of legionella with polymerase chain reaction and gene probe methods. *Mol. Cell. Probes.* **4,** 175–187.
44. Lisby, G. and Dessau, R. (1994) Construction of a DNA amplification assay for clinical diagnosis of *Legionellae* species. *Eur. J. Clin. Microbiol.* **13,** 225–231.
45. Rosa, P. A. and Schwan, T. G. (1989) A specific and sensitive assay for the Lyme disease spirochete *Borrelia burgdorferi* using the polymerase chain reaction. *J. Infect. Dis.* **169,** 1018–1029.
46. Jaulhac, B., Nicolini, P., Piemont, Y., and Monteil, H. (1991) Detection of *Borrelia burgdorferi* in cerebrospinal fluid of patients with lyme borreliosis. *N. Engl. J. Med.* **324,** 1440.
47. Bernet, C., Garret, M., De Barbeyrac, B., Bebear, C., and Bonnet, J. (1989) Detection of *Mycoplasma pneumoniae* by using the polymerase chain reaction. *J. Clin. Microbiol.* **27,** 2492–2496.

48. Jensen, J. S., Uldum, A. S., Søndergård-Andersen, J., Vuust, J., and Lind, K. (1991) Polymerase chain reaction for detection of *Mycoplasma genitalium* in clinical samples. *J. Clin. Microbiol.* **29,** 46–50.
49. Dutilh, B., Bebear, C., Rodriguez, P., Vekris, A., Bonnet, J., and Garret, M. (1989) Specific amplification of a DNA sequence common to all chlamydia trachomatis serovars using the polymerase chain reaction. *Res. Microbiol.* **140,** 7–16.
50. Holland, S. M., Gaydos, C. A., and Quinn, T. C. (1990) Detection and differentiation of *Chlamydia trachomatis, Chlamydia psittaci*, and *Chlamydia pneumoniae* by DNA amplification. *J. Infect. Dis.* **162,** 984–987.
51. Border, P. M., Howard, J. J., Plstow, G. S., and Siggens, K. W. (1990) Detection of *Listeria* species and *Listeria monocytogenes* using polymerase chain reaction. *Lett. Appl. Microbiol*. **11,** 158–162.
52. McDonough, K. A., Schwan, T. G., Thomas, R. E., and Falkow, S. (1988) Identification of a *Yersinia pestis*-specific DNA probe with potential for use in plague surveillance. *J. Clin. Microbiol.* **26,** 2515–2519.
53. Wren, B. W. and Tabaqchali, S. (1990) Detection of pathogenic *Yersenia enterocolitica* by the polymerase chain reaction. *Lancet* **336,** 693.
54. Lampel, K. A., Jagow, J. A., Truclcsess, M., and Hill, W. E. (1990) Polymerase chain reaction for detection of invasive *Shigella flexneri* in food. *Appl. Envir. Microbiol.* **56,** 1536–1540.
55. Olive, D. M., Atta, A. I., and Setti, S. K. (1988) Detection of toxigenic *Escherichia coli* using biotin-labeled DNA probes following enzymatic amplification of the heat labile toxin gene. *Mol. Cell. Probes* **2,** 47–57.
56. Victor, T., du Toit, R., van Zyl, J., Bester, A. J., and van Helden, P. D. (1991) Improved method for the routine identification of toxigenic *Escherichia coli* by DNA amplification of a conserved region of the heat-labile toxin A subunit. *J. Clin. Microbiol.* **29,** 158–161.
57. Karch, H. and Meyer, T. (1989) Single primer pair for amplifying segments of distinct shiga-like-toxin genes by polymerase chain reaction. *J. Clin. Microbiol.* **27,** 2751–2757.
58. Pollard, D. R., Johnson, W. M., Lior, H., Tyler, S. D., and Rozee, K. R. (1990) Rapid and specific detection of verotoxin genes in *Escherichia coli* by the polymerase chain reaction. *J. Clin. Microbiol.* **28,** 540–545.
59. van Ketel, R. J., de Wever, B., and van Alphen, L. (1990) Detection of *Haemophilus influenzae* in cerebrospinal fluids by polymerase chain reaction DNA amplification. *J. Med. Microbiol.* **33,** 271–276.
60. Kristiansen, B. E., Ask, E., Jenkins, A., Fermer, C., Radstrøm, P., and Skød, O. (1991) Rapid diagnosis of meningococcal meningitis by polymerase chain reaction. *Lancet* **337,** 1568,1569.
61. Houard, S., Hackel, C., Herzog, A., and Bollen, A. (1989) Specific identification of *Bordetella pertussis* by the polymerase chain reaction. *Res. Microbiol.* **140,** 477–487.
62. Burstain, J. M., Grimprel, E., Lukehart, S. A., Norgard, M. V., and Radolf, J. D. (1991) Sensitive detection of *Treponema pallidum* by using the polymerase chain reaction. *J. Clin. Microbiol.* **29,** 62–69.

63. Grimprel, E., Sanchez, P. J., Wendel, G. D., Burstain, J. M., McCracken, G. H., Radolf, J. D., and Norgard, M. V. (1991) Use of polymerase chain reaction and rabbit infectivity testing to detect *Treponema pallidum* in amniotic fluid fetal and neonatal sera and cerebrospinal fluid. *J. Clin. Microbiol.* **29,** 1711–1718.
64. Hoshina, S., Kahn, S. M., Jiang, W., and Green, P. H. R. (1990) Direct detection and amplification of *Helicobacter pylori* ribosomal 16S gene segments from gastric endoscopi biopsies. *Diagn. Microbiol. Infect. Dis.* **13,** 473–479.
65. Hill, W. E., Keasler, S. P., Trucksess, M. W., Feng, P., Kaysner, C. A., and Lampel, K. A. (1991) Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. *Appl. Envir. Microbiol.* **57,** 707–711.
66. Pollard, D. R., Johnson, W. M., Lior, H., Tyler, S. D., and Rozee, K. R. (1990) Detection of the aerolysin gene in *Aeromonas hydrophila* by the polymerase chain reaction. *J. Clin. Microbiol.* **28,** 2477–2481.
67. Wren, B., Clayton, C., and Tabaqchali, S. (1990) Rapid identification of toxigenic *Clostridium difficile* by polymerase chain reaction. *Lancet* **335,** 423.
68. Wilson, I. G., Cooper, J. E., and Gilmour, A. (1991) Detection of enterotoxigenic *Staphylococcus aureus* in dried skimmed milk: use of the polymerase chain reaction for amplification and detection of *Staphylococcal* enterotoxin genes entB and entC1 and the thermonuclease gene nuc. *Appl. Envir. Microbiol.* **57,** 1793–1798.
69. Duggan, D. B., Ehrlich, G. D., Davey, F. P., and Kwok, S. (1988) HTLV-I Induces lymphoma mimicking Hodgkin's disease. Diagnosis by polymerase chain reaction amplification of specific HTLV-I sequences in tumor DNA. *Blood* **71,** 1027–1032.
70. Lisby, G. (1993) Search for an HTLV-I-like retrovirus in patients with multiple sclerosis by enzymatic DNA amplification. *Acta. Neurol. Scand.* **88,** 385–387.
71. Lee, H., Swanson, P., Shorty, V. S., Zack, J. A., Rosenblatt, J. D., and Chen, I. S. Y. (1989) High rate of HTLV-II infection in seropositive IV drug abusers in New Orleans. *Science* **244,** 471–475.
72. Murakawa, G. J., Zaia, J. A., Spallone, P. A., and Stephens, D. A. (1988) Direct detection of HIV-l RNA from AIDS and ARC patient samples. *DNA* **7,** 287–295.
73. Holodniy, M., Katzenstein, D. A., Sengupta, S., and Wang, A. M. (1991) Detection and quantification of human immunodeficency virus RNA in patient serum by use of the polymerase chain reaction. *J. Infect. Dis.* **163,** 862–866.
74. Jackson, J. B., Kwok, S., Sninsky, J., and Hopsicker, J. S. (1990) Human immunodeficiency virus type 1 detected in all seropositive symptomatic and asymptomatic individuals. *J. Clin. Microbiol.* **28,** 16–19.
75. Rayfield, M., De Cock, K., Heyvvard, W., and Goldstein, L. (1988) Mixed human immunodeficiency virus (HIV) infection in an individual: demonstration of both HIV type 1 and type 2 proviral sequences by using polymerase chain reaction. *J. Infect. Dis.* **158,** 1170–1176.
76. Bevan, I. S., Daw, R. A., Day, P. J. R., Ala, F. A., and Walker, M. R. (1991) Polymerase chain reaction for detection of human cytomegalovirus infection in a blood donor population. *Br. J. Haemat.* **78,** 94–99.
77. Jiwa, N. M., Van Gemert, G., Raap, A. K., and Van de Rijke, F. M. (1989) Rapid detection of human cytomegalovirus DNA in peripheral blood leukocytes of

viremic transplant recipients by the polymerase chain reaction. *Transplant.* **48,** 72–76.

78. Cao, M., Xiao, X., Eghert, B., Darragh, T. M., and Yen, T. S. B. (1989) Rapid detection of cutaneous herpes simplex virus infection with the polymerase chain reaction. *J. Invest. Dermatol.* **82,** 391,392.
79. Aurelius, E., Johansson, B., Skoldenberg, B., Staland, A., and Forsgren, M. (1991) Rapid diagnosis of herpes simplex encephalitis by nested polymerase chain reaction assay of cerebrospinal fluid. *Lancet* **337,** 189–192.
80. Koropchak, C. M., Graham, G., Palmer, J., and Winsberg, M. (1991) Investigation of Varicella-Zoster virus infection by polymerase chain reaction in the immunocompetent host with acute varicella. *J. Infect. Dis.* **163,** 1016–1022.
81. Jilg, W., Sieger, E., Alliger, P., and Wolf, H. (1990) Identification of type A and B isolates of Epstein-Barr virus by polymerase chain reaction. *J. Virol. Meth* **30,** 319–322.
82. Buchbinder, A., Josephs, S. F., Ablashi, D., Salabuddin, S. Z., and Klotman, M. E. (1988) Polymerase chain reaction amplification and in situ hybridization for the detection of human B-lymphotropiic virus. *J. Virol. Meth.* **21,** 191–197.
83. Jarrett, R. F., Clark, D. A., Josephs, S. F., and Onions, D. E. (1990) Detection of human herpesvirus-6 DNA in peripheral blood and saliva. *J. Med. Virol.* **32,** 73–76.
84. Margolis, H. S. and Nainan, O. V. (1990) Identification of virus components in circulating immune complexes isolated during hepatitis A virus infection. *Hepatol.* **11,** 31–37.
85. Jansen, R. W., Siegl, G., and Lemon, S. M. (1990) Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. *Proc. Natl. Acad. Sci. USA* **87,** 2867–2871.
86. Kaneko, S., Miller, R. H., Feinstone, S. M., and Unoura, M. (1989) Detection of serum hepatitis B virus DNA in patients with chronic hepatitis using the polymerase chain reaction assay. *Proc. Natl. Acad. Sci. USA* **86,** 312–316.
87. Garson, J. A., Tedder, R. S., Briggs, M., and Tuke, P. (1990) Detection of hepatitis C viral sequences in blood donations by "nested" polymerase chain reaction and prediction of infectivity. *Lancet* **335,** 1419–1422.
88. Weiner, A. J., Kuo, G., Bradley, O. W., and Bonino, F. (1990) Detection of hepatitis C viral sequences in non-A non-B hepatitis. *Lancet* **335,** 1–3.
89. Zignego, A. L., Deny, P., Feray, C., and Ponzetto, A. (1990) Amplification of hepatitis delta virus RNA sequences by polymerase chain reaction: a tool for viral detection and cloning. *Mol. Cell. Probes* **4,** 43–51.
90. Reyes, G. R., Purdy, M. A., Kim, J. P., and Luk, K. C. (1990) Isolation of a cDNA from the virus responsible for enterically transmitted non-A non-B hepatitis. *Science* **247,** 1335–1339.
91. Shibata, D. K., Arnheim, N., and Martin, W. J. (1988) Detection of human papilloma virus in paraffin-embedded tissue using the polymerase chain reaction. *J. Exp. Med.* **167,** 225–230.
92. Anceschi, M. M., Falcinelli, C., Pieretti, M., and Cosmi, E. V. (1990) Multiple primer pairs polymerase chain reaction for the detection of human papillomavirus types. *J. Virol. Meth.* **28,** 59–66.

93. Ho-Terry, L., Terry, G. M., and Londesborough, P. (1990) Diagnosis of foetal rubella virus infection by polymerase chain reaction. *J. Gen. Virol.* **71,** 1607–1611.
94. Olive, D. M., Al-Mufti, S., Al-Mulla, W., and Khan, M. A. (1990) Detection and differentiation of picornaviruses in clinical samples following genomic amplification. *J. Gen. Virol.* **71,** 2141–2147.
95. Rotbart, H. A., Kinsella, J. P., and Wasserman, R. L. (1990) Persistent enterovirus infection in culture-negative meningoencephalitis: demonstration by enzymatic RNA amplification. *J. Infect. Dis.* **161,** 787–791.
96. Ermine, A., Larzul, D., Ceccaldi, P. E., Guesdon, J. L., and Tsiang, H. (1990) Polymerase chain reaction amplification of rabies virus nucleic acids from total mouse brain RNA. *Mol. Cell. Probes* **4,** 189–191.
97. Arthur, R. R., Dagostin, S., and Shah, K. V. (1989) Detection of BK virus and JC virus in urine and brain tissue by the polymerase chain reaction. *J. Clin. Microbiol.* **27,** 1174–1179.
98. Carman, W. F., Williamson, C., Cunliffe, B. A., and Kidd, A. H. (1989) Reverse transcription and subsequent DNA amplification of rubella virus RNA. *J. Virol. Methods* **25,** 21–30.
99. Godec, M. S., Asher, D. M., Swoveland, P. T., and Eldadah, Z. A. (1990) Detection of measles virus genomic sequences in SSPE brain tissue by the polymerase chain reaction. *J. Med. Virol.* **30,** 237–244.
100. Yamada, A., Imanishi, J., Nakajima, E., Nakajima, K., and Nakajima, S. (1991) Detection of influenza viruses in throat swab by using polymerase chain reaction. *Microbiol. Immunol.* **35,** 259–265.
101. Hyypiä, T., Auvinen, P., and Maaronen, M. (1989) Polymerase chain reaction for human picorna-viruses. *J. Gen. Virol.* **70,** 3261–3268.
102. Torgersen, H., Skern, T., and Blaas, D. (1989) Typing of human rhinoviruses based on sequence variations in the 5' non-coding region. *J. Gen. Virol.* **70,** 3111–3116.
103. Gow, J. W., Behan, W. M. H., Clements, G. B., and Woodall, C. (1991) Enteroviral RNA sequences detected by polymerase chain reaction in muscle of patients with post-viral fatigue syndrome. *Br. Med. J.* **302,** 692–696.
104. Gouvea, V., Glass, R. I., Woods, P., and Taniguchi, K. (1990) Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. *J. Clin. Microbiol.* **28,** 276–282.
105. Allard, A., Girones, R., Juto, P., and Wadell, G. (1990) Polymerase chain reaction for detection of adenoviruses in stool samples. *J. Clin. Microbiol.* **28,** 2659–2667.
106. Salimans, M. M. M., Van de Rijke, F. M., Raap, A. K., and van Elsacker-Niele, A. M. W. (1989) Detection of parvovirus B19 DNA in fetal tissues by *in situ* hybridisation and polymerase chain reaction. *J. Clin. Pathol.* **42,** 525–530.
107. Laille, M., Deubel, V., and Sainte-Marie, F. F. (1991) Demonstration of concurrent dengue 1 and dengue 3 infection in six patients by the polymerase chain reaction. *J. Med. Virol.* **34,** 51–54.
108. Eldadah, Z. A., Asher, D. M., Godec, M. S., and Pomeroy, K. L. (1991) Detection of Flaviviruses by reverse-transcriptase polymerase chain reaction. *J. Med. Virol.* **33,** 260–267.

109. Morita, K., Tanaka, M., and Igarashi, A. (1991) Rapid identification of dengue virus serotypes by using polymerase chain reaction. *J. Clin. Microbiol.* **29,** 2107–2110.
110. Giebel, L. B., Zoller, L., Bautz, I. K. F., and Darai, G. (1990) Rapid detection of genomic variations in different strains of hantaviruses by polymerase chain reaction techniques and nucleotide sequence analysis. *Virus Res.* **16,** 127–136.
111. Lunkenheimer, K., Hufert, F. T., and Schmitz, H. (1990) Detection of lassa virus RNA in specimens from patients with lassa fever by using the polymerase chain reaction. *J. Clin. Microbiol.* **28,** 2686–2692.
112. Wakefield, A. E., Pixley, F. J., Banerji, S., and Sinclair, K. (1990) Detection of *Pneumocystis carinii* with DNA amplification. *Lancet* **336,** 451–453.
113. Burg, J. L., Grover, C. M., Pouletty, P., and Boothroyd, J. C. (1989) Direct and sensitive detection of a pathogenic protozoan *Toxoplasma gondii* by polymerase chain reaction. *J. Clin. Microbiol.* **27,** 1787–1792.
114. Holliman, R. E., Johnson, J. D., and Savva, D. (1990) Diagnosis of cerebral toxoplasmosis in association with AIDS using the polymerase chain reaction. *Scand. J. Infect. Dis.* **22,** 243–244.
115. Foote, S. J., Kyle, D. E., Martin, R. K., and Oduola, A. M. J. (1990) Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum. Nature* **345,** 255–258.
116. Kain, K. C. and Lanar, D. E. (1991) Determination of genetic variation within *Plasmodium falciparum* by using enzymatically amplified DNA from filter paper disks impregnated with whole blood. *J. Clin. Microbiol.* **29,** 1171–1174.
117. Tannich, E. and Burchard, G. D. (1991) Differentiation of pathogenic from nonpathogenic *Entamoeba histolytica* by restriction fragment analysis of a single gene amplified in vitro. *J. Clin. Microbiol.* **29,** 250–255.
118. Mahbubani, M. H., Bej, A. K., Perlin, M. H., and Schaefer, F. W. III (1992) Differentiation of *Giardia duodenalis* from other giardia species by using polymerase chain reaction and gene probes. *J. Clin. Microbiol.* **30,** 74–78.
119. Carl, M., Tibbs, C. W., Dobson, M. E., Paparello, S., and Dasch, G. A. (1990) Diagnosis of acute typhus infection using the polymerase chain reaction. *J. Infect. Dis.* **161,** 791–793.
120. Kelly, D. J., Marana, D. P., Stover, C. K., Oaks, E. V., and Carl, M. (1990) Detection of *Rickettsia tsutsugamushi* by gene amplification using polymerase chain reaction techniques. *Ann. New York Acad. Sci.* **590,** 564–571.
121. Tzianabos, T., Anderson, B. E., and McDade, J. E. (1989) Detection of *Rickettsia rickettsii* DNA in clinical specimens by using polymerase chain reaction technology. *J. Clin. Microbiol.* **27,** 2866–2868.
122. Moser, D. R., Kirchhoff, L. V., and Donelson, J. E. (1989) Detection of *Trypanosoma cruzi* by DNA amplification using the polymerase chain reaction. *J. Clin. Microbiol.* **27,** 1477–1482.
123. Moser, D. R., Cook, G. A., Ochs, D. E., and Bailey, C. P. (1989) Detection of *Trypanosoma congolense* and *Trypanosoma brucei* subspecies by DNA amplification using the polymerase chain reaction. *Parasitology* **99,** 57–66.

124. Gottstein, B. and Mowatt, M. R. (1991) Sequencing and characterization of an *Echinococcus multilocularis* DNA probe and its use in the polymerase chain reaction. *Mol. Biochem. Parasitol.* **44,** 183–194.
125. McLaughlin, G. L., Vodkin, M. H., and Huizinga, H. W. (1991) Amplification of repetitive DNA for the specific detection of *Naegleria fowleri. J. Clin. Microbiol.* **29,** 227–230.
126. Gasser, R. B., Morahan, G., and Mitchell, G. F. (1991) Sexing single larval stages of Schistosoma mansoni by polymerase chain reaction. *Mol. Biochem. Parasitol.* **47,** 255–258.
127. Gottstein, B., Deplazes, P., Tanner, I., and Skaggs, J. S. (1991) Diagnostic identification of *Taenia saginata* with the polymerase chain reaction. *Trans. Roy. Soc. Trop. Med. Hyg.* **85,** 248,249.
128. Zarlenga, D. S., McManus, D. P., Fan, P. C., and Cross, J. H. (1991) Characterization and detection of a newly described Asian *Taeniid* using cloned ribosomal DNA fragments and sequence amplification by the polymerase chain reaction. *Exp. Parasitol.* **72,** 174–183.
129. Lopez-Berestein, G., Bodey, G. P., Fainstein, V., and Keating, M. (1989) Treatment of systemic fungal infections with liposomal amphotericin B. *Arch. Intern. Med.* **149,** 2533–2536.
130. Buchman, T. G., Rossier, M., Merz, W. G., and Charache, P. (1990) Detection of surgical pathogens by in vitro DNA amplification. Part I rapid identification of *Candida albicans* by in vitro amplification of a fungus-specific gene. *Surgery* **108,** 338–347.
131. Vilgalys, R. and Hester, M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *J. Bacteriol.* **172,** 4238–4246.
132. Kemker, B. J., Lehmann, P. F., Lee, J. W., and Walsh, T. J. (1991) Distinction of deep versus superficial clinical and nonclinical isolates of *Trichosporon beigelii* by isoenzymes and restriction fragment length polymorphisms of rDNA generated by polymerase chain reaction. *J. Clin. Microbiol.* **29,** 1677–1683.
133. Woese, C. R. (1987) Bacterial evolution. *Microbiol. Rev.* **51,** 221–271.
134. Chen, K., Neimark, H., Rumore, P., and Steinman, C. R. (1989) Broad range DNA probes for detection and amplifying eubacterial nucleic acids. *FEMS Microbiol. Lett.* **57,** 19–24.
135. Böddinghaus, B., Rogall, T., Flohr, T., Blöcker, H., and Böttger, E. C. (1990) Detection and identification of mycobacteria by amplification of rRNA. *J. Clin. Microbiol.* **28,** 1751–1759.
136. Kirschner, P., Rosenau, J., Springer, B., Teschner, K., Feldmann, K., and Böttger, E. (1996) Diagnosis of mycobacterial infections by nucleic acid amplification: 18 month prospective study. *J. Clin. Microbiol.* **34,** 304–312.
137. Relman, D. A., Schmidt, T. M., MacDermott, R. P., and Falkow, S. (1992) Identification of the uncultured bacillus of Whipple's disease. *N. Engl. J. Med.* **327,** 293–301.
138. Relman, D. A., Loutit, J. S., Schmiidt, T. M., Falkow, S., and Tompkins, L. S. (1990) The agent of bacillary angiomatosis. *N. Engl. J. Med.* **323,** 1573–1580.

139. Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Res.* **18,** 6531–6535.
140. Welsh, J. and McClelland, M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acid Res.* **18,** 7213–7218.
141. Williams, J. G. K., Hanafey, M. K., Rafalski, J. A., and Tingey, S. V. (1993) Genetic analysis using random amplified polymorphic DNA markers. *Methods Enzymol.* **218,** 704–740.
142. Akopyanz, N., Bukanov, N. O., Westhlum, T. U., Kresovich, S., and Berg, D. E. (1992) DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucleic Acids Res.* **20,** 5137–5142.
143. Kersulyte, D., Woods, J. P., Keath, E. J., Goldman, W. E., and Berg, D. E. (1992) Diversity among clinical isolates of *Histoplasma capsulatum* detected by polymerase chain reaction with arbitrary primers. *J. Bacteriol.* **174,** 7075–7079.
144. Wang, G., Whittam, T. S., Berg, C. M., and Berg, D. E. (1993) RAPD (arbitrary primer) PCR is more sensitive than multilocus enzyme electrophoresis for distinguishing related bacterial strains. *Nucleic Acid Res.* **21,** 5930–5933.
145. Berg, D. E., Akopyants, N. S., and Kersulyte, D. (1994) Fingerprinting microbial genomes using the RAPD or AP-PCR method. *Methods Mol. Cell. Biol.* **5,** 13–24.
146. Thanos, M., Schonian, G., Meyer, W., Schweynoch, C., Graser, Y., Mitchell, T. G., Presber, W., and Tietz, H. J. (1996) Rapid identification of *Candida* species by DNA fingerprinting with PCR. *J. Clin. Microbiol.* **34,** 615–621.
147. Mahenthiralingam, E., Campbell, M. E., Foster, J., Lam, J. S., and Speert, D. P. (1996) Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. *J. Clin. Microbiol.* **34,** 1129–1135.
148. Lin, A. W., Usera, M. A., Barret, T. J., and Goldsby, R. A. (1996) Application of random amplified polymorphic DNA analysis to differentiate strains of *Salmonella enteritidis. J. Clin. Microbiol.* **34,** 870–876.
149. Weyant, R. S., Edmonds, P., and Swaminathan, B. (1990) Effect of ionic and nonionic detergents on the taq polymerase. *BioTechniques* **9,** 308,309.
150. Beutler, E., Gelbart, T., and Kuhl, W. (1990) Interference of heparin with the polymerase chain reaction. *BioTechniques* **9,** 166.
151. Holodnly, M., Kim, S., Katzenstein, D., Konrad, M., Groves, E., and Merigan, T. C. (1991) Inhibition of human immunodeficiency virus gene amplification by heparin. *J. Clin. Microbiol.* **29,** 676–679.
152. Thin, N. and Stafford, D. W. (1976) Isolation of high-molecular-weight DNA. *Nucleic Acid Res.* **3,** 2303–2308.
153. Millar, D. S., Withey, S. J., Tizard, M. L. V., Ford, J. G., and Hermon-Taylor, J. (1995) Solid phase hybridisation-capture of low abundance target DNA sequences: application to the PCR detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp. *silvaticum. Analyt. Biochem.* **226,** 325–330.
154. Mangiapan, G., Vokurka, M., Schouls, L., Cadranel, J., Lecossier, D., van Embden, J., and Hance, A. (1996) Sequence capture-PCR improves detection of mycobacterial DNA in clinical specimens. *J. Clin. Microbiol.* **34,** 1209–1215.

155. Loeffelholz, M. J., Lewinski, C. A., Silver, S. R., Purohit, A. P., Herman, S. A., Buonagurio, D. A., and Dragon, E. A. (1992) Detection of *Chlamydia trachomatis* in endocervical specimens by polymerase chain reaction. *J. Clin. Microbiol.* **30,** 2847–2851.
156. Ishizawa, M. Y., Kobayashi, T., and Matsuuva, S. (1991) Simple procedure of DNA isolation from human serum. *Nucleic Acid Res.* **19,** 5792.
157. Lanciotti, R. S., Calisher, C. H., Ciubler, D. J., Chang, G. J., and Vornadam, A. V. (1992) Rapid detection and typing of Dengue viruses from clinical samples by using reverse transcription-polymerase chain reaction. *J. Clin. Microbiol.* **30,** 545–551.
158. Shankar, P., Manjunath, N., Mohan, K. K., Prasad, K., Behari, M., Shriniwas, P., and Ahuja, G. K. (1991) Rapid diagnosis of tuberculous meningitis by polymerase chain reaction. *Lancet* **337,** 5–7.
159. Lisby, G., Dessau, R., Andersen, C. B., and Ladefoged, S. (1994) Polymerase chain reaction as a rapid diagnostic assay for cytomegalovirus infection in renal transplant recipients. *APMIS* **102,** 690–694.
160. Higuchi R. (1989) Simple and rapid preparation of samples for PCR, in *PCR Technology: Principles and Applications for DNA Amplification* (Erlich, H. A., ed.), Stockton, New York, pp. 31–38.
161. Cousins, D. V., Wilton, S. D., Francis, B. R., and Gow, B. L. (1992) Use of polymerase chain reaction for rapid diagnosis of tuberculosis. *J. Clin. Microbiol.* **30,** 255–258.
162. Frankel, G., Riley, L., Giron, J. A., Valmassoi, J., Freidmann, A., Stokbine, N., Falkow, S., and Schoolnik, G. K. (1990) Detection of *Shigella* in feces using DNA amplification. *J. Infect. Dis.* **161,** 1252–1256.
163. Kwok, S. (1990) Procedures to minimize PCR-carry over, in *PCR Protocols: A Guide to Methods and Applications* (Innis, M. A., Gelfand, D. H., Sninsky, J. J., White, T. J., eds.), Academic, San Diego, pp. 142–145.
164. Dragon, E. A., Spadoro, J. P., and Madej, R. (1993) Quality control of polymerase chain reaction, in *Diagnostic Molecular Microbiology—Principles and Applications* (Persing, D. H., Smith, T. F., Tenover, F. C., and White, T. J., eds.), American Society for Microbiology, Washington, DC, pp. 160–168.
165. Sheppard, H. W., Ascher, M. S., Busch, P. R., and Sohmer, P. R. (1991) A multicenter proficiency trial of gene amplification (PCR) for the detection of HIV-1. *J. Acquir. Immun. Defic. Syndr.* **4,** 277–283.
166. Zaaijer, H. L., Cuypers, H. T. M., Reesink, H. W., and Winkel, I. N. (1993) Reliability of polymerase chain reaction for detection of hepatitis C virus. *Lancet* **341,** 722–724.
167. Jackson, J. B., Drew, J., Lin, H. J., and Otto, P. (1993) Establishment of a quality assurance program for Human Immunodeficiency Virus type 1 DNA polymerase chain reaction assays by the AIDS clinical trials group. *J. Clin. Microbiol.* **31,** 3123–3128.
168. Mahony, J. B., Luinstra, K. E., Waner, J., and McNab, G. (1994) Interlaboratory agreement study of a double set of PCR plasmid primers for detection of *Chlamydia trachomatis* in a variety of genitourinary specimens. *J. Clin. Microbiol.* **32,** 87–91.
169. Barany, F. (1991) The ligase chain reaction in a PCR world. *PCR Methods Applic.* **1,** 5–16.

170. Barany, F. (1991) Genetic disease detection and DNA amplification using cloned thermostable ligase. *Proc. Natl. Acad. Sci. USA* **88,** 189–193.
171. Abravaya, K., Carrino, J. J., Muldoon, J., and Lee, H. H. (1995) Detection of point mutations with a modified ligase chiain reaction (Gap-LCR). *Nucleic Acids Res.* **23,** 675–682.
172. Dille, B. J., Butzen, C. C., and Birkenmeyer, L. G. (1993) Amplification of *Chlamydia trachomatis* DNA by ligase chain reaction. *J. Clin. Microbiol.* **31,** 729–731.
173. Birkenmeyer, L. G. and Armstrong, A. S. (1992) Preliminary evaluation of the ligase chain reaction for specific detection of *Neisseria gonorrhoeae. J. Clin. Microbiol.* **30,** 3089–3094.
174. van Doornum, G. J. J., Buimer, M., Prins, M., Henquet, C. J. M., Coutinho, R. A., Plier, P. K., Tomazic-Allen, S., Hu, H., and Lee, H. (1995) Detection of *Chlamydia trachomatis* infection in urine samples from men and women by ligase chain reaction. *J. Clin. Microbiol.* **33,** 2042–2047.
175. Guatelli, J. C., Whitfield, K. M., Kwoh, D. Y., and Barringer, K. J. (1990) Isothermal in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication. *Proc. Natl. Acad. Sci. USA* **87,** 1874–1878.
176. Gingeras, T. R., Whitfield, K. M., and Kwoh, D. Y. (1990) Unique features of the self-sustained sequence replication (3SR) reaction in the in vitro amplification of nucleic acids. *Ann. Biol. Clin.* **48,** 498–501.
177. Fahy, E., Kwoh, D. Y., and Gingeras, T. R. (1991) Self-sustained sequence replication (3SR): An isothermal transcription-based amplification system alternative to PCR. *PCR Methods Appl.* **1,** 25–33.
178. Compton, J. (1991) Nucleic acid sequence-based amplification. *Nature* **350,** 91,92.
179. Kwoh, D. Y., Davis, G. R., Whitfield, K. M., Chappelle, H. L., DiMichele, L. J., and Gingeras, T. R. (1989) Transcription-based amplification system and detection of amplified human deficiency virus type 1 with a bead-based sandwich hybridization format. *Proc. Natl. Acad. Sci. USA* **86,** 1173–1177.
180. Gingeras, T. R. and Kwoh, D. Y. (1992) In vitro nucleic acid target amplification techniques: issues and benefits. *Praxis Biotechnol.* **4,** 403–429.
181. Zaaijer, H. L., Kok, W., ten-Veer, J. H., Reesink, H. W., Foolen, H., Winkel, I. N., Huisman, J. G., Cuypers, H. T., Kievits, T., and Lelie, P. N. (1995) Detection of HIV-1 RNA in plasma by isothermal amplification (NASBA) irrespective of the stage of HIV-1 infection. *J. Virol. Methods.* **52,** 175–181.
182. Vandamme, A. M., Van-Dooren, S., Kok, W., Goubau, P., Fransen, K., Kievits, T., Schmit, J. C., De-Clercq, E., and Desmyter, J. (1995) Detection of HIV-1 RNA in plasma and serum samples using the NASBA amplification system compared to RNA-PCR. *J. Virol. Methods.* **52,** 121–132.
183. Abe, C., Hirano, K., Wada, M., and Kazumi, Y. (1993) Detection of *Mycobacterium tuberculosis* in clinical specimens by polymerase chain reaction and Gen-Probe amplified Mycobacterium tuberculosis direct test. *J. Clin. Microbiol.* **31**, 3270–3274.
184. Miller, N., Hernandez, S. G., and Cleary, T. J. (1994) Evaluation of Gen-Probe amplified *Mycobacterium tuberculosis* direct test and PCR for direct detection of *Mycobacterium tuberculosis* in clinical specimens. *J. Clin. Microbiol.* **32,** 393–397.

185. Kern, D. G., Sheridan, P. J., Stempien, M. S. III, and Yeghiazarian, Y. (1992) Quantitation of HIV-1 RNA in plasma using branched DNA technology. 15th AIDS Clinical Trial Group Meeting, Washington, DC, November 3–6.
186. Todd, J., Stempien, M., Kojima, E., and Shirasaka, T. (1993) Quantitation of HIV plasma RNA using the branched DNA (bDNA) and reverse transcription coupled polymerase chain reaction (RT-PCR) assays. IXth International Conference on AIDS, Berlin, Germany, June 7–11.
187. Cao, Y., Ho, D. D., Todd, J., Kokka, R., Urdea, M., Lifson, J. D., Piatak, M., Jr., Chen, S., Hahn, B. H., and Saag, M. S. (1995) Clinical evaluation of branched DNA signal amplification for quantifying HIV type l in human plasma. *AIDS Res. Hum. Retrovir.* **11,** 353–361.
188. Mellors, J. W., Kingsley, L. A., Rinaldo, C. R., Todd, J. A., Hoo, B. S., Kokka, R. P., and Gupta, P. (1995) Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion. *Ann. Intern. Med.* **122,** 573–579.
189. Martell, M., Esteban, J. I., Quer, J., and Genesca, J. (1992) Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. *J. Virol.* **66,** 3225–3229.
190. Sherman, K. E., O'Brien, J., Gutierrez, A. G., and Harrison, S. (1993) Quantitative evaluation of hepatitis C virus RNA in patients with concurrent human immunodeficiency virus infections. *J. Clin. Microbiol.* **31,** 2679–2682.
191. Lau, J. Y., Davis, G. L., Kniffen, J., and Qian, K. P. (1993) Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. *Lancet* **341,** 1501–1504.
192. Yamada, G., Takatani, M., Kishi, F., Takahashi, M., Doi, T., Tsuji, T., Shin, S., Tanno, M., Urdea, M. S., and Kolberg, J. A. (1995) Efficiacy of interferon alpha therapy in chronic hepatitis C patients depend primarily on hepatitis C virus RNA level. *Hepatology* **22,** 1351–1354.
193. Watabe, Y., Hino, K., Iino, S., and Suzuki H. (1993) Detection of HBV DNA by DNA probe method and its clinical usefulness. International Symposium on Viral Hepatitis and Liver Disease, Tokyo, Japan, May 10–14.
194. Hendricks, D. A., Stowe, B. J., Hoo, B. S., Kolberg, J., Irvine, B. D., Neuwald, P. D., Urdea, M. S., and Perrillo, R. P. (1995) Quantitation of HBV DNA in human serum using a branched DNA (bDNA) signal amplification assay. *Am. J. Clin. Pathol.* **104,** 537–546.
195. Urdea, M. (1993) Synthesis and characterization of branched DNA (bDNA) for the direct and quantitative detection of CMV, HBV, HCV, and HIV. *Clin. Chem.* **39,** 725,726.

2

Detection of Rodent Parvoviruses by PCR

David G. Besselsen

1. Introduction

The rodent parvoviruses include mouse parvovirus (MPV), minute virus of mice (MVM), H-1 parvovirus (H-1), Kilham rat virus (KRV), rat parvovirus (RPV), and hamster parvovirus (HaPV). Recent reports suggest LuIII, an autonomous parvovirus of unknown host origin, may also belong to the rodent parvovirus group ***(1,2)***. In general, rodent parvoviruses cause subclinical infections in their host of origin, although naturally occurring clinical disease may occasionally occur secondary to KRV or HaPV infection ***(3,4)***. Probably of more importance to the scientific community are the potential adverse effects these agents may have on research that utilizes rodents with inapparent and undetected infections. Subclinical rodent parvovirus infections may alter the immune response or cause fetal resorption or oncosuppression ***(5)***. In addition, rodent parvoviruses can cause persistent infections in cell culture and transplantable tumor lines ***(6,7)***, and therefore can adversely affect experiments performed in vitro.

The diagnosis of rodent parvovirus infections in both rodents and biological materials has relied primarily upon serologic assays that detect antibodies directed against rodent parvoviruses ***(5)***. One pitfall associated with serologic detection of parvovirus infections in rodents is that a diagnosis cannot be made in acute epizootic infections until seroconversion has occurred, which may prevent containment of the epizootic in an animal facility. Detection of parvovirus infections in biological materials currently relies on rodent antibody production tests or virus isolation. These methods are labor-intensive, expensive to perform, and have a slow turnaround time.

Given the limitations of methods for detecting rodent parvovirus infections in animals and biological materials, we developed a series of PCR assays that

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

enable rapid detection of any rodent parvovirus and subsequent differentiation among the various strains of rodent parvoviruses ***(8–10)***. All PCR primer sets were designed on the basis of DNA sequence comparisons of the rodent parvoviruses for which sequence data were available (this excludes RPV). Initially, a PCR primer set was designed from a portion of the nonstructural protein coding region that is conserved among the rodent parvovirus genomes. This primer set will amplify the targeted viral DNA from DNA preparations of cell cultures or rodent tissues infected with any of the rodent parvoviruses. Additional PCR primer sets were then designed on the basis of heterologous portions of the capsid protein-coding region of the parvovirus genomes to enable differentiation among the rodent parvoviruses. Four primer sets were designed and were subsequently shown to be specific for MVM, H-1, KRV, and the MPV/HaPV/LuIII group, respectively. Each of these primer sets will amplify its targeted viral DNA only from DNA preparations of cell cultures or rodent tissues infected with the specifically targeted rodent parvovirus(es). All of these PCR assays are able to detect as little as 10 pcg of the targeted viral DNA in the presence of 1.25 μg of genomic DNA from the respective rodent host. The sample preparation and amplification parameters are the same for each PCR assay; the only difference among the PCR assays is the primer set used in each particular assay.

2. Materials

2.1. Tissue Collection and DNA Extraction

1. Necropsy instruments (scissors and forceps).
2. Bleach diluted 1:10.
3. Whirlpak bags or sterile 1.5-mL microcentrifuge tubes.
4. Sterile scalpel blades and toothpicks.
5. QiAmp tissue kit (Qiagen, Chatsworth, CA).

2.2. PCR Amplification

1. Sterile water.
2. 10X PCR buffer: 100 m*M* Tris-HCl, 15 m*M* $MgCl_2$, and 500 m*M* KCl (Boehringer Mannheim, Indianapolis, IN).
3. 10 m*M* stocks of deoxynucleotides dATP, dTTP, dCTP, and dGTP (Boehringer Mannheim).
4. Working stock of deoxynucleotides that contains 1.25 m*M* of each dNTP. Prepare by adding 125 μL of each 10 m*M* dNTP stock to 500 μL sterile water for a total volume of 1 mL.
5. Oligonucleotide primers diluted in sterile water to 20 μ*M* (*see* **Table 1**).
6. Sterile 0.2-mL PCR reaction tubes.
7. *Taq* polymerase (5 U/μL).
8. Positive control DNA template diluted to 20 μg/mL (*see* **Note 1**).

Table 1
Oligonucleotide Primers Used for the Rodent Parovirus PCR Assays

Primers	Sequence (5' to 3')	Position[a]	Product size
Generic rodent parovirus			334 bp
1458f	ACCAGCCAGCACAGGCAAATCTAT	1458–1481	
1791r	CATTCTGTCTCTGATTGGTTGAGT	1791–1768	
MVM-specific			639 bp
4072f	TGGGATTCTTACAAATGCAA	4072–4090	
4710r	TATCTTGTTATTCCAAAG	4710–4693	
MPV/HaPV/LuIII-specific			260 bp
3759f	GCAGCAATGATGTAACTGAAGCT	3759–3781	
4018r	CCATCTGCCTGAATCATAGCTAA	4018–3996	
H-1-specific			254 bp
3479f	CTAGCAACTCTGCTGAAGGAACTC	3479–3502	
3732r	TAGTGATGCTGTTGCTGTATCTGATG	3732–3707	
KRV-specific			281 bp
3691f	GCACAGACAACCAAACAGGAACTCTCC	3691–3717	
3971r	AGTCTCACTTTGAGCGGCTG	3971–3952	

[a]The nucleotide positions within each respective viral sequence that corresond to the 5' and 3' ends of each primer. The genomic sequence of MPV-1 ***(1)*** was used to indicate the positions of the MPV/HaPV/LuIII primers.

2.3. Agarose Gel Electrophoresis

1. NuSieve agarose (FMC BioProducts, Rockland, ME).
2. Ethidium bromide (5 mg/mL) in sterile water.
3. 1X TBE buffer: Prepare a 5X TBE solution by adding 54 g Tris base, 27.5 g boric acid, and 20 mL of 0.5 *M* EDTA (pH 8.0) to 1 L of dd H_2O ***(11)***.
4. Gel loading dye: 0.25% bromophenol blue, 80% glycerol (v/v), prepared in sterile water.
5. DNA markers. Any of a number of commercially available DNA marker preparations that contain fragments ranging from 100- to 1000-bp will work. We routinely use DNA Marker VI from Boehringer Mannheim, which contains 11 fragments ranging from 154 to 2176 bp.
6. Parafilm.

3. Methods

3.1. Tissue Collection and DNA Extraction

1. Decontaminate necropsy instruments and the necropsy table surface with a 1:10 dilution of bleach before tissue collection from each animal (*see* **Note 2**).
2. Collect tissues aseptically from freshly euthanized rodents (*see* **Note 3**). Place each tissue into a separate, labeled whirlpack or sterile microcentrifuge tube and store at –20°C until use.

3. Alternatively, aseptically collect approx 10^7 cells from a cell culture to be tested and pellet the cells by centrifugation at 500*g* for 10 min. Resuspend the pellet in 1 mL of 1X PBS and transfer the suspension to a sterile microcentrifuge tube. Repellet the cells by microcentrifugation for 2 min and decant the supernatant.
4. Mince approx 25 mg of each tissue sample with a sterile scalpel blade (one scalpel blade per tissue sample) and place the sample into a labeled, sterile microcentrifuge tube (*see* **Note 4**). Sterile toothpicks can be used to assist in the handling of the tissue sample.
5. Extract DNA from the animal tissue or cell cultures with a QiAmp Tissue Kit (Qiagen) according to the manufacturer's instructions (*see* **Note 5**).
6. Determine the DNA content and purity of the tissue DNA extracts by measuring the A_{260}/A_{280} optical density ratio with a UV-vis spectrophotometer.
7. Calculate the volume of each sample which contains 1.25 µg of DNA. Subtract this volume from 32 µL to determine the amount of sterile water that will need to be added to each PCR reaction.

3.2. PCR Amplification

1. Prepare a master mix of PCR reagents containing (per PCR reaction) 5 µL 10X PCR buffer, 8 µL of 1.25 m*M* dNTP working stock, and 2.5 µL of each of the two primers from the selected primer set (*see* **Note 6**). Make enough for one extra PCR reaction to ensure there is enough for all samples.
2. Add the volume of sterile water required for each sample to labeled 0.2 mL PCR tubes as determined by **Subheading 3.1., step 7**. Add 27 µL sterile water for the positive control reaction and 32 µL sterile water for the negative control reaction.
3. Add 18 µL of the master mix to each PCR tube.
4. Add 1.25 µg of sample DNA to the appropriately labeled PCR tube (*see* **Note 7**). Add 100 ng of positive control DNA to the positive control PCR tube.
5. Add 0.4 µL (2.0 U) of *Taq* polymerase to each PCR tube.
6. Cap the PCR tubes and microfuge them briefly.
7. Load the tubes into the thermal cycler block (*see* **Note 8**).
8. Amplify the samples according to the following cycling program:
   Initial denaturation:
   94°C, 30 s
   Followed by 35 cycles of:
   94°C, 2 s
   55°C, 2 s (use a 40°C annealing temperature for the MVM-specific primer set)
   72°C, 30 s
   Hold: 4°C.
9. Store PCR products at –20°C until ready for electrophoretic analysis.

3.3. Agarose Gel Electrophoresis

1. Prepare a 3% NuSieve agarose gel prestained with ethidium bromide (*see* **Note 9**).
2. Remove the well comb, place the gel into the gel box, and fill the gel box with 1X TBE buffer until the gel is covered.

3. Place a 10 µL drop of loading dye onto a strip of parafilm for each marker and PCR product that will be subjected to electrophoresis. Add 5 µL of DNA marker or 10 µL of PCR product from each reaction to the drops of loading dye in the order that they will be loaded into the wells of the gel (*see* **Note 10**).
4. Load the samples into the wells and run the gel on constant voltage at 80 V until the dye front is about to run off the end of the gel (about 2 h) (*see* **Note 11**).
5. Photograph the gel on a UV transilluminator with a Polaroid camera through a yellow filter onto Polaroid type 52 film. A 0.5-s exposure at f-stop 5.6 works well.
6. Check the control lanes to ensure the positive and negative control reactions worked. If the controls worked properly, examine the sample lanes for DNA fragments of the appropriate size for the primer set that was used (*see* **Table 1**).

4. Notes

1. MVM(p), LuIII, H-1, and KRV are easily propagated to high titers, work well as positive controls for the rodent parvovirus PCR assays, and all are available from American Type Culture Collection (ATCC). MVM(p) is grown in murine fibroblasts (A9, ATCC CCL 1.4), LuIII and H-1 parvoviruses are grown in NB324K simian virus 40-transformed human newborn kidney cells ***(12)***, and Kilham rat virus (KRV) is grown in rat glial tumor cells (C_6 Glial, ATCC CCL 107). All cells are grown in Dulbecco's modified Eagle's medium containing 10% Serumplus (JRH Biosciences, Lenexa, KS) at 37°C in a 10% CO_2 atmosphere, and cells are inoculated with virus at a multiplicity of infection of 0.1. Cell pellets are collected by centrifugation (10 min at 500*g*) when approx 90% of the cells exhibit cytopathic effect. Each cell pellet is resuspended in a 1/10 volume of Tris-EDTA (50 m*M* Tris, 10 m*M* EDTA, pH 8.5) and subjected to four freeze-thaw cycles. Cellular debris are then removed by centrifugation (10 min at 1000*g*). The DNA concentration of the purified viral preparation is determined spectrophotometrically or by ethidium bromide fluorescent quantitation ***(11)***. Postive control viral preparations should be diluted at least 1:10 in sterile water prior to addition to the PCR reaction vial or the Tris-EDTA may inhibit DNA amplification.
2. To remove any potential DNA contamination, two sets of instruments are allowed to soak in a 1:10 dilution of bleach for at least 20 min prior to use. One set of instruments is used per animal. If multiple tissues are harvested from an animal, the instruments are rinsed in the bleach solution between collection of each tissue from that animal. Once tissue collection for an animal is completed, the used set of instruments is placed into the bleach solution and allowed to soak while tissue is collected from the next animal with the other set of instruments.
3. Proper selection of tissues and the age of animal to be tested can play important roles in the ability of these PCR assays to detect viral DNA. For example, a higher percentage of MPV-infected mice tested positive by the MPV-PCR assay when intestinal DNA was used as template as compared to when kidney DNA was used as template. Testing of 5- to 6-wk-old rodents is recommended since the rodents become more predisposed to parvovirus infection as maternal antibodies wane during the postweaning period.

4. Mincing of the tissue can be eliminated if one allows the proteinase K digestion step to occur overnight. When coupled with overnight proteinase K digestion, placement of the proper sized tissue sample (e.g., 25 mg) directly into a microcentrifuge tube at the time of tissue collection allows for less manipulation of the sample. The advantages of this are a decreased potential for sample contamination and a decreased technician time required. The disadvantages are a longer processing time and the lack of additional tissue for retesting.
5. Tissues that contain high levels of RNA (e.g., liver and kidney) are treated with RNase A (20 mg/mL) to allow for more accurate quantitation of DNA.
6. Aerosol-resistent pipetor tips are used for all preparation and handling of PCR reagents and DNA samples.
7. Change pipetor tips between addition of each sample and between addition of *Taq* polymerase to each PCR tube.
8. The author uses an automated Perkin-Elmer model 2400 or model 9600 thermocycler.
9. For mini-gels (7 × 10 cm) add 1.5 g NuSieve agarose to a flask containing 50 mL 1X TBE buffer. For mini-wide gels (15 × 10 cm) add 3.75 g NuSieve agarose to 125 mL 1X TBE buffer. Cover the flask opening with parafilm, heat the suspension in the microwave until the agarose is dissolved, and then allow the agarose to cool briefly. Add 1 μL of a 5 mg/mL ethidium bromide solution per 50 mL of the melted agarose, swirl to evenly disperse the ethidium bromide, and pour the agarose into a gel-casting stand with a well comb in place. Allow the gel to solidify over a period of 30 min.
10. A suggested order is marker, positive control, and negative control, followed by samples 1 through N. A marker lane is needed for each gel if multiple gels are required.
11. It is especially important to run the dye front to the edge of the gel when examining hamster tissues for the presence of HaPV DNA. This particular reaction generates a nonspecific band that is slightly smaller than the product resulting from amplification of the targeted viral DNA, and therefore requires maximum band separation to prevent false positive interpretation.

Acknowledgments

I thank Lela Riley, Cindy Besch-Williford, Dave Pintel, Greg Purdy, and Rick Knowles for their considerable contributions towards the development and optimization of these assays. This work was supported by NIH grants DHHS5 PO1 RR08624 and DHHS5 T32 RR07004.

References

1. Ball-Goodrich, L. J. and Johnson, E. (1994) Molecular characterization of a newly recognized mouse parvovirus. *J. Virol.* **68,** 6476–6486.
2. Besselsen, D. G., Pintel, D. J., Besch-Williford, C. L., Purdy, G. A., Franklin, C. L., Hook, R. R., Jr., and Riley, L. K. (1996) Molecular characterization of newly recognized rodent parvoviruses. *J. Gen. Virol.* **77,** 899–911.

3. Besselsen, D. G., Gibson, S. V., Besch-Williford, C. L., Purdy, G. A., Knowles, R. L., Wagner, J. E., Pintel, D. J., Franklin, C. L., Hook, R. R., Jr., and Riley, L. K. (1998) Naturally occurring novel parovirus infection in Syrian hamsters (*Mesocricetus auratus*), submitted for publication.
4. Jacoby, R. O., Bhatt, P. N., and Jonas, A. M. (1979) Viral disease, in *The Laboratory Rat*, vol. 1 (Baker, H. J., Lindsey, J. R., and Weisbroth, S. H., eds.), Academic, New York, pp. 272–306.
5. Jacoby, R. O., Ball-Goodrich, L. J., Besselsen, D. G., McKisic, M. D., Riley, L. K., and Smith, A. L. Rodent parvovirus infections. *Lab. Anim. Sci.* **46,** 370–380.
6. Collins, M. J., Jr. and Parker, J. C. (1972) Murine virus contaminants of leukemia viruses and transplantable tumors. *J. Natl. Cancer Inst.* **49,** 1139–1143.
7. Nicklas, W., Kraft, V., and Meyer, B. (1993) Contamination of transplantable tumors, cell lines, and monoclonal antibodies with rodent viruses. *Lab. Anim. Sci.* **43,** 296–300.
8. Besch-Williford, C., Purdy, G., Riley, L., and Pintel, D. (1991) Detection of rodent parvoviruses (MVM, H-1, and RV) by polymerase chain reaction. *AALAS Bull.* **30,** 24 (abstract P06).
9. Besselsen, D. G., Besch-Williford, C. L., Pintel, D. J., Franklin, C. L., Hook, R. R., Jr., and Riley, L. K. (1995) Detection of newly recognized rodent parvoviruses by PCR. *J. Clin. Microbiol.* **33,** 2859–2863.
10. Besselsen, D. G., Besch-Williford, C. L., Pintel, D. J., Franklin, C. L., Hook, R. R., Jr., and Riley, L. K. (1995) Detection of H-1 parvovirus and Kilham rat virus by PCR. *J. Clin. Microbiol.* **33,** 1699–1703.
11. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
12. Shein, M. and Enders, J. F. (1962) Multiplication and cytopathogenicity of simian vacuolating virus 40 in cultures of human tissues. *Proc. Soc. Exp. Biol. Med.* **109,** 495–500.

3

The SURF Technique

Selective Genetic Analysis of Microscopic Tissue Heterogeneity

Darryl Shibata

1. Introduction

1.1. Selective Ultraviolet Radiation Fractionation

Selective ultraviolet radiation fractionation (SURF) is a simple technique for the isolation of histologically defined microscopic tissue regions ***(1,2)***. Very small numbers (100–400) of cells can be rapidly isolated with relatively crude equipment. The isolated cells can be analyzed genetically by PCR, thereby allowing a direct comparison between microscopic phenotype with genotype. The ability to compare genotype between different tissue areas provides opportunities to analyze many disease processes, including the heterogeneity expected of multistep tumor progression.

1.2. Tissue Microdissection Techniques

Tissues are complex mixtures of different cell types. This heterogeneity can hinder analysis if the cells of interest are only a minority of all cells. Conversely, the high sensitivity of many molecular techniques may allow detection of rare sequences that are absent from most cells. Tissue analysis can be improved by first refining the target such that only the desired cells are examined. Toward this goal, various investigators have used physical microdissection techniques to isolate specific cells (for example **refs. *3–5***, and many others).

An alternative to physical isolation are *in situ* hybridization techniques. Unfortunately, the sensitivity of *in situ* techniques are limited and not generally useful for mutation analysis of single-copy genes. *In situ* amplification techniques have been useful for the analysis of viral infections ***(6,7)***.

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer © Humana Press Inc., Totowa, NJ

1.3. Advantages of SURF

Tissue microdissection can be tedious and requires great skill. The operator must be able to cut the desired region away from the undesired cells and the underlying microscope slide. He/she must also lift the isolated tissue and place it into the appropriate isolation tube. Multiple dissections require great care to prevent crosscontamination.

An alternative to the direct isolation of desired cells is the elimination of undesired cells. This approach has multiple technical advantages. First, the elimination of the unwanted cells greatly reduces the chance of contamination. Second, this approach is more amenable to technical innovation. For example, an elegant method to eliminate undesired DNA utilized a computer-controlled laser *(8)*. Essentially, a laser destroys everything on a microscopic slide except the small areas of interest. The SURF approach is less complicated and mimics the masking techniques used for the mass production of consumer electronic microcircuits. Electronic integrated chips are small and complex, and direct physical dissection would be extremely inefficient. Instead, a photographic mask or picture of the desired pattern is projected onto a photosensitive material. The optical pattern is converted into a physical pattern with the exposed areas eliminated whereas the protected areas remain. Extremely fine structures can be constructed because of the high resolution of light.

Microscopic tissue sections are "photosensitive" because the DNA present in the slides can be destroyed by ultraviolet light. Therefore, analogous to electronic circuits, masking techniques can be used to protect the cells of interest and ultraviolet light can be used to eliminate the DNA present in all other undesired cells. The resolution can be theoretically greater than conventional microscopy since ultraviolet light has a shorter wavelength than visible light. Hence, we have the technique of SURF (**Fig. 1**).

SURF requires some practice and modification of existing tools. One primary requirement is the ability to recognize histologic features. As such, the technique is ideal for pathologists although most individuals can achieve competence after several days or weeks of study. SURF places the emphasis on which areas to analyze because the subsequent isolation is greatly simplified. Of note: real surfing is much harder than SURF.

Before PCR, genetic analysis required large amounts of fresh tissue since hybridization probes required weeks to detect 1–10 μg of DNA. Now, over a decade after the PCR revolution, large amounts of DNA are still extracted from bulk tissues even though PCR allows the genetic analysis of small numbers of molecules. Depending on the situation, it is both unnecessary and unwise to perform genetic studies on bulk extracted DNA, unless it can be safely assumed that tissue heterogeneity is either not present or unimportant.

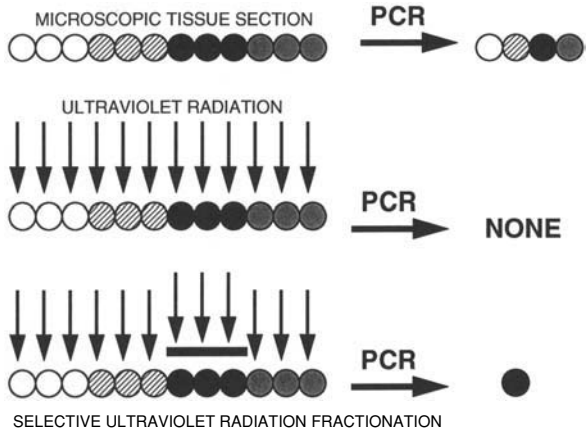


Fig. 1. Principles of SURF.

2. Materials

2.1. Preparations for SURF

1. Formalin-fixed, paraffin embedded tissues (*see* **Note 1**).
2. Plastic slides (*see* **Note 2**).
3. Stainless steel metal tray.
4. 90°C oven.
5. Hematoxylin and eosin stains (*see* **Table 1**).
6. "Sharpie" (Sanford Corporation, Bellwood, IL) marking pen.

2.2. SURF

1. Microdotter (*see* **Note 3**).
2. Ink from a Sharpie marking pen (*see* **Note 4**).
3. Inverted microscope (*see* **Note 5**) with photodocumentation system.

2.3. After SURF

1. Short-wave (254- or 302-nm) ulltraviolet transilluminator.
2. Photocopier.
3. Sterile scissors and forceps.
4. Sterile microfuge tubes (500 µL).
5. DNA extraction solution: 100 m*M* Tris-HCl, 2 m*M* EDTA, pH 8.0.
6. 20 mg/mL Proteinase K.
7. 42 to 56°C water bath.

3. Methods

3.1. Preparations for SURF

1. Select an appropriate formalin-fixed, paraffin-embedded tissue (*see* **Note 1**).

2. Place a single 5-μ thin tissue section on a plastic slide (*see* **Note 2**) using standard histology techniques. The plastic slides should be handled by their edges only.
3. A Sharpie marking pen is used to label the slides.
4. The slides (tissue-face-up) are place on a flat metal tray over small water drops. The water drops provide surface tension to prevent curling of the slides.
5. The slides are heated on the metal plate in a 90°C oven for 5–8 min. This baking melts the paraffin and firmly adheres the tissue to the slide. The slides should remain flat.
6. The slides are stained with conventional histologic hematoxylin and eosin reagents (**Table 1**). For some tissues that tend to fall off, each step may be shortened because only light staining is necessary.
7. The stained slides can be stored at room temperature. Coverslips are not used because they result in the loss of histologic detail. However, histologic features are usually adequate to distinguish between cells of different phenotypes.

3.2. SURF

1. The stained plastic slides are taped (transparent tape) onto glass slides so they can be moved by the microscope stage.
2. Areas of interest are identified by histologic microscopic criteria.
3. Ink dots are placed either manually or using a modified microdotter attached to a micromanipulator (*see* **Note 3** and **Fig. 2**). The dots are placed directly on the tissue. A "good" dot is thick enough to totally prevent the passage of light. Multiple small dots are placed to cover 100–400 cells (**Fig. 3**). Fewer cells can be covered but a larger number of cells prevents false allelic dropout and allows the use of less-robust PCR assays.
4. Photography allows the documentation of the tissues covered with each dot (*see* **Note 6**).
5. Approximately 10–20 areas per slide can be protected ("dotted") in 10–30 min.

3.3. After SURF

1. After the ink dots dry, the plastic sections, still attached to the glass slides, are placed face-down on a photocopier and then copied. Ideally the tissues are magnified by 200%. The photocopies allow a precise documentation of the location of each dot. Each dot is given an identifying number or letter. Note that at this point, only the back of the plastic slide must be protected against contamination because the front will be sterilized by UV radiation.
2. The plastic slides are removed from the glass slide and then placed directly (tissue-side-down) on a short-wave UV transilluminator. Both 254-nm and 302-nm wavelengths work fine. The time of illumination varies with the transilluminator and must be increased as the transilluminator ages (from 90 min for a new transilluminator to 3–4 h with an older one). The protection by the dots is almost complete so excess UV exposure does not appear to be a problem.
3. The slides are moved around every 20–30 min to ensure uniform exposure.
4. Afterwards, the plastic with attached allots are cut out with sterile scissors (2–4 mm squares) and placed directly into 0.5-mL microfuge tubes. In this way, there can be little doubt that the desired cells are indeed placed into the tube.

Table 1
Staining of Plastic Slides

Reagent	Interval	Purpose
Clear-Rite 3[a]	3 min	Deparaffinize
Ethanol 100%	6 dips	Wash
Ethanol 95%	6 dips	Wash
Water	6 dips	Wash
Hematoxylin	4 dips	Stain
Water	6 dips	Wash
0.5% Ammonium hydroxide	2 dips	Darken stain
Water	2 dips	Wash
Eosin	3 dips	Stain
Ethanol 95%	6 dips	Wash
Ethanol 100%	6 dips	Wash
Shake and air-dry	2 min	Dry

[a]Clear-Rite 3 (Richard-Allen Medical, Richland, MI). Each dip is approx 2–4 s.

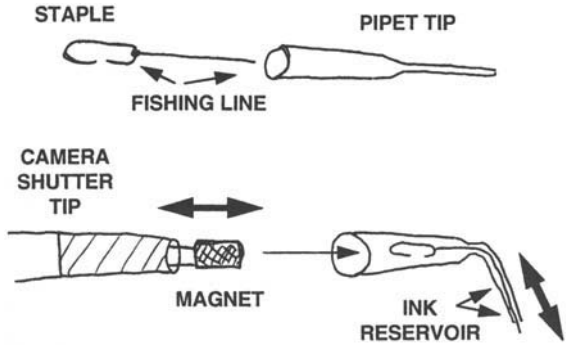


Fig. 2. A simple apparatus for placing protective dots. The bend in the pipet tip is induced with an autoclave. A micromanipulator is attached at the camera shutter tip to allow the precise placement of the dotter above the microscope slide.

5. The DNA is extracted (33 µL of 100 m*M* Tris-HCl, 2 m*M* EDTA, pH 8.0, with 0.7 µL of 20 mg/mL Proteinase K) overnight at 42°C or 4 h at 56°C.
6. The tubes are boiled for 5–7 min, vortexed, centrifuged, and stored at –20°C.
7. The dots should be scraped off with a pipet tip if they have not already fallen off to ensure complete extraction. If desired, the entire fraction including the ink dot and plastic can be subjected to PCR. Typically 8–10 µL are used in a 50 µL PCR so that multiple loci can be analyzed from the same dissected cells. PCR of many different targets (*see* **Note 7**) with appropriate controls is possible (*see* **Note 8**).

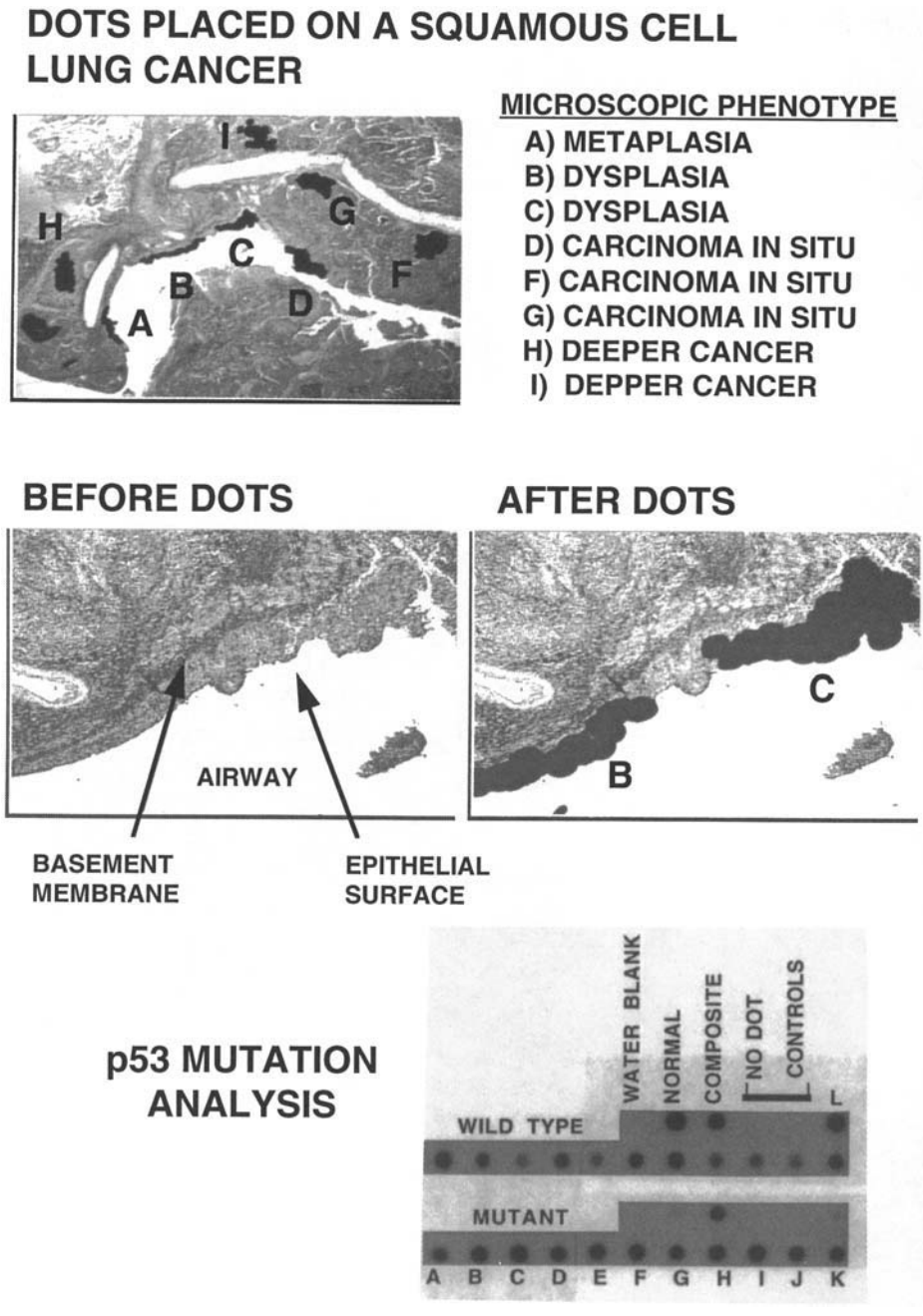


Fig. 3. Example of SURF on a squamous cell lung cancer ***(13)***. The p53 point mutation was detected using radiolabeled allelic specific hybridization probes in a dot blot format. The p53 mutation was present in metaplastic, dysplastic and throughout the tumor tissue, but was absent from normal tissue (L). Loss of heterozygosity is not evident in this example.

4. Notes

1. SURF is best performed on conventional formalin-fixed, paraffin-embedded tissue. Tissues are sometimes preserved with other fixatives. Fixatives that prevent subsequent PCR analysis are "B-5", Zenkers, Carnoy's, and Bouin's ***(9)***. Optimal fixatives are 10% buffered formalin (used almost universally for human tissues) and ethanol-based fixatives. Prolonged fixation (>5 d) in formalin degrades the DNA and the usual overnight fixation typically yields amplification in >90% of cases. Very old blocks paraffin blocks can be used ***(10)***.
2. Plastic slides have the optical properties of glass but the attached tissue and plastic can be cut by scissors. Specific isolation of tissue is greatly facilitated by this approach and the investigator can be confident that the desired tissues are indeed present in the desired tubes. Plastic slides are currently not commercially available. In art stores, "acetate" sheets can be purchased. The thickness should be about "10 mil" or 0.1 cm. These sheets are also called "desk protectors" in stationary stores. The sheets should be purchased flat and not extensively rolled.

   Using gloves, the sheets are cut into "microscope" slides with sterile scissors. These slides are dipped into a 0.1% poly-L-lysine adhesive solution (Catalog Number P 8920, Sigma Diagnostics, St. Louis, MO) for 5 min and air-dried. Conventional slide carriers that hold slides vertically (long dimension-up) appear to work best when dipping or staining plastic slides. The coated slides are stored at room temperature.
3. Dots can be place manually using a fine point Sharpie pen. However, a micromanipulator greatly facilitates placing the dots on the desired areas. The current setup in my laboratory consists of an inverted microscope and a video camera and recorder. The "dotter" is a custom designed camera shutter cord mounted on a simple micromanipulator (**Fig. 2**). The parts are:
   a. Stapled staple with 1 lb fishing line (monofilament) tied around it.
   b. Autoclaved gel loader pipet tip bent at about 60°.
   c. Flexible camera shutter cord with a small magnet at the tip.

   The tips (**Notes 1** and **2**) are autoclaved for 1 h in aluminum foil. The bend is induced when the tips are placed in the aluminum foil and becomes "permanent" after autoclaving. The sterile tips are placed on the camera shutter cord and the magnet provides a direct control link.

   After assembly, modifications to the pipette tip can be made with a sterile scissors. A Sharpie pen is broken open and its wick (covered with plastic) is squeezed to place a drop of ink onto a clean glass slide. The ink is usually too thin and is usually aged for 5–15 min, until the correct consistency is reached (depends on temperature and humidity, with differences present between pens). The dotter tip is placed into the ink pool and 0.2–0.4 cm (length) of ink is drawn up, by capillary action. With the dotter about 0.2 to 0.3 cm above the slide, the shutter is depressed to move the fishing line out of the ink reservoir and onto the slide (**Fig. 2**). The fishing line is flexible so that it is usually impossible to dislodge the tissue from the slide. Therefore, a single dotter can be used to dot multiple regions. The shape and consistency of the dots will change with time as the ink slowly dries (about 30–50 min).

4. Various UV protective ink umbrellas have been tried without success (many inks contain substances that inhibit subsequent PCR). The ink in Sharpie marking pens works well because it blocks UV radiation, produces sharp dots, and does not inhibit PCR.
5. An inverted microscope allows the direct visualization of the dotting process because one can observe the approach of the microdotter or ink pen towards the tissue section.
6. It is essential to document the site and phenotype of each dot. This can be done several ways. First, each dot is assigned an identification number. A photograph is taken before and after each dot to document the exact number and types of cells covered by the ink dot. A video camera with a digital frame grabber (made by various companies) is most convenient since many pictures are required for each slide. Second, the phenotype of each dot (normal, dysplastic, cancer, and so forth) is written down (**Fig. 3**).
7. Radioactive techniques and a large number of PCR cycles (36–48) are usually needed since the number of starting molecules is low. The PCR products from the fractions are analyzed by conventional techniques including dot-blot hybridization, restriction enzyme digestion, SSCP, direct sequencing, and electrophoresis (such as microsatellite size analysis) ***(11,12)***. Loss of heterozygosity studies are also possible but require careful attention to PCR conditions since many PCR cycles are necessary to detect the low numbers of starting molecules.

   Virtually any target can be analyzed with SURF and PCR. The biggest concern is the size of the PCR product. It must be short enough (<200 bp with <160 bp better) to be preserved in the fixed tissue but long enough (5100 bp) to be readily inactivated by UV radiation.
8. The following controls should be used: No dot control: An adjacent square of similar but unprotected tissue should also be isolated and analyzed by PCR. It should demonstrate no PCR products. If PCR products are detected with this negative control, a longer PCR target or greater exposure to the UV radiation is necessary. Duplicates: Each tissue section should be SURFed at least twice to verify the distribution of each mutation. Positive controls: If difficulty is encountered in getting detectable PCR products, the tissue not exposed to UV radiation should be amplified to verify that its DNA is intact. A shorter PCR target usually corrects this problem unless the tissue has been fixed in B-5, Bouin's, or Zenkers.

Acknowledgments

The author thanks the many talented students and technicians who have gone SURFing in my laboratory, including Zhi-Hua Li, Jenny Tsao, Qioping Shu, Susan Leong, and Bridgette Duggan, Antonio Hernandez, and Adrian Ireland.

References

1. Shibata, D., Hawes, D., Li, Z. H., Hernandez, A., Spruck, C. H., and Nichols, P. W. (1992) Specific genetic analysis of microscopic tissue after selective ultraviolet radiation fractionation and the polymerase chain reaction. *Am. Pathol.* **141**, 539–543.

2. Shibata, D. (1993) Selective ultraviolet radiation fractionation and polymerase chain reaction analysis of genetic alterations. *Am. J. Pathol.* **143,** 1523–1526.
3. Meltzer, S. J., Yin, J., Huang, Y., McDaniel, T. K., Newkirk, C., Iseri, O., Volgelstein, B., and Resau, J. H. (1991) Reduction to homozygosity involving p53 in esophageal cancers demonstrated by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **88,** 4976–4980.
4. Bianchi, A. B., Navone, N. M., and Conti, C. J. (1991) Detection of loss of heterozygosity in formalin-fixed paraffin-embedded tumor specimens by the polymerase chain reaction. *Am. J. Pathol.* **138,** 279–284.
5. Volgelstein. B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakumura, Y., White, R., Smits, A. M. M., and Bos, J. L. (1988) Genetic alterations during colorectal-tumor development. *N. Engl. J. Med.* **319,** 1525–1532.
6. Haase, A. T., Retzel, E. F., and Staskur, K. A. (1990) Amplification and detection of lentiviral DNA inside cells. *Proc. Natl. Acad. Sci. USA* **87,** 4971–4975.
7. Nuovo, G. J., MacConnell, P., Forde, A., and Delvenne, D. P. (1991) Detection of human papillomavirus DNA in formalin-fixed tissues by *in situ* hybridization after amplification by polymerase chain reaction. *Am. J. Pathol.* **139,** 847–854.
8. Hadano, S., Watanabe, M., Yokai, H., Kogi, M., Kondo, I., Tsuchiya, H., Kanazawa, I., Wakasa, K., and Ikeda, J. E. (1991) Laser microdissection and single unique primer PCR allow generation of regional chromosome DNA clones from a single human chromosome. *Genomics* **11,** 364–673.
9. Greer, C. E., Peterson, S. L., Kiviat, N. B., and Manos, O. M. (1991) PCR amplification from paraffin-embedded tissues. *Am. J. Clin. Pathol.* **95,** 117–124.
10. Shibata, D., Martin, W. J., and Arnheim, N. (1988) Analysis of DNA sequences in forty-year-old paraffin-embedded thin-tissue sections: a bridge between molecular biology and classical histology. *Cancer Res.* **48,** 4564–4566.
11. Shibata, D., Schaeffer, J., Li, Z. H., Capella, G., and Perucho, M. (1993) Genetic heterogeneity of the c-K-ras locus in colorectal adenomas but not adenocarcinomas. *J. Natl. Cancer Inst.* **85,** 1058–1063.
12. Shibata, D., Peinadoa, M. A., Ionov, Y., Malkhosyan, S., and Perucho, M. (1991) Genomic instability in simple repeat sequences is a very early somatic event in colorectal tumorigenesis that persists after transformation. *Nature Genetics* **6,** 273–281.
13. Zheng, J., Shu, Q., Li, Z. H., Tsao, J. L., Weiss, L. M., and Shibata, D. (1994) Patterns of p53 mutations in squamous cell carcinoma of the lung: early acquisition at a relatively early age. *Am. J. Pathol.* **145,** 1444–1449.

4

Prenatal and Presymptomatic Diagnosis of Marfan Syndrome Using Fluorescence PCR and an Automated Sequencer

Mei Wang and Maurice Godfrey

1. Introduction

The Marfan syndrome (MFS) is an autosomal dominant heritable connective tissue disorder characterized by variable and pleiotropic manifestations primarily in the skeletal, ocular, and cardiovascular systems ***(1)***. Molecular defects in fibrillin-1, a 350 kDa glycoprotein, encoded by the FBN1 gene located on chromosome 15 are now well documented in individuals and families with MFS (*see* **ref.** ***2*** for a review). The variability in phenotypic expression of MFS is significant not only between families, but also within a family, while the effective treatment of patients with MFS relies heavily on early and accurate diagnosis. It, therefore, seems likely that prenatal and presymptomatic molecular genetic analysis would prove a valuable adjunct to clinical diagnosis for purposes of risk assessment ***(3–5)***. Mutations have been found along the entire 10 kb coding region of fibrillin and in most of its 65 exons. In addition, most families appear to have unique mutations. Therefore, the routine use of mutation screening for prenatal and presymptomatic diagnosis is impractical in this disorder. We have used intragenic polymorphic markers in FBN-1 ***(4)*** to perform linkage analyses in MFS families. We have adapted a "one-step'' or "two-step" fluorescence PCR and automated sequencer to accurately, reproducibly, and rapidly perform prenatal or presymptomatic diagnosis of MFS in informative families.

2. Materials

1. DNA extracted from peripheral whole blood, cultured dermal fibroblasts, or chorionic villus sample. Extraction procedures such as those in **ref.** ***6*** are appropriate.

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

2. *Taq* DNA polymerase (5 U/μL).
3. 10 X PCR buffer: 200 m*M* Tris-HCl (pH 8.4), 500 m*M* KCl, and 50 m*M* $MgCl_2$ (Gibco-BRL).
4. Deoxynucleotides (1.25 m*M*) are prepared by diluting and mixing the concentrated 10 m*M* dATP, dCTP, dGTP, and dTTP purchased from USB.
5. The oligonucleotide primers (10 μ*M*) used for amplification of FBN1 microsatellite markers are (*see* **Note 1**):
   MtS1/forward: 5' CAA CAA AGA AGG AGA AAC AG 3'
   MtS1/reverse: 5' GAC AAT GTA TTC CAG AGG C 3'
   MtS2/forward: 5' GTA GTT GTT ATC TTG CAG A 3'
   MtS2 / reverse: 5' CTG CCC TCT AGG ACT CTA AG 3'
   MtS3/forward: 5' GAG TAC ATA GAG TGT l-l-l AGG G 3'
   MtS3/reverse: 5' CCT GGC TAC CAT TCA ACT CCC 3'
   MtS4/forward: 5' GAT GTC CCT ATT GCC ATC AC 3'
   MtS4/reverse: 5' CCT GTG CAG GGT AAG ACA AG 3'
   Generic MtS1/forward: 5' TGT AAA ACG ACG GCC AGT CAA CAA AGA AGG AGA AAC AG 3'
   Generic MtS2/forward: 5' TGT AAA ACG ACG GCC AGT GTA GTT GTT ATC TTG CAG A 3'
   Generic MtS3/forward: 5' TGT AAA ACG ACG GCC AGT GAG TAC ATA GAG TGT TTT AGG G 3'
   Generic MtS4/forward: 5'TGT AAA ACG ACG GCC AGT GAT GTC CCT ATT GCC ATC AC 3'
   Gene Label JOE (Green): 5' - JOE - TGT AAA ACG ACG GCC AGT 3'
   Gene Label FAM (Blue): 5' - FAM - TGT AAA AGG ACG GCC AGT 3'
   Gene Label TAMRA (Yellow): 5' - TAMRA - TGT AAA AGG ACG GCC AGT 3'
   Gene Label JOE, Gene Label FAM, and Gene Label TAMRA, which are used in the two step procedure, can be purchased from Applied Biosystem. MtS series forward primers, used in the one step method, were fluorescence labeled at the 5' end when synthesized.
6. Stock solution for polyacrylamide gel: 40% solution containing 38% acrylamide and 2% bisacrylamide. Store at 4°C.
7. Stock electrophoresis running buffer (10X TBE): 1.33 *M* Tris-HCl, pH 7.6, 0.45 *M* boric acid, 25 m*M* EDTA.
8. 10% Ammonium persulfate (prepare fresh stock weekly). Store at 4°C.
9. Sample loading buffer: 50 mm EDTA and 0.05% crystal violet.
10. Deionized formamide.
11. Gene Scan–500 TAMRA internal lane size standard purchased from Applied Biosystems.
12. DNA thermal cycler. We use the Perkin-Elmer 4800 model.
13. An automated sequencer. We use a model 373A manufactured by Applied Biosystems.

3. Methods

3.1. One-Step PCR Procedure (*Fig. 1*; see *Note 2*)

1. Add the following components to a sterile 0.5 mL microcentrifuge tube (*see* **Note 3**):

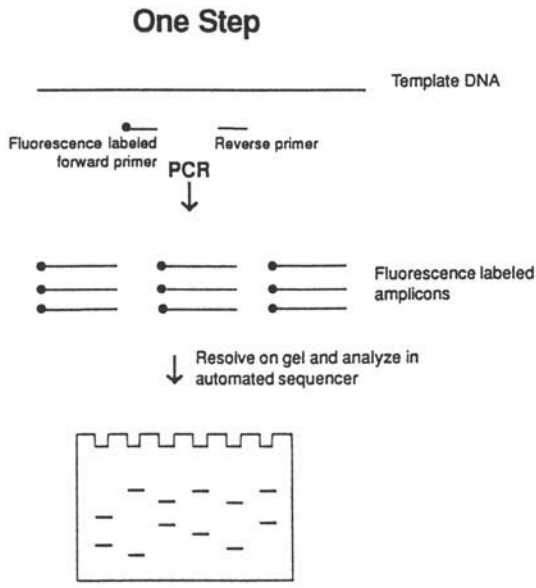


Fig. 1. Schematic diagram of the one step method (not drawn to scale).

Component	Volume	Final concentration
10X PCR buffer	2.5 μL	1X
1.25 m*M* dNTP mixture	4.0 μL	0.2 m*M*
50 m*M* $MgCl_2$	0.75 μL	1.5 m*M*
MtS forward primer (fluorescence labeled, 10 m*M*)	2.5 μL	1.0 μ*M*
MtS reverse primer (10 m*M*)	2.5 μL	1.0 μ*M*
Template DNA	1–2 μL (500 ng)	–
Autoclaved distilled water	21.75 μL	–

2. Mix contents of tube and overlay with 50 μL of mineral oil.
3. Cap tubes and microfuge briefly.
4. Incubate tubes in a thermocycler at 94°C for 5 min to completely denature the template.
5. Mix 0.25 μL of *Taq* DNA polymerase (5U/μL) with 3 μL of autoclaved distilled water for each reaction tube. Add 3.25 μL of mixture to each tube (*see* **Note 4**).
6. PCR amplification for 30 cycles is run as follows:

Denature	94°C for 1 min
Anneal	53°C for 2 min (MtS1, MtS2)
	58°C for 2 min (MtS3, MtS4)
Extend	72°C for 30 s

7. A final extension is performed for an additional 10 min at 72°C after which the reaction can be maintained at 4°C. Samples can be stored at –20°C in the dark until used.

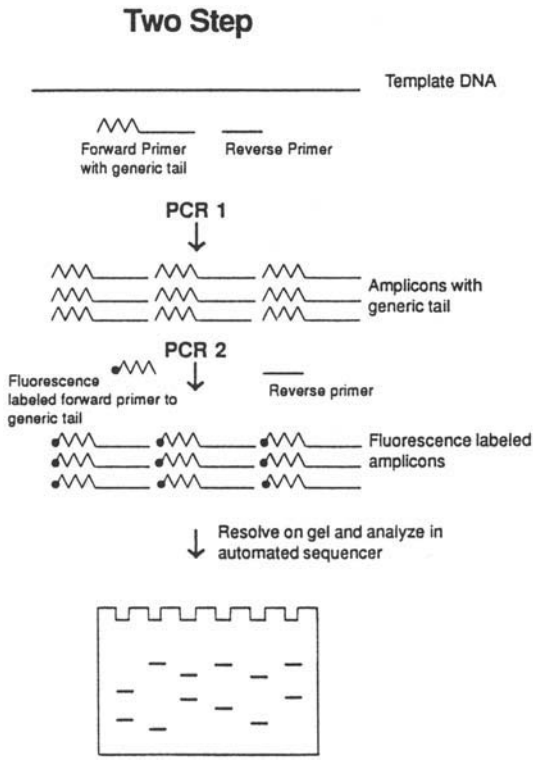


Fig. 2. Schematic diagram of the two step method (not drawn to scale).

3.2. Two-Step PCR Procedure (*Fig. 2*; **see** *Note 2*)

1. The first step PCR is set up exactly as described for the one step procedure except for the MtS forward primer used. In the two step method, the MtS forward primer has been synthesized with a generic oligonucleotide tail instead of a fluorescence label.
2. Following the first PCR, one microliter (1 μL) of the amplified material is removed and used as the template for the second step PCR. The second step PCR is also set up exactly as noted in the one step method. Here, however, the forward primer used is no longer specific to the MtS polymorphism. The forward primer is fluorescence labeled and its sequence is identical to that of the generic tail used in the first PCR. The same reverse primer is used in both amplifications in the two step method.

3.3. Analysis of Amplified Products

1. Gel preparation: A 6% polyacrylamide gel containing 8 *M* urea and 1X TBE should be prepared using 36-cm glass plates, spacers, and 24 well-forming combs, supplied by Applied Biosystems. The detailed instructions can be found in the User's Manual. Let the gel polymerize for 2 h after pouring the gel.

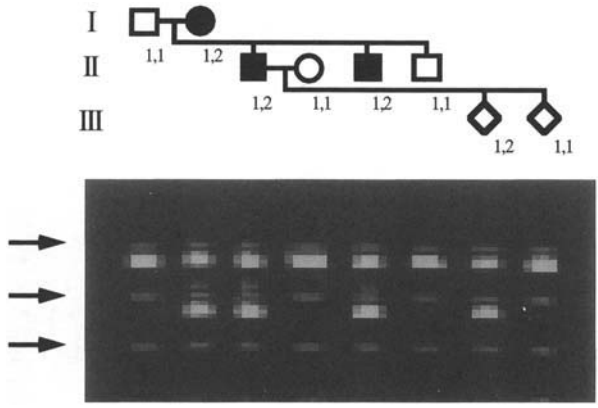


Fig. 3. Pedigree, fluorescence scan of labeled fibrillin MtS3 polymorphic marker, and MtS3 genotype in a family in whom prenatal diagnosis was performed on two separate occasions. The Marfan syndrome (MFS) cosegregates with the "2" allele in this family. Therefore, the fetus whose genotype was "1 2", was predicted to be affected, while the "1 1" fetus was predicted not to be affected with MFS. Molecular weight markers (arrows) are shown in each lane. Symbols: □ unaffected male; ■ affected male; ○ unaffected female; ● affected female; ◇ fetus.

2. Prerun the gel at 30 W constant power for 15–30 min before loading samples.
3. Sample preparation: Mix 1 μL of PCR product with a mixture of the following items: 3 μL of deionized formamide, 0.5 μL of Gene Scan–500 TAMRA internal lane size standard, and 0.5μL of sample loading buffer.
4. Sample loading: heat the samples at 90°C for 2 min and load the entire 5 μL volume.
5. Gel running: Fluorescence labeled PCR products are run at 2500 V, 30 W, for 14 h on a model 373A automated sequencer and analyzed by Gene Scan 672 Software (Applied Biosystems) (*see* **Fig. 3** for an example of linkage analysis using the MtS 3 polymorphism in a family for prenatal diagnosis.)

4. Notes

1. Primers can be purchased from a commercial source or synthesized by a core laboratory within an academic center.
2. There are advantages to both the one step and two step methods for automated linkage analysis using fluorescence labeled oligonucleotide primers. The one step method is certainly faster since only one amplification cycle is needed. We would recommend this method if one performs many repeat analyses of a fairly small number of markers. On the other hand, if a laboratory performs few analyses or uses numerous markers on an occasional basis, then the two step procedure is preferable. This is true because the shelf-life of the fluorescence labeled primer is only one to two years. Adding a fluorescence label to a primer increases its cost by about $50, however, a generic tail adds less than half that amount to the cost of synthesis.

3. If desired, a master mix can be prepared for the multiple PCR reactions. This helps to minimize reagent loss and to enable more accurate pipeting.
4. While adding *Taq* DNA polymerase to each PCR reaction tube, maintain the temperature at 80°C. This can be done by setting a cycle at 80°C between the "hot-start" (94°C for 5 min) and 30 cycles of PCR amplification.

Acknowledgments

This study was supported in part by a Clinical Research Grant 6-FY94-0012 from the March of Dimes Birth Defects Foundation and a grant from the National Heart, Lung, and Blood Institute of the National Institutes of Health, (HL48126-01) (M. G.). Maurice Godfrey is an Established Investigator of the American Heart Association.

References

1. Godfrey, M. (1993) The Marfan syndrome, in *McKusick's Heritable Disorders of Connective Tissue* (Beighton, P., ed.), C.V. Mosby, St. Louis, MO, pp. 51–135.
2. Dietz, H. C. and Pyeritz, R. E. (1995) Mutations in the human gene for fibrillin-1 (FBN1) in the Marfan syndrome and related disorders. *Human Mol. Genet.* **4,** 1799–1809.
3. Godfrey, M., Vandemark, N., Wang, M., Velinov, M., Wargowski, D., Tsipouras, P., Han, J., Becker, J., Robertson, W., Droste, S., and Rao, V. H. (1993) Prenatal diagnosis and a donor splice site mutation in fibrillin in a family with the Marfan syndrome. *Am. J. Hum. Genet.* **53,** 472–480.
4. Pereira, L., Levran, O., Ramirez, F., Lynch, J. R., Sykes, B., Pyeritz, R., and Dietz, H. C. (1994) A molecular approach to the stratification of cardiovascular risk in families with Marfan's syndrome. *N. Engl. J. Med.* **331,** 148–153.
5. Wang, M., Mata, J., Price, C. E., Iversen, P. L., and Godfrey, M. (1995) Prenatal and presymptomatic diagnosis of the Marfan syndrome using fluorescence PCR and an automated sequencer. *Prenatal Diagn.* **15,** 499–507.
6. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

5

Measurement of TNF and iNOS mRNA Using cDNA-Equalized Reverse Transcriptase PCR

Jay K. Kolls and Jianming Xie

1. Introduction

Since the polymerase chain reaction (PCR) for DNA amplification was first introduced in 1985 ***(1)***, the combination of reverse transcription with subsequent PCR amplification of the cDNA (RT-PCR) has been an increasingly utilized technique to analyze gene expression ***(2,3)***. In order for this procedure to be reasonably quantitative, however, appropriate controls must be applied to all steps, including the quantitation of the original RNA, the reverse transcription, and the PCR itself. Several investigators have published methods on quantitative RT-PCR that involve varying cDNA input into the PCR ***(3–7)***, varying cycle number ***(3,4,8,9)***, or the use of a competitive template as an internal standard ***(10,11)***. However, only a few of the competitive PCR methods take into account the efficency of the reverse transcription phase of RT-PCR ***(4,7,11)***, which may vary from 10–50% ***(12,13)***. In the former methods, it would also be necessary to amplify another control gene in parallel (e.g., actin) to control for RNA input and reverse transcription.

The efficiency of reverse transcription is more variable in small or mildly degraded RNA preparations that are otherwise ideal for analysis by RT-PCR. Since the existing PCR procedures require accurate quantitation of the input RNA, we have recently developed a method to quantitate gene expression for TNF and iNOS by RT-PCR in small clinical specimens, which accurately quantitates the cDNA resulting from first strand synthesis reactions from RNA samples that are too small for accurate RNA quantitation. It also compensates for variable efficiencies and lengths in the reverse transcription reaction. In this method, random primed cDNA is labeled with α-^{32}P d-CTP, an aliquot is electrophoresed on a polyacrylamide sequencing gel and quantitated using image analysis software. The samples are normalized based on the cDNA content prior to quantitative PCR. Since the cDNA is randomly primed,

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

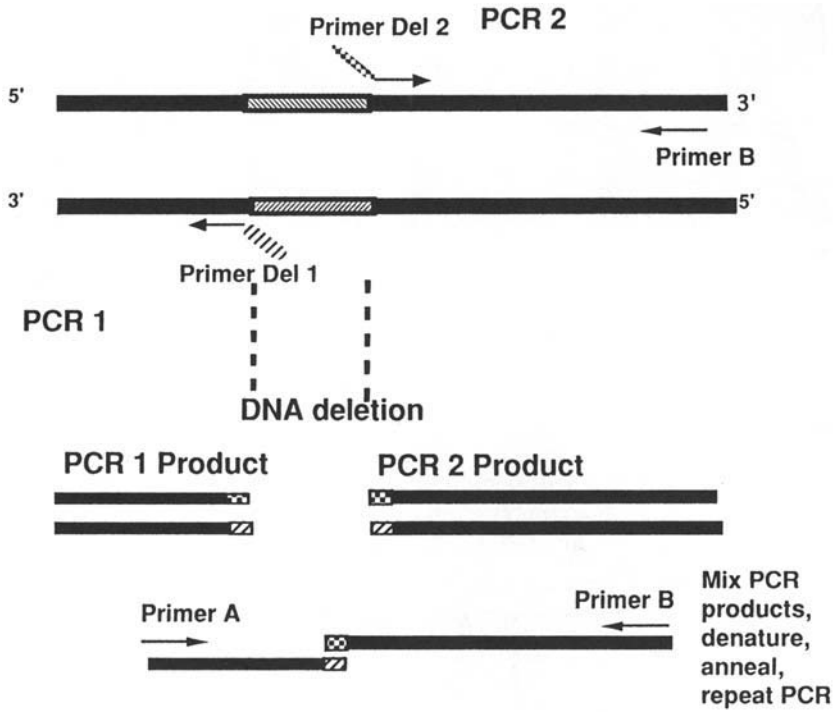


Fig. 1. This schematic demonstrates the generation of a competitive fragment using PCR-based methodology as described by Higuchi ***(18)***. Two separate PCR reactions are performed (labeled PCR1 and PCR2). PCR1 is performed with the 5' oligo and a deletion oligo which has 15 bp of homologous sequence on it's 3'-end and at least a 15 bp overhang which is homologous to the 5' end of Del2. PCR 2 is performed with Del 2 and Primer B. The two PCR products are checked on an agarose gel and then gel purified. The fragments are eluted from the gel slice, mixed, denatured, reannealed, and then undergo PCR again with Primer A and B to yield the PCR product with the appropriate deletion.

each RNA will be represented in the proportions originally present in the RNA sample. To validate this method, we varied input cDNA into the RT reaction and performed standard and competitive PCR for two cytokine mRNAs, murine tumor necrosis factor-α (TNF) and human tissue growth factor-β, (TGF-β_1) ***(13)***. We have subsequently developed an assay for iNOS ***(14–17)***. For both TNF and iNOS we generated competitive templates by using oligonucleotides and PCR to make small deletions in the PCR product (**Fig. 1**) ***(18)***. Thus, the PCR product of interest and the competitor can easily be separated by polyacrylamide electrophoresis. Using deletions to create a mutant competitor template has the theoretical problem of heterodimer formation between the mutant and wild-type PCR product ***(1)***.

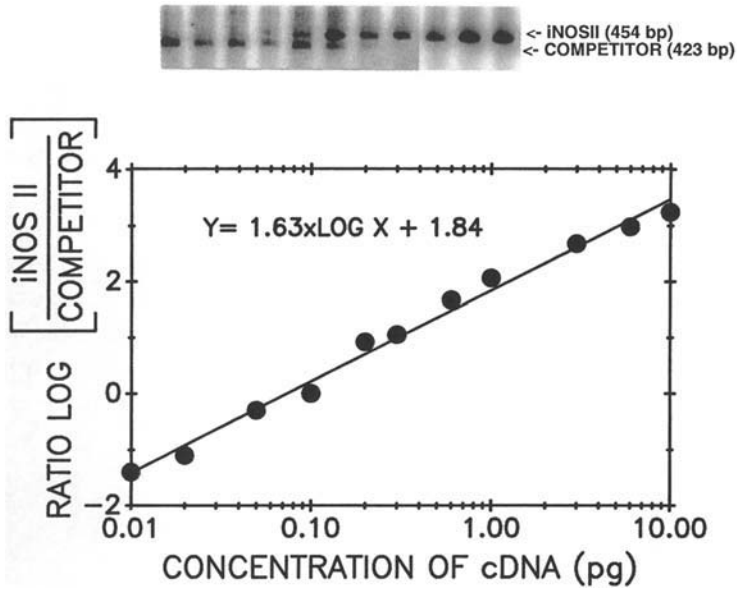


Fig. 2. **(Top)** A representative polyacrylamide gel of standard curve for iNOS created with competitive cERT-PCR. The top bands are signals from a series dilution of positive cDNA for iNOS (0.01–10 pg). The bottom band is the signal for the competitive fragment (0.03 pg added to each reaction). **(Bottom)** The ordinate is the ratio of iNOS vs competitor and the abscissa is the concentration of cDNA for iNOS. This particular concentration of competitor is linear over four orders of magnitude of cDNA concentrations.

However, with the two genes we have focused on, this has not been a problem in achieving linear standard curves over a large range of concentrations of input cDNA (**Fig. 2**) ***(19)***. For many genes of interest, there are now commercially available primer pairs as well as competitive fragments of which the latter share no internal homology to the desired PCR product and thus, reduce the chance of heterodimer formation.

In this chapter, we will outline the assay we call cERT-PCR (cDNA equalized reverse transcription-polymerase chain reaction) to quantitate both TNF and iNOS transcripts. We have applied this to both lavaged lung cells as well as peripheral blood cells and this assay is ideal for quantitating transcripts in small tissue samples.

2. Materials

2.1. Tissue and Cell Lines

Any tissue or cell line can be used as starting materials. For our studies, we used alveolar macrophages (AM) obtained by whole lung lavage from specific

pathogen-free Balb/c mice or Sprague-Dawley rats as previously described ***(13)***. Briefly, animals were given a lethal dose of pentobarbital (400 mg/kg) and exsanguinated by aortic transection. The trachea was exposed, opened, and cannulated with a polyethylene catheter. The lung was lavaged with warmed, calcium- and magnesium-free PBS (Gibco-BRL, Bethesda, MD) containing 0.6 m*M* EDTA in aliquots (0.5 mL for mice, 5 mL for rats) up to a total volume of 11 mL for mice and 30 mL for rats. Cells can be pelleted at 500*g* for 5 min at 4°C and washed twice with cold PBS. Cells can then be immediately frozen in liquid nitrogen for RNA isolation, resuspended in complete medium for culture, or extracted immediately for RNA. Cells are counted in a hemacytometer and viability is checked to be >98% as determined by trypan blue exclusion. Differential cell counts can be performed by cytospin preps.

2.2. RNA Isolation

1. Lysis buffer: Total cellular RNA is obtained by lysing the cells in RNazol (Biotecx Laboratories, Friendsville, TX) or Tri-zol (Gibco-BRL).
2. Chloroform.
3. 5 *M* NaCl or 3 *M* Na acetate (pH 5.2) for precipitation.
4. Absolute ehanol.

2.3. cDNA Synthesis Reagents

1. 10X PCR buffer: 500 m*M* KCl, 100 m*M* Tris-HCl, and 25 m*M* $MgCl_2$, pH 8.3.
2. DEPC-H_2O: 2 mL of diethylpyrocarbonate is added to 1 L of distilled, deionized water (ddH_2O), shaken, and allowed to sit at room temperature for 4 h. Residual DEPC is removed by autoclaving.
3. Random hexamers: Lyophilized random hexamers (pd$[N]_6$ Pharmacia, Uppsala, Sweden) are resuspended at 1 μg/mL concentration in DEPC-H_2O.
4. Deoxynucleotides: A 20 m*M* stock of dATP-dGTP-dTTP is made by diluting 100 m*M* stocks of individual nucleotides (Pharmacia) with DEPC-H_2O. For 100 μL, we add 20 μL each of dATP, dGTP, and dTTP and add 40 μL DEPC-H_2O.This is aliquoted and stored at –20°C.
5. Dithiothreitol 0.1 m*M* (Gibco-BRL), which is supplied with MMLV-RT (*see* **item 7**).
6. RNasin from Promega (Madison, WI).
7. Moloney-Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) from Gibco-BRL.
8. 0.06 m*M* dCTP: made from 100 m*M* stock of dCTP (Pharmacia) in DEPC-H_2O.
9. 3000 Ci/m*M* α-^{32}dCTP (Dupont NEN Research, Wilmington, DE).

2.4. cDNA Quantitation

1. 10X TBE: 108 g Tris base, 55 g boric acid, 9.3 g Na_2 EDTA·H_2O, in 1 L of ddH_2O, and autoclave. The pH should be 8.3.
2. 30% Stock acrylamide (19:1): 28.5 g acrylamide, 1.5 g *bis*-acrylamide. Add ddH_2O to 100 mL Filter through a 0.22 μm filter before use.

3. 6% acrylamide solution (denaturing): Combine 63 g of urea, 15 mL of 10X TBE, and 30 mL of 30% acrylamide stock. Bring volume up to 150 mL with ddH_2O, heat to dissolve and filter through a 0.22 μm filter. This solution is good for 3 mo if stored at 4°C in a light-protected bottle.
4. 25% ammonium persulfate: dissolve 2.5 g of ammonium persulfate in ddH_2O and adjust volume up to 10 mL Syringe filter through a 0.22 μm filter.
5. TEMED: available from many vendors.
6. Sample buffer: 10 mL formamide, 10 mg xylene cyanol, 10 mg bromphenol blue, 0.2 mL 0.5 *M* EDTA, pH 8.0. We use high-quality formamide (from Fluka) which does not require deionization.
7. Vertical gel apparatus, pipets, sequencing tips. A gel dryer is preferred, however gels can be exposed wet with adequate results.
8. Phosphorimaging system with image analysis software. At our institution, we utilize a core laboratory with a PhosphorImager (Molecular Dynamics, Mountainview, CA) that is networked to several laboratories, thus, scanned images can be downloaded directly to the investigator's computer for image analysis. Alternatively, it is possible to use autoradiography and densitometry to quantitate cDNA in the gel, but this will be slower and is subject to nonlinearity in the film response compared to the phosphor screen.

2.5. Polymerase Chain Reaction

1. 10X PCR buffer: *see* **Subheading 2.3.**
2. Deoxynucleotide triphosphates: 1.25 m*M* dNTPs are diluted from 100 m*M* stocks with ddH_2O. For 1 mL, we mix 12.5 μL of dATP, dGTP, dCTP, dTTP, and 940 μL of ddH_2O, aliquot and freeze at –20°C.
3. Primers: primer pairs are commercially available or can be synthesized by several different Biotech companies. We typically aliquot our primers at 30 pmol/μL in ddH_2O. If possible, we design primers that span an intron to allow discrimination of the RT-PCR product from contaminating genomic DNA. Primers for TNF and iNOS are listed in **Table 1**.
4. *Taq* DNA polymerase (5 U/μL) from Perkin-Elmer (Emeryville, CA).
5. 3000 Ci/m*M* α-^{32}dCTP (Dupont NEN Research, Wilmington, DE).
6. Thermocycler.
7. Mineral oil.

3. Methods

3.1. RNA Extraction

1. Total RNA from cells is isolated by RNazol B (Biotech Laboratories, Houston, TX) following the manufacturer's protocol.
2. If sufficient cells (more than 3–4 million cells) are available for the RT-PCR assay, the total RNA can be quantitated by using a spectrophotometer. Measure the OD reading at 260 nm and 280 nm wavelength. Calculate the concentration of RNA based on the following formula:

Table 1
Primers Used for Competitive TNF and iNOS mRNA Assay[a]

TNFA AAGTTCCCAAATGGCCTCCCTCTCATC	TNFB GGAGGTTGACTTTCTCCTGGTATGAGA
TNF del1 GGCTGGCACCACTAGTTTGCTCCTCCACTTGGTG	TNF del2 CACCAAGTGGAGGAGCAAACTAGTGGTGCCAGCC
iNOSA AATGGCAACATCAGGTCGGCCATCACT	iNOSB GCTGTGTGTCACAGAAGTCTCGAACTC
iNOSdel1 ATGCTCCATGGTCACCTCAGCTTGCAAGACCAGAGG	iNOSdel2 CCTCTGGTGTTGCAAGCTGAGGTGACCATGGAGCAT

[a]All sequences are written 5' to 3'

$$\text{RNA (mg/mL)} = (0.064\ A_{260} - 0.031\ A_{280}) \times \text{diluting factor} \qquad (1)$$

Then an equal amount of RNA from each sample can be used for the RT-PCR reaction. However, the following protocol can be used when the number of cells is limited (as few as 100,000 alveolar macrophage) or for clinical samples in which the quality of RNA may not be ideal. By random priming the cDNA in the presence of ^{32}P-dCTP, not only are the molecules present in the RNA sample represented in an equal proportion to that present in the original sample, but one can also quantitate the RNA at a much greater sensitivity (down to the picogram range) than by measuring RNA concentration using spectrophotometry. Thus, if this latter method is chosen, an equal amount of cDNA can be analyzed by PCR.

3.2. First-Strand cDNA Synthesis/RT Reaction

In this part of the protocol the amount of cDNA synthesized in the RT reaction is quantitated by labeling the cDNA by the addition of 1 μCi of ^{32}P-dCTP. The incorporation of label into appropriately sized cDNA is measured by first separating unincorporated from incorporated label by electrophoresing an aliquot of the RNA reaction on a denaturing polyacrylamide gel and scanning the gel on a Phosphorlmager (Molecular Dynamics). A known amount of cDNA (usually 10 ng/reaction) will then be placed into the PCR reaction.

1. Dissolve the RNA pellet with 20 μL DEPC-treated water. (It is sometime helpful to pretreat RNA sample at 95°C for 5 min and then quick-chill on ice. Presumably the heat treatment breaks up RNA aggregates and some secondary structures that may inhibit the priming step).
2. Make the mastermix for the RT reaction by using the above stock solutions. When preparing the master mix, one should prepare enough mastermix for the number of samples plus a water control and two more reactions. This ensures enough master mix for the reactions since there are some losses resulting from pipeting. The following formula is the mastermix for one sample:

Stocks	Volume	Final concentration
10X PCR buffer	4 μL	1X
Dithiothreitol (DTT, 100 m*M*)	0.4 μL	1 m*M*
dATP, dGTP, and dTTP (20 m*M*)	2 μL	1 m*M*
dCTP (1.25 m*M*)	1 μL	0.03 m*M*

Stocks	Volume	Final concentration
Random hexamer (1 μg/μL)	5 μL	0.125 mg/mL
RNase inhibitor (40 U/μL)	0.5 μL	0.5 U/μL
RTase (200 U/μL)	2 μL	10 U/μL
[^{32}P]-dCTP (10 μCi/μL)	0.1 μL	1 μCi/reaction
Deionized H_2O	5 μL	
Total volume	20 μL	

3. Add 20 μL of the mastermix to the tube containing 20 μL of RNA sample. Mix briefly. The above reaction conditions have the highest yield of cDNA when the total amount of RNA is in the range of 1–3 μg.
4. Incubate the tube at 37°C for 60 min.
5. To stop the reaction, heat the sample at 95°C for 3 min and then quick chill on ice.
6. While the RT reaction is incubating, a 6% denaturing polyacrylamide gel can be poured. First clean glass plates with 75% ethanol and air-dry. The plates can also be siliconized using Sigmacote (Sigma, St. Louis, MO) in a fume hood. Prepare the gel mix with 1 μL/mL 25% APS and TEMED and pour the gel following the gel manufacturer's instructions.
7. Mix 1.5 μL of the reaction with 1.5 μL of sample buffer and load on the 6% denaturing polyacrylamide gel. It is also helpful to run a labeled marker. We typically use a 1 kB ladder that has been labeled with γ-^{32}P ATP (>3000 Ci/m*M*) by T4 polynucleotide kinase. Run the gel at 10 V/cm and dry the gel. Gel drying is optional, wet polyacrylamide gels can be exposed for 24 h with little diffusion of signal. A portion (1.5 μL) of the water control reaction should also be spotted on a Whatman 3MM filter paper to determine the total radioactivity in the reaction. If desired, this standard can be run in duplicate and the counts averaged.
8. Expose the gel and standards overnight and scan the gel on a Phosphorlmager (Molecular Dynamics). A sample gel is pictured in **Fig. 3**.
9. Quantitate the density of each lane of the gel using image analysis software. We typically draw a rectangle from just below the top of the lane to just below the predicted size of the PCR product. If the cDNA is going to be used for several different PCR's with different size PCR products, we use the lowest size product to determine the lower position of the rectangle. This rectangle is copied and pasted over each sample and a final rectangle is drawn over the standards. Usually it is not necessary to subtract background since it is close to zero. The concentration of the cDNAs can be calculated by the following formula:

$$\text{Total cDNA (ng)} = (\text{counts of sample/counts of standard}) \times 1.0\ (\text{m}M) \times 330 \times 4 \quad (2)$$

(*see* **Note 1**)

10. A known amount of cDNA can be placed into the PCR reaction. For TNF and iNOS, 5–10 ng of cDNA gives a reproducible standard curve using competitive or noncompetitive PCR ***(13)***.

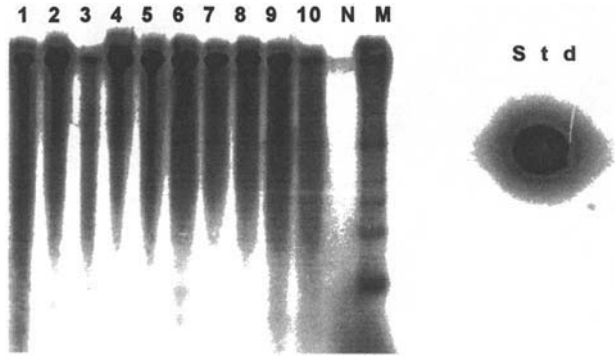


Fig. 3. A representative polyacrylamide gel of labeled cDNA derived from alveolar macrophages. Note that there is significant less cDNA in sample 3 than the rest of the samples. Abbreviations: N = water control and M = marker.

3.3. Creation of Competitive Templates

1. Depending on the expression of the gene of interest, varying the amount of input cDNA (as quantitated above) gives a linear relationship ($r = 0.96$) between PCR product and input cDNA. However, above 15 ng of input cDNA, PCR reactions without a competitor may plateau significantly. Thus, this plateau can be eliminated by the addition of a competitive template.
2. To serve as a competitive template for our murine TNF assay, a 46-bp deletion was created using the overlapping primer method described by Higuchi *(18)* (**Fig. 1**). PCR was performed using a PUC-9 plasmid encoding the cDNA for murine TNF-α (obtained from DrBruce Beutler) as a template with TNF-A and TNF del1, which yielded a 144-bp fragment and TNF-B and TNF del2, which yielded a 155-bp fragment (primer sequences are listed in **Table 1**).
3. These two fragments were gel purified and combined and denatured at 95°C for 5 min and than allowed to reanneal at room temperature.
4. PCR was then carried out with TNF-A and TNF-B and a 265-bp fragment with the resulting 46-bp deletion was obtained, gel purified and quantified by A_{260} and DNA fluorometry (Hoefer Scientific, San Francisco, CA) with good agreement.
5. For the gene of interest, it is necessary to choose deletion oligonucleotides with at least 15 bp of homologous DNA and at least 15 bp of 5' nonhomologous DNA to facilitate reannealing. One may also incorporate restriction enzyme sites in the 5' end to introduce new mutations in the competitive fragment. To validate the above competitor, a dilution series should be performed against the gene of interest as outlined in **Subheading 3.4.1.** (**Fig. 2**).

3.4. Polymerase Chain Reaction

3.4.1. Validation of Competitor

1. If noncompetitive PCR is chosen, a dilution series of cDNA should be subjected to PCR and quantitated to determine the linear range for the PCR conditions chosen

and for the amount of cDNA to be analyzed. The amount of cDNA should include at least two orders of magnitude such as 20, 10, 3, 1, 0.3, and 0.1 ng. Depending on the expression of the gene, lower concentrations may need to be added.

2. If a competitive assay is desired, a dilution series of competitor can also be run against a dilution series of cDNA in addition to the above reactions. Once an ideal amount of competitor is identified (typically 0.1–1 pg), the amount of competitor can be added to the mastermix and validated once more with PCR against a dilution series of cDNA. In this latter experiment, at least four orders of magnitude of cDNA should be assayed.

3.4.2. PCR Assay

Below is the competitive PCR assay we use for iNOS:

1. Aliquot 10 ng of cDNA. Add water, if necessary, to make a final volume of 25 μL. Pretreat the samples at 95°C for 5 min and maintain at 72°C while adding the mastermix.
2. Make the mastermix for PCR reactions by using the above stock solutions. Again set up enough master mix to run the samples plus a water control, a positive control and two additional samples. The following formula is the mastermix for one sample:

Stocks	Volume	Final concentration
10X PCR buffer	5 μL	1X
dNTP (1.25 m*M*)	8 μL	0.2 m*M*
Primer A (30 p*M*/μL)	0.5 μL	0.3 p*M*/μL
Primer B (30 p*M*/μL)	0.5 μL	0.3 p*M*/μL
Taq DNA polymerase (5 U/μL)	0.1 μL	0.5 U/reaction
[^{32}P]-dCTP (10 μCi/μL)	0.3 μL	3 μCi/reaction
Competitor (1 pg/μL)	1 μL	1 pg/reaction
Deionized H_2O	9.6 μL	
Total volume	25 μL	

3. Add 25 μL of mastermix to the tube containing 25 μL (10 ng) of cDNA sample.
4. Run 30 amplification cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min.
5. At the end of the reaction, extend the sample at 72°C for 10 min.
6. Load a 10 μL aliquot of the reaction on a 6% denaturing polyacrylamide gel. Electrophorese and dry the gel.
7. Expose the gel overnight and scan the gel on a Phosphorlmager (Molecular Dynamics).
8. Quantitate the iNOS and competitor counts using image analysis software from each lane of the gel. The amount of mRNA for iNOS can be calculated by the following formula:

$$\text{mRNA (pg/ng cDNA)} = (\text{density of iNOS/density of competitor}) \times \text{pg competitor/ng cDNA} \quad (3)$$

4. Notes

1. For the formula in **Subheading 3.2.**, 1.0 m*M* is the concentration of dNTP, 330 is the average molecular weight of nucleotide, 4 is the number of nucleotides in the cDNA fragments.
2. This protocol is somewhat labor intensive. However, the increased availability of commercial oligonucleotides and competitive templates for many genes of interest has made this technology more widely available. It is important to realize, however, that these kits often do not quantitate the cDNA synthesized nor do they validate the competitive fragment over a wide range of cDNA concentrations. More recently several labs have developed plasmid-based competitive fragment constructs ***(20,21)*** however, these competitors suffer from the same pitfall. That is, a standard curve should be run with cDNA derived from the tissue or cell line of interest to determine the range of cDNA concentrations in which the competitor competes in a linear fashion.
3. The author uses a dedicated set of pipetors for PCR as well as aerosol resistant tips.
4. A separate set of pipetors (non-PCR) are used for any positive control DNA fragments or plasmids.
5. A marker should be run with the cDNA synthesis reaction. The authors use 1 kB ladder (from Gibco-BRL) labeled with γ ^{32}P-ATP and T4 polynucleotide kinase.
6. The quality of the RNA is critical to the cDNA reaction. Routine procedures to prevent RNase contamination during the RNA isolation should be performed. All tubes and pipes tips should be sterile and RNase-free and gloves should be worn throughout the isolation procedure.
7. All reagents used in the master mixes can be made in bulk and stored in aliquots and frozen at –20°C. Then, if contamination becomes a problem the aliquots that are in current use can be discarded.

Acknowledgments

These studies were supported by a Parker B. Francis Pulmonary Research Fellowship award (J. K. K.) and PHS grants R29-M10384 (J. K. K.) R01-M09816 (J. X.) and the LSU Alcohol Research Center (M-09803). We would also like to thank Prescott Deininger for careful review of the manuscript.

References

1. Saiki, R. K., Scharf, S., Faloona, E., Mullis, K. B., Horn, G., Erlich, H. A., and Arnheim, N. (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230,** 1350–1354.
2. Murakawa, G. J., Zaia, J. A., Spallone, P. A., Stephens, D. A., Kaplan, B. E., Wallace, R. B., and Rossi, J. J. (1988) Direct detection of HIV-1 RNA from AIDS and ARC patient samples. *DNA* **7,** 287–295.
3. Terada, Y., Moriyama, T., Martin, B. M., Knepper, M. A., and Garcia-Perez, A. (1992) RT-PCR microlocalization of mRNA for guanylyl cyclase-copled ANF receptor in rat kidney. *Am. J. Physiol.*

4. Wang, A., Doyle, M. V., and Mark, D. F. (1989) Quantitation of mRNA by the polymaerase cahin reaction. *Proc. Natl. Acad. Sci. USA* **86,** 9717–9721.
5. Harbarth, P. and Vosberg, H. (1988) Enzymatic amplification of myosin heavy-chain mRNA sequences in vitro. *DNA* **7,** 297–306.
6. Rappolee, D. A., Wang, A., Mark, D., and Werb, Z. (1989) Novel method for studying mRNA phenotypes in single or small number of cells. *J. Cell. Biochem.* **39,** 1–11.
7. Murphy, L. D., Herzog, C. E., Rudick, J. B., Fojo, A. T., and Bates, S. E. (1990) Use of the polymerase chain reaction of mar-1 gene expression. *Biochemistry* **29,** 10,351–10,356.
8. Golde, T. E., Estus, S., Usiak, M., Younkin, L. H., and Younkin, S. G. (1990) Expression of β-amyloid protein precursor mRNAs: recognition of a novel alternatively spliced form and quantitation in Alzheimer's disease using PCR. *Neuron* **4,** 253–267.
9. Hoof, T., Riordan, J. R., and Tummler, B. (1991) Quantitation of mRNA by the kinetic polymerase chain reaction assay: a tool for monitoring P-glycoprotein gene expression. *Anal. Biochem.* **196,** 161–169.
10. Gilliand, G., Perrin, S., Blanchard, K., and Bunn, H. F. (1992)Analysis of cytokine mRNA and DNA: detection and quantitaion by competitive polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **87,** 2725–2729.
11. Becker-Andre, M. and Hahlbrock, K. (1989) Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR-aided transcript titration assay (PATTY). *Nucl. Acids. Res.* **17,** 9437–9446.
12. Gerard, G. F. (1987) Making effective use of cloned M-MLV reverse transcriptase. *Focus* **9,** 5,6.
13. Kolls, J., Deininger, P., Cohen, J. C., and Larson, J. (1993) cDNA equalization for reverse transcription-polymerase chain reaction quantitation. *Anal. Biochem.* **208,** 264–269.
14. Kolls, J., Xie, J., LeBlanc, R., Malinski, T., Nelson S., Summer, W., and Greenberg, S. S. (1994) Rapid induction of messenger RNA for nitric oxide synthase 11 in rat neutrophils in vivo by endotoxin and its suppression by prednisolone. *Proc. Soc. Exp. Biol. Med.* **205,** 220–229.
15. Spolarics, Z., Spitzer, J. J., Wang, J. F., Xie, J., Kolls, J., and Greenberg, S. (1993) Alcohol administration attenuates LPS-induced expression of inducible nitric oxide synthase in Kupffer and hepatic endothelial cells. *Biochem. Biophys. Res. Commun.* **197,** 606–611.
16. Greenberg, S. S., Xie, J., Kolls, J. K., Malinski, T., Nelson, S., and Summer, W. R. (1994) Ethanol decreases mRNA for nitric oxide synthase 11 in alveolar macrophages stimulated with interferon gamma and endotoxin. *Alcohol* **11,** 539–547.
17. Greenberg, S. S., Xie, J., Kolls, J. K., Mason, C., and Didier, P. (1994) Ethanol suppresses Mycobacteria tuberculosis-induced mRNA for nitric oxide synthase in alveolar macrophages, in vivo. *Alcohol Clin. Exp. Res.* **19,** 394–401.
18. Higuchi, R. (1989) Using PCR to engineer DNA, in *PCR Technology* (Erlich, H. A., ed.), Stockton, New York, pp. 61–70.

19. Xie, J., Kolls, J., Bagby, G., and Greenberg, S. S. (1995) Independent suppression of nitric oxide and TNF-alpha in the lung of conscious rats by ethanol. *FASEB. J.* **9,** 253–261.
20. Reiner, S. L., Zheng, S., Corry, D. B., and Locksley, R. M. (1993) Constructing polycompetitor cDNAs for quantitative PCR. *J. Immunol. Methods* **165,** 37–46.
21. Siegling, A., Lehmann, M., Platzer, C., Emmrich, F., and Yolk, H. (1994) A novel multispecific competitor fragment for quantitative PCR analysis of cytokine gene expression in rats. *J. Immunol. Methods* **177**, 23–28.

6

PCR Diagnosis of the Bovine Immunodeficiency-Like Virus

David L. Suarez and Cecelia A. Whetstone

1. Introduction

The bovine immunodeficiency-like virus (BIV), also known as the bovine lentivirus or the bovine immunodeficiency virus, causes a persistent viral infection of cattle, and is found in cattle populations around the world ***(1,2)***. A closely related lentivirus, Jembrana disease virus, causes an acute disease in *Bos javanicus* cattle, and while it also infects *Bos taurus* and *Bos indicus* cattle the infection is generally asymptomatic ***(3)***. Strong evidence has not been presented that links BIV to a specific cattle disease, but BIV has been associated with decreased milk yield and with a variety of disease conditions including clinical immunodeficiency, encephalitis, bovine paraplegic syndrome, skin infections, and emaciation ***(4–6)***.

Even though BIV was first isolated over 25 yr ago, our knowledge of its disease potential is limited. Several serological surveys have reported an estimated prevalence for BIV in different areas of the United States and other countries, but only a single epidemiological study examining milk yield in cattle with serological evidence of BIV infection has been reported ***(5)***. Relying solely on serological tests to diagnose BIV may be underestimating the true prevalence of BIV infection because, at least experimentally, the levels of antibody in cattle and sheep to the major gag protein, p26, can decrease to undetectable levels in animals persistently infected with BIV ***(7–10)***. The p26 gag protein is the most important antigen in the diagnosis of BIV in Western blot assays ***(11)***, and in some serological studies only recombinant *gag* antigens were used for the detection of BIV specific antibody ***(6,12)***. For reliable epidemiological data related to disease conditions, it is crucial to have a sensitive and specific diagnostic test, and current evidence suggests that the p26-

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

based serological tests may underdetect longterm infected animals. This underestimation of longterm infected animals may confound the relationships between BIV infection and disease, especially if BIV is similar to many other lentiviruses in that a long incubation period occurs before clinical disease becomes apparent. Further research into the nature of antibody loss to BIV is necessary and possibly a change in the types of antigens used in serological tests may be needed before serology can be a reliable diagnostic method.

Virus isolation is a routine laboratory test commonly used in the diagnosis of many viral pathogens. The isolation of BIV from field cases is very difficult, and only four successful isolations have been described. Each report used different cell types for isolation including fetal bovine spleen cells, fetal bovine lung cells, and embryonic rabbit epithelial cells. All four isolation procedures used cocultivation techniques of peripheral blood WBCs or mononuclear cells from cattle ***(2,13,14)***. BIV, specifically the R29-derived isolates, was permissive for a number of cell lines ***(15)***. Experimental inoculations with the Florida isolates also suggested a wide host cell range, since they infected a number of different cell types in peripheral blood samples as identified by PCR ***(16)***. The R29 isolate and the two Florida isolates required multiple blind passages of the cell cultures before evidence of CPE, including syncytia, became apparent. The patterns of CPE were different for R29 and the Florida isolates. Destruction of the cell sheet occurred sooner and larger syncytia with more nuclei were observed with the R29-derived isolates ***(13,14)***. A Costa Rican isolate, CR1, appeared to have similar culture characteristics as the R29 isolate, with CPE occurring earlier in cell culture and having syncytia with large numbers of nuclei ***(2)***. When FL491, FL112, and the R29-derived isolates were used in experimental inoculations, virus was consistently recovered from infected cattle at early times post infection. Over time it became more difficult to isolate BIV, with an increased number of blind passages required before CPE was observed ***(9)***. For the R29-1203 virus inoculated into calf 289, one blind passage at 7 d postinoculation (PI) was needed to isolate virus compared with three blind passages at 291 d PI. The Florida isolates inoculated into different cattle were isolated after three blind passages at 7 d PI, but required up to nine blind passages at 470 d PI ***(9)***. It is likely that this represents a decreased viral replication rate in the animal, but selection to a less tissue culture adapted virus in the host is also possible. It is not known why BIV from naturally infected animals is so difficult to culture, but our current methods for isolation of BIV are underdeveloped.

Diagnosis by PCR remains a promising method to identify BIV infected cattle, and five different PCR tests were described for use with cattle ***(9,17,18)***. The nested *pol* and *env* PCR tests were compared experimentally to the Western blot assay and virus isolation as potential diagnostic tests. In those studies,

the pol and env PCR tests appeared comparable in their ability to detect infected cattle, identifying infected cattle at 3 d PI, sooner than both virus isolation at 5 d and serological methods at 17 d PI. Virus isolation appeared to be a reliable diagnostic test in these experimental studies ***(9)***, but in naturally infected cattle virus isolation has been extremely difficult. The most important comparison was between the Western blot test and PCR test later in infection. Antibody levels to p26 declined to undetectable levels in one calf after only 6 mo PI, and to borderline detectable levels in another calf after 1.4 yr PI. In both cases, serological diagnosis became questionable in cattle that were consistently positive by two different nested PCR tests and virus isolation methods ***(9)*** In these experimentally infected cattle, the nested PCR tests were the only reliable tests for the diagnosis of BIV.

None of the PCR tests described have been widely used to diagnose naturally infected cattle, and in comparison studies large differences in sensitivity and specificity were apparent ***(19)***. For sensitivity issues, the PCR tests can be divided into extended cycle single-step PCR and nested two-step PCR tests. All tests target proviral DNA, and although the infection rate of cells in peripheral blood samples is not known, infected cells appear rare and the single-step PCR tests appear to suffer from poor sensitivity. The nested PCR tests had the best sensitivity with the samples tested. The DLS nested *env* PCR test appeared to have the best specificity, but because of the poor sensitivity of three of the other PCR tests, it was difficult to determine the true specificity of the different primer combinations ***(19)***. Three of the PCR tests were targeted to the *pol* gene region of the BIV genome, which is thought to be one of the most conserved regions of the viral genome. Unexpectedly these primer combinations appeared less specific than the nested *env* PCR test. Without a true "gold" standard for comparisons of the different PCR results, caution must be used in interpreting these comparison results.

2. Materials

*2.1. DNA Extraction-Method 1: Commercial Salt Precipitation (***see** *Note 1)*

1. EDTA blood collection tubes.
2. Puregene DNA Extraction Kit (Gentra Systems, Minneapolis, MN).
3. Isopropanol.
4. 70% Ethanol.

2.2. DNA Extraction-Method 2: SDS/Proteinase K Digestion-Phenol/Chloroform Extraction

1. EDTA blood collection tubes.
2. 2X Ca^{2+} and Mg^{2+} free Hank's solution: 1.6 g NaCl, 0.08 g KCl, 0.02 g KPO_4 monobasic, 0.254 g $NaHCO_3$, 0.02 g Na_2HPO_4, 0.4 g glucose; to 100 mL with sterile distilled water.

3. TNE: 10 m*M* Tris-HCl, 1.0 m*M* EDTA, 100 m*M* NaCl, pH adjusted to 7.6 with 1% sodium dodecyl sulfate (SDS).
4. Proteinase K (100 μg/mL).
5. 25:24:1 Phenol/chloroform/isoamyl alcohol.
6. 24:1 Chloroform/isoamyl alcohol.
7. 7.5 *M* Ammonium acetate.
8. 95% Ethanol.
9. 70% Ethanol.

2.3. PCR Amplification for All Methods

1. 10X PCR buffer: 100 m*M* Tris-HCl, 15 m*M* $MgCl_2$, and 500 m*M* KCl (Boehringer Mannheim, Indianapolis, IN).
2. 10 m*M* stocks of deoxynucleotides dATP, dTTP, dCTP, and dGTP (Boehringer Mannheim). The stock solutions are diluted to 1.0 m*M* for working stocks. All four dNTPs can be combined in a single tube with 4 μL being used in each 50 μL PCR reaction.
3. 25 m*M* $MgCl_2$. Additional $MgCl_2$ needs to be added to the PCR reaction to bring the final Mg concentration to 3.0 m*M*. The 10X PCR buffer listed already contains 1.5 m*M* Mg and an additional 3 μL needs to be added in a 50 μL PCR reaction to obtain the proper concentration.
4. Oligonucleotide primers (**Table 1**) are diluted in molecular biology grade (MBG) water; 20 pmol of each primer are used.

3. Methods

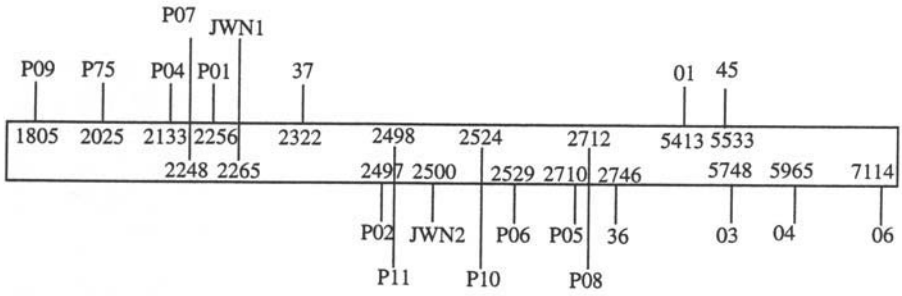
3.1. DNA Extraction Method 1

1. Collect blood into EDTA tubes.
2. Use 1 mL of whole blood with Puregene DNA extraction kit according to kit instructions.
3. Determine DNA concentration.

3.2. DNA Extraction Method 2

1. Collect 10 mL of blood, centrifuge at 1000*g* for 20 min. Collect buffy coat layer by pipeting and place into a 15 mL conical centrifuge tube (*see* **Note 2** for tissue sample specimens).
2. Lyse red blood cells with 6 mL sterile distilled water and mix. Immediately add 6 mL of 2X Hank's solution. Centrifuge tube at 1000*g* for 5–10 min. Pour off supernatant. Repeat step one or two more times or until pellet is white.
3. Add 1 to 2 mL of TNE with 1% SDS and vortex.
4. Add 1 mL of proteinase K per 10 mL of solution. Vortex (gently) and incubate in 37°C water bath for 3 h to overnight. Mix occasionally.
5. Add equal amounts of phenol/chloroform/isoamyl alcohol to sample. Vortex for 10 s and centrifuge at 2500*g* for 3–5 min (*see* **Note 3**). Remove the top aqueous layer with a Pasteur pipet, avoiding the interface, and place in new microcentrifuge tube (*see* **Note 4**).

Table 1
Nucleotide Positions of the 5' Nucleotide of the pol and env Primers Relative to the BIV R29-127 Proviral Genome



Pol Primers		Env Primers	
Primer 36	CATTTCTTGGGTGTGAGCTC	Primer 01	TCTATGGTATCTCTGGCTGC
Primer 37	CCTTACCCTCCAGGAATTAA	Primer 45	ATCAATAACGGTGAGATCCA
Primer P01	ATGCTAATGGATTTTAGGGA	Primer 03	GTTCCTGCCATAGTGAGAGT
Primer P02	CATCCTTGTGGTAGAACATT	Primer 04	CTATGGATCAGGACCTAGAC
Primer P03	ACACTGGAGCAGATGAGGTA	Primer 06	GCCAGCACAAGCAGGAATAT
Primer P04	CAGGCTCTTAAGGAAATTGT		
Primer P05	ACTCTCTCTTCCTGGACCTT		
Primer P06	TGGTAGTCTGATAAATGGCA	Actin Primers	
Primer P07	GATGGAGGATGCTAATGGAT	Actin 1	AGAAGCTGTGCTACGTCGC
Primer P08	TCACTCTCTCTTCCTGGACCT	Actin 2	CCAGACAGCACTGTGTTGG
Primer P09	CACTGGAGCAGATGAGGTAGT		
Primer P10	GTCTGATARATGGCAGGRCT		
Primer P11	CCATCCTTGTGGTAGAACATT		
Primer 75	ACTTGCTTCACCCTACTTGT		
Primer JWN1	GATTTTAGGGAATTAAATAA		
Primer JWN2	ACCCATCCTTGTGGTAGAAC		

[a]Positive sense primers are located on the top strand and negative sense primers are located on the bottom strand.

6. Add equal amounts of chloroform/isoamyl alcohol to sample and vortex for 10 s. Centrifuge at 2500*g* for 3–5 min. Remove the aqueous layer and place in new microcentrifuge tube.
7. Precipitate DNA with the addition of 0.5 vol of ammonium acetate, and 2.5 vol of 95% ethanol and gently invert tube multiple times. Store sample at –20°C for 30 min to overnight. Centrifuge sample at 8000*g* for 30 min.
8. Gently pour off supernatant. Invert tube and air dry for 15 min. Resuspend sample in 200–400 µL of MBG water and transfer to clean tube.
9. Determine DNA concentration of sample.

3.3. *Nested* env *PCR*

1. For the first step of the nested PCR reaction, add 5 µL 10X PCR buffer, 4 µL dNTPS, 3 µL $MgCl_2$, 20 pmol of primers 04 and 06 (at 20 p*M*/µL add 1 µL),

1.25 U (0.25 μL) of *Taq* polymerase, 0.5 μg sample DNA (*see* **Note 5**) and q.s to 50 μL total volume.
2. The reactants are cycled for 1 cycle at 94°C for 2 min, 51°C for 15 s, and 72°C for 2 min, followed by 30 cycles of 94°C for 45 s, 51°C for 15 s, and 72°C for 1 min, with a final extension step of 72°C for 10 min.
3. For the second step of the nested PCR reaction, add in a separate tube 5 μL 10X PCR buffer, 4 μL dNTPS , 3 μL $MgCl_2$, 20 pmol of primers 01 and 45 (at 20 p*M*/μL add 1 μL), 1.25 U (0.25 μL) of *Taq* polymerase, 34 mL of MBG H_2O, and 2 μL of the product from the first step PCR reaction (*see* **Note 6**).
4. The reactants are cycled for 1 cycle at 94°C for 2 min, 61°C for 15 s, and 72°C for 2 min, followed by 30 cycles of 94°C for 45 s, 61°C for 15 s, and 72°C for 1 min, with a final extension step of 72°C for 10 min.
5. An oligonucleotide probe, 03, was used to confirm some of the env PCR product as being BIV specific using a chemiluminescent (ECL, Amersham, Arlington Heights, IL) Southern blot hybridization detection system of the PCR product (*see* **Note 7**).
6. Negative PCR controls using no DNA in the amplification reaction were used in all PCR runs.
7. PCR negative samples may also be screened with actin primers to assure the samples fitness for amplification (*see* **Note 8**).

3.4. Original Nested pol PCR

1. Original nested *pol* PCR used primers P01 and 36 for the first step and primers 37 and P02 (**Table 1**) for the second step using cycling and reaction conditions as described above for the *env* PCR.

3.5. Improved Nested pol PCR

1. The recommended BIV nested *pol* PCR test includes primers P06 and P09 in the first step of the nested PCR test and primers P11 and P04 in the second step reaction. Alternative primers and primer combinations are listed in **Table 2**. Several of these primer combinations provide improved specificity and are therefore recommended over the original nested PCR procedure. (For a discussion of how these new primer combinations were evaluated *see* **Note 9.**)
2. Cycling conditions are the same for the first step reaction for all *pol* PCR primer combinations tested, but the second step PCR used a lower annealing temperature of 51°C for 15 s, instead of 61°C for 15 s.

3.6. Primer Design

1. All primers should have the general characteristics of balanced GC/AT ratios, complementary melting temperatures, an absence of inherent secondary structure, and little primer self annealing.
2. Select primers from conserved regions (*see* **Note 10**).
3. Select primers so that the 3' nucleotide is in the first or second codon position in the proper reading frame (*see* **Note 11**).

Table 2
Nested *pol* Primer Combinations

First step primers	Second step primers	Isolates identified
P06, P09	P11, P04	5[a]
P06, P09	P11, P07	5[a]
P06, P09	P11, 37	5[a]
P04, P06	P07,P02	4[b]
P04, P06	P07, P11	4[b]
P04, P06	P07, JWN2	4[b]
P04, P06	P01, JWN2	4[b]
P04, P06	JWN1, JWN2	4[b]
P04, P06	JWN1, P01	4[b]
P09, P08, 36	P10, P07	4[b]
P09, P08, 36	P02, P04	4[b]
P09, P08, 36	JWN1, JWN2	4[b]
P09, P10, P06, P04	P02, 37	5[a]
P09, P10, P06, P04	P02, P07	5[a]

[a]Isolates R29-289, FL491-1268, FL112-1275, OK94, and 846.
[b] Isolates R29-289, FL491-1268, FL112-1275, and OK94.

4. Notes

1. Two alternative methods of extraction of DNA for PCR amplification are provided for use with blood samples. The main advantages to using the Puregene system is the decrease in the use of hazardous chemicals, specifically phenol. The Puregene kit can also reduce processing time and reagent costs for the preparation of the DNA. However, for the extraction of DNA from tissue samples obtained by biopsy or necropsy, the SDS/proteinase K digestion followed by phenol/chloroform extraction is recommended because it provides better digestion of the sample that frees more DNA for extraction.
2. For tissue samples, mince a small piece of tissue with sterile scissors and proceed to TNE/SDS step. Digestion times with proteinase K need to be increased over WBC samples. Overnight digestion or longer is the norm.
3. The digested sample may be up to 2 mL in volume. I routinely use only 0.5 mL so that the procedure can be performed in a 1.5 mL microcentrifuge tube. The remaining digested solution can be stored long-term at –20°C for later extractions if necessary.
4. The sample is placed in a clean microcentrifuge tubes at multiple points in the extraction process to help reduce the carryover of the chemicals used for extraction.
5. The amount of DNA that is used per PCR reaction can be changed. The standard protocol uses 0.5 μg of DNA, however 1 μg, and rarely 2 μg can improve the results. BIV, from all indications, does not replicate to high levels in cattle and especially after long-term infection the number of infected cells is small. This is the reason why a nested PCR test is the preferred method for PCR diagnosis, and the additional DNA is occasionally necessary to amplify BIV from clinical samples.

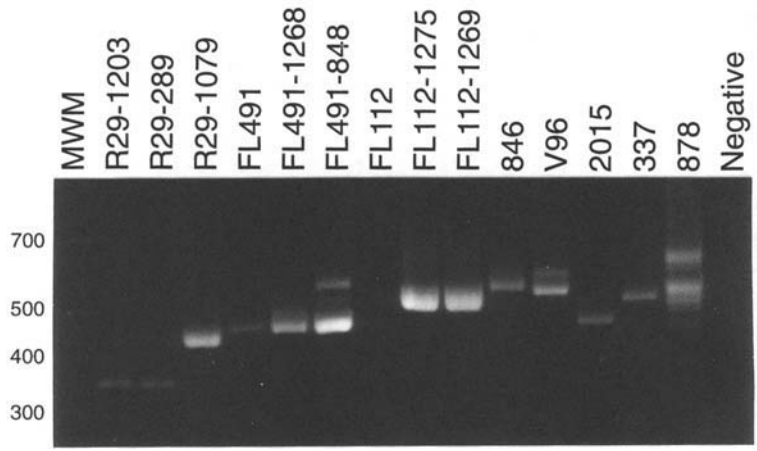


Fig. 1. PCR amplification of the surface *env* gene from DNA samples from cell culture, R29-1203, FL112, and FL491, and from BIV-infected and BIV-suspect cattle. The PCR product varies greatly in size, highlighting the size variability found in the BIV SU gene. MWM = Molecular weight marker.

6. The most likely source of false positive reactions caused by contamination from carryover of PCR product will occur when adding the 2 μL of product from the first PCR reaction to the second tube for the second round of amplification. To reduce the possibility of contamination, place all the PCR components for the second step PCR reaction, except for the 2 μL of DNA from the first step, in a tube in the same flow hood used for setting up the first reaction. Then move these samples to another flow hood and transfer the 2 μL of template. This procedure allows all stock reagents to be kept away from any amplified DNA samples. Other standard practices to reduce contamination, e.g., clean gloves and filtered pipet tips, are also used.
7. The product of the nested env PCR shows size variation of up to 200 bp between some isolates (**Fig. 1**). Primers 45 and 01 span the second hypervariable gene region which was shown to have high sequence divergence as well as size variation ***(20)***. The primers are located in conserved areas around this hypervariable region that allows amplification of the product. The quasispecies phenomenon was evident in several samples that had multiple DNA bands representing different genotypes of the virus isolate. All the bands hybridized to the oligonucleotide probe 03 and were BIV specific (data not shown).
8. A serious concern in the reliability of using PCR as a routine diagnostic test is false-negative results. While false-negatives can be caused by several reasons, one common reason is having nonspecific inhibitors in your template. These inhibitors can range from incomplete removal of phenol or ethanol during the extraction steps, having excessively high salt concentrations, or other unidentified inhibitors. The use of primers targeted to cellular genes reduces the number of unaccounted for false-negatives. Amplification of the DNA sample in a sepa-

rate reaction with the actin gene primers using the same buffer, $MgCl_2$, and dNTPS provides assurance that the sample is suitable for PCR amplification.

9. Previous sequence comparisons have provided evidence that the reverse transcriptase *pol* gene is more conserved than the SU *env* gene ***(20)***. However, the overall increased sequence conservation has not translated into greater specificity using the different *pol* PCR tests. The nested *pol* PCR appears to have equal sensitivity to the nested *env* PCR test, but was unable, in comparison studies, to identify several white blood cell (WBC) samples that were positive by *env* PCR ***(19)***. Different primers were selected to try to enhance the nested *pol* PCR test to improve specificity (**Fig. 2**). A total of 29 different primer combinations were tried with a small panel of WBC samples that were PCR positive with another test (**Table 2**). Four of these samples were known to be BIV based on sequencing, including R29-289, FL112-1275, FL491-1268, and OK 94. Some of the other samples had serological evidence to support that they were from BIV positive cattle. Several different primer combinations had basically equivalent specificity, identifying 5 of the 8 BIV isolates examined (**Table 3**). Most of the remaining combinations of primers amplified only 4 of the 8 samples. Several combinations of four and five different *pol* primers for the first step reaction provided better results, but this improved specificity was later duplicated by using the appropriate two primers. Although specificity was improved with several new primer combinations, the nested *pol* primers could not identify all of the samples that were detected with nested *env* PCR test. It remains a vexing problem why the nested *pol* PCR cannot be adapted to have at least equal specificity of the nested *env* PCR.
10. Most RNA viruses have regions of their genomes that can tolerate variation and areas that remain highly conserved. Ideally, the sequences from a number of diverse isolates can be compared to determine these variable and conserved regions and primers can be selected from the conserved regions. However, with BIV only two infectious molecular clones from the R29 isolate have been completely sequenced. Sequence data from parts of the *pol* and *env* gene are available from several other BIV isolates, and enough sequence homology exists in the *pol* gene between BIV and the Jembrana disease virus for useful sequence comparisons to be made ***(3,20,21)***. However, for most regions of the BIV genome other factors need to be used to select regions from which primers are selected. For example to predict conserved areas in the *env* gene, antigenic indexes were calculated from sequence information from R29-127, and the *env* primers 01, 06, and 45 were selected in areas predicted to be poorly antigenic.

    It is assumed that conserved areas of the *env* gene are preserved because they are important for the structure of the protein or they are in an active site of the protein. In vitro evidence of the function of the tat protein exists in the region where the *tat* gene overlaps, in another reading frame, the 5' end of the SU gene where primer 04 is located. In vitro experimentation has documented 8 aa in this overlap region that are critical for the RNA binding function of the tat protein ***(22)***. The in vitro data are supported by comparing the aa sequence of different *tat* genes from a number of different BIV isolates including the related Jembrana disease virus (**Fig. 3**) ***(3,20,21)***. Some nucleotide variation occurs in this region, but with the aa important in RNA binding, only synony-

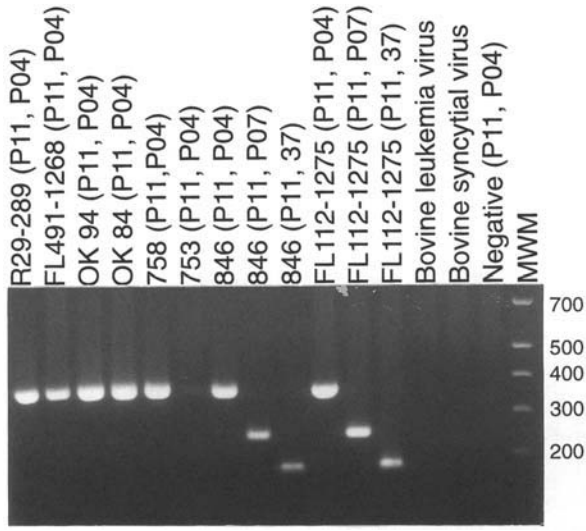


Fig. 2. PCR amplification of the reverse transcriptase *pol* gene from DNA samples from BIV-infected and BIV-suspect cattle. Different nested primer combinations were used with some of the samples. The first step primers for these reactions are P06 and P09, and the second step reactions primers vary and are indicated in parentheses.

Table 3
BIV Samples Used for *pol* PCR Screening

Isolate	Source	Evidence of BIV infection
R29-289	Louisiana dairy cow	Cultured isolate, sequence data
FL491-1268	Florida dairy cow	Cultured isolate, sequence data
FL491-1275	Florida dairy cow	Cultured isolate, sequence data
OK-94	Oklahoma cow	Sequence data, serology
846	Louisiana dairy cow	Serology, *env* PCR (+)
ND1	North Dakota cow	*env* PCR (+)
849	Florida dairy calf	*gag* PCR (+)
147	Michigan dairy cow	*gag* PCR (+)

mous changes occur in the *tat* gene, with the same changes causing nonsynonymous changes in the SU gene. Some aa that are less critical in RNA binding have several nonsynonymous changes with the same changes causing synonymous changes in the SU gene. This provides evidence why this area of the SU gene is conserved, since important segments of overlapping genes are present.

11. When using *Taq* polymerase in the PCR process, the 3' nucleotide is the most important nucleotide for binding to the template allowing successful amplification. Mis-

Peptide[a]	SGPRP**RGTRGK**G**RRI**RR
R29-Derived[b]	SGPRPRGTRGKGRRIRR T---ASGGDQRREADS
FL491, FL112	SGSRPRGTRGRGRKIRR TPTPDSGGPRQREANH
JDV[c]	GRRKKRGTRGKGRKIHY ARSITESGGQRAPNCA
OK14[d],OK02[d],OK20[d]	*****RGTRGRGRKIRR TPNPASRGHRTRQANH
OK 40[d]	*****RRTRGRGRKIRR TPNPASRGHRTRQANH

a Chen, L. and A.D. Frankel, 1995
b R29-127, R29-106, R29-1079, R29-1203, R29-289, R29-1078, R29-Davis
c Jembrana Disease Virus
d Clones from OK 94 isolate

Fig. 3. Amino acid sequence comparison of a region of the BIV *tat* gene. Experimental evidence for critical aa residues were conducted with a 17 aa peptide in RNA binding studies, and eight (bold) aa were found to be important ***(6)***. These same aa were highly conserved when many BIV isolates and the closely related Jembrana disease virus are compared, providing further support for the conserved nature of these aa. The 04 *env* primer overlaps this conserved region and it helps explain why this primer has broad specificity. Sequence homology deteriorates shortly downstream of this conserved region. Symbols: *** (No sequence data available); — (Gaps in alignment).

Primer 45

5'	aca	tgg	ATC	AAT	AAC	GGT	GAG	ATC	CAt	agt	tgg	cag 3'
3'	tgt	acc	tag	tta	ttg	cca	ctc	tag	gta	tca	acc	gtc 5'
aa	T	W	I	N	N	G	E	I	**H**	S	W	Q

Primer 01

5'	ata	ttg	gca	gcc	aga	gat	acc	ata	gat	tgg	tgg	ctc 3'
3'	tat	aac	CGT	CGG	TCT	CTA	TGG	TAT	CTa	acc	acc	gag 5'
aa	I	L	**A**	A	R	D	T	I	D	W	W	L

Fig. 4. The PCR primers were designed to have their 3' nucleotide end in the first or second position of the codon in the proper reading frame. Primer 45 (capital letters) is a positive sense primer and the 3' nucleotide is in the second position in the histidine (bold) codon. Primer 01 (capital letters) is a negative sense primer and its 3' nucleotide is in the first position of the alanine (bold) codon. aa = amino acid.

matches at the 3' nucleotide position can greatly decrease the efficiency of the annealing process ***(23–25)***. One strategy for decreasing a mismatch at the 3' nucleotide is to have the primer's 3' nucleotide be in the first or second amino acid (aa) codon position (**Fig. 4**) ***(9)***. By avoiding the wobble codon position, there is less chance of a synonymous codon change affecting the results. This strategy can be expanded so that the aa coded for at the 3' end is one with a lower probability of accepting mutations. However, trying to incorporate all these criteria into selecting a primer may not be possible and compromises are often necessary. The primers in this study were selected so that their 3' nucleotide was not in the wobble position (**Fig. 4**).

Acknowledgments

The authors thank Janice Miller, Martin Van Der Maaten, and Nicole Gueniat for providing samples used in the PCR analysis; and J. R. Emmanuelli for technical support. This work was supported by the Agricultural Research Service CRIS 3630-32000-001-00D.

References

1. Suarez, D. L., Whetstone, C. A., Miller, J. M., and Van Der Maaten, M. J. (1994) Bovine lentivirus (BIV): diagnosis, prevalence and pathogenesis. Proc. 98th Annual Meeting of the United States Animal Health Association, pp. 33–42.
2. Hidalgo, G., Flores, M., and Bonilla, J. A. (1995) Detection and isolation of bovine immunodeficiency-like virus (BIV) in dairy herds of Costa Rica. *J. Vet. Med.* **B 42,** 155–161.
3. Chadwick, B. J., Coelen, R. J., Wilcox, G. E., Sammels, L. M., and Kertayadnya, G. (1995) Nucleotide sequence analysis of Jembrana disease virus: a bovine lentivirus associated with an acute disease syndrome. *J. Gen. Virol.* **76,** 1637–1650.
4. Gonda, M. A., Luther, D. G., Fong, S. E., and Tobin, G. J. (1994) Bovine immunodeficiency virus: molecular biology and virus-host interactions. *Virus Res.* **32,** 155–181.
5. McNab, W. B., Jacobs, R. M., and Smith, H. E. (1994) A serological survey for bovine immunodeficiency-like virus in Ontario cattle and associations between test results, production records and management practices. *Can. J. Vet. Res.* **58,** 36–41.
6. Walder, R., Kalvatchev, Z., Tobin, G. J., Barrios, M. N., Garzaro, D. J., and Gonda, M. A. (1995) Possible role of bovine immunodeficiency virus in bovine paraplegic syndrome: evidence from immunological, virological and seroprevalence studies. *Res. Virol.* **146,** 313–323.
7. Isaacson, J. A., Roth, J. A., Wood, C., and Carpenter, S. (1995) Loss of Gag-specific antibody reactivity in cattle experimentally infected with bovine immunodeficiency-like virus. *Viral Immunology* **8,** 27–36.
8. Smith, H. E., Jacobs, R. M., and Mallard, B. (1994) Cell mediated and humoral immunity in sheep exposed to bovine immunodeficiency-like virus. *Comp. Immun. Microbiol. Infect. Dis.* **17,** 29–39.
9. Suarez, D. L., Van Der Maaten, M. J., and Whetstone, C. A. (1995) Improved early and long-term detection of bovine lentivirus by a nested polymerase chain reaction test in experimentally infected calves. *Amer. J. Vet. Res.* **56,** 579–586.
10. Whetstone, C. A., Van Der Maaten, M. J., and Black, J. W. (1990) Humoral immune response to the bovine immunodeficiency-like virus in experimentally and naturally infected cattle. *J. Virol.* **64,** 3557–3561.
11. Van Der Maaten, M. J. and Whetstone, C. A. (1990) Bovine lentivirus. 94th Annual Meeting of the United States Animal Health Assoc. pp. 81–88.
12. St. Cyr Coats, K., Pruett, S. B., Nash, J. W., and Cooper, C. (1994) Bovine immunodeficiency virus: incidence of infection in Mississippi dairy cattle. *Vet. Micro.* **42,** 181–189.
13. Suarez, D. L., Van Der Maaten, M. J., Wood, C., and Whetstone, C. A. (1993) Isolation and characterization of new wild-type isolates of bovine lentivirus. *J. Virol.* **67,** 5051–5055.

14. Van Der Maaten, M. J., Boothe, A. D., and Seger, C. L. (1972) Isolation of a virus from cattle with persistent lymphocytosis. *J. Natl. Cancer Inst.* **49,** 1649–1657.
15. Gonda, M. A., Braun, M. J., Carter, S. G., Kost, T. A., et al. (1987) Characterization and molecular cloning of a bovine lentivirus related to human immunodeficiency virus. *Nature* **320,** 388–389.
16. Whetstone, C. A., Suarez, D. L., Miller, J. M., Pesch, B. A., and Harp, J. A. (1997) Bovine lentivirus (BIV) induces early transient B cell proliferation in experimentally inoculated cattle and appears to be pantropic. *J. Virol.* **71,** 640–644.
17. Nadin-Davis, S. A., Chang, S. C., Smith, H., and Jacobs, R. M. (1993) Detection of bovine immunodeficiency-like virus by the polymerase chain reaction. *J. Virol. Meth.* **42,** 323–336.
18. Nash, J. W., Hanson, L. A., and St. Cyr Coats, K. (1995) Detection of bovine immunodeficiency virus in blood and milk-derived leukocytes by use of polymerase chain reaction. *Amer. J. Vet. Res.* **56**, 445–449.
19. Suarez, D. L. and Whetstone, C. A. (1997) Comparison of different PCR tests to detect bovine lentivirus in cell culture and experimentally and naturally infected cattle. *J. Vet. Diagn Invest.* **9,** in press.
20. Suarez, D. L. and Whetstone, C. A. (1995) Identification of hypervariable and conserved regions in the surface envelope gene in the bovine lentivirus. *Virology* **212,** 728–733.
21. Garvey, K. J., Oberste, M. S., Elser, J. E., Braun, M. J., and Gonda, M. A. (1990) Nucleotide sequence and genome organization of biologically active proviruses of the bovine immunodeficiency-like virus. *Virology* **175,** 391–409.
22. Chen, L. and Frankel, A. D. (1995) A peptide interaction in the major groove of RNA resembles protein interactions in the minor groove. *Proc. Natl. Acad. Sci. USA* **92,** 5077–5081.
23. Huang, M., Arnheim, N., and Goodman, M. (1992) Extension of base mispairs by *Taq* polymerase: implications for single nucleotide discrimination in PCR. *Nucleic Acid Res.* **20,** 4567–4573.
24. Kwok, S., Kellogg, D. E., McKinney, D., Spasic, D., Goda, L., and Sninsky, J. J. (1990) Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. *Nucleic Acid Res.* **18,** 999–1005.
25. Kwok, S., Chang, S., Sninsky, J. J., and Wang, A. (1994) A guide to the use of mismatched and generate primers. *PCR Meth. App.* **3,** S39–S47.

7

PCR Analysis for the Identification of Porcine Reproductive and Respiratory Syndrome Virus in Boar Semen

Jane Christopher-Hennings and Eric A. Nelson

1. Introduction

In 1987, a disease syndrome characterized by reproductive and respiratory symptoms in pigs was first described in the United States ***(1)***. Late-term abortions, increased numbers of stillborn and weak pigs, poor conception in breeding herds, along with respiratory distress and high mortality in suckling, weaned, and grow-finish pigs was observed ***(2,3)***. Similar outbreaks were also described in Europe in 1990 ***(4)***. In 1991, the etiologic agent was identified as an enveloped, single-stranded RNA (ssRNA) virus and tentatively classified in the Arteriviridae family, which includes lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus ***(2,5,6)***. The porcine reproductive and respiratory syndrome virus (PRRSV) or Lelystad virus (the European PRRSV isolate) has a genome comprised of seven open reading frames (ORFs). ORFs 1a and 1b; ORFs 2-6 and ORF 7 are predicted to encode for the RNA polymerase, viral membrane associated proteins, and nucleocapsid, respectively ***(7)***.

The primary route of PRRSV transmission is thought to be through direct contact with infected pigs because PRRSV has been isolated from secretions such as saliva, urine, feces, and semen. Airborne transmission of PRRSV has been implicated in epidemiologic studies, but has been difficult to reproduce experimentally ***(8)***. Transmission of PRRSV through semen is increasing in importance as the swine industry becomes more reliant on artificial insemination to introduce new genetic information and minimize new animal introduction into high health status herds. Boar semen may be sent to several recipient herds so that a PRRSV contaminated semen sample may be widely dissemi-

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

nated. Experimental studies have shown transmission of PRRSV from infected boar semen via artificial insemination ***(9,10)***.

Identification of viruses in semen by conventional methods, such as virus isolation, is difficult because of the cytotoxicity of semen samples. Therefore, a "swine bioassay" was developed to detect PRRSV. In this assay, 13–15 mL of semen was injected intraperitoneally into a 4- to 8-wk-old pig, which was then monitored weekly for seroconversion to PRRSV ***(11)***. If seroconversion occurred, this would indicate that the semen contained PRRSV. However, this method was time-consuming, laborious, and expensive. The polymerase chain reaction (PCR) had been previously used in identifying other viruses such as human immunodeficiency virus ***(12)***, bovine herpesvirus 1 ***(13,14)*** and equine arteritis virus ***(15)*** in human, bovine, and equine semen, respectively. Therefore, we adapted PCR for the identification of PRRSV in boar semen.

Boar semen samples previously tested for the presence of PRRSV by the "swine bioassay" were used to evaluate the PCR assay ***(16)***. Three important steps were necessary to obtain reliable results when PCR was used for detection of PRRSV RNA in boar semen. First, it was necessary to use low-speed centrifugation to obtain the cell fraction (sperm and nonsperm cells) from semen for PCR analysis. When seminal plasma or whole semen was used, PCR results failed to consistently correlate with the "swine bioassay." Since 150–500 mL of boar semen can be obtained from a single collection, a method of concentrating the virus-rich fraction of semen by centrifugation was necessary to identify PRRSV. Preliminary experiments using immunohistochemistry and vasectomized boars have determined that PRRSV is present in nonsperm cells. Secondly, it was important to use at least 6 mL of whole semen for centrifugation. By using a larger amount of whole semen, a higher nonsperm cell concentration was obtained. The final step was to use a lysis buffer without a reducing agent, such as 2-mercaptoethanol, since reducing agents can cause chromatin decondensation of sperm DNA ***(17)*** and may lead to false-negative PCR reactions ***(13,14)***.

Using these three steps, along with nested PCR methodology, we found that 63 of 67 semen samples correlated with the "swine bioassay" ***(16)***. Four samples were found to contain PRRSV by PCR, but not by the "swine bioassay." This may indicate a greater detection sensitivity with PCR. Alternatively, the "swine bioassay" is designed to detect replicating virus, whereas PCR detects a portion of the viral genome, which is not necessarily indicative of replicating virus. However, it is unlikely that ssRNA, which is susceptible to enzymes present in semen, would remain intact without the protection of exterior viral proteins. Therefore, we concluded that in most cases, the detection of PRRSV RNA in boar semen by PCR is indicative of infectious virus.

Subsequent to the development of this PCR assay, the usefulness of PCR to detect PRRSV in boar semen became apparent. We conducted experiments

that detected PRRSV in semen from boars given a modified-live PRRSV vaccine ***(18)***; identified shedding of PRRSV in semen from boars naturally exposed to PRRSV in field outbreaks; and performed a 5 mo study that illustrated the relationships between viremia, serostatus, and PRRSV shedding in semen, as well as identified the persistent shedding of PRRSV in semen through 92 d postinoculation ***(19)***.

2. Materials

2.1. Primers and Probe

The outer sense and antisense primers from ORF 1b of the Lelystad virus were 5'-CCGTCACCAGTGTGTCCAA-3' (nucleotides 8752–8770) and 5'-CCGTTCTGAAACCCAGCAT-3' (nucleotides 9003 to 8985), respectively. The seminested sense and antisense primers were 5'-ACATGGTATTGTCGGCCTT-3' (nucleotides 8803–8821) and 5'-CGTTCTGAAACCCAGCATC-3' (nucleotides 9002–8984), respectively. The primers and probe from ORF 7 were designed in our laboratory and derived from the ATCC VR-2332 sequence (a U.S. PRRSV isolate) ***(20)***. The outer sense and antisense primers were 5'-TCGTGTTGGGTGGCAGAAAAGC-3' (nucleotides 2763–2784) and 5'-GCCATTCACCACACATTCTTCC-3' (nucleotides 3247–3226), respectively. The nested sense and antisense primers were 5'-CCAGATGCTGGG TAAGATCATC-3' (nucleotides 2885–2906) and 5'-CAGTGTAACTTA TCCTCCCTGA-3' (nucleotides 3120–3099), respectively. The internal probe was also derived from ORF 7 of the VR-2332 isolate and was 5'-TGTCAGACATCACTTTACCC-3' (nucleotides 3002–3021).

2.2. RNA Extraction Materials

1. Lysis Buffer: 4 *M* guanidine thiocyanate, 25 m*M* sodium citrate (pH 7.0), 0.5% *N*-lauryl-sarcosine.
2. Ultra-pure Tris-saturated phenol.
3. Chloroform-isoamyl alcohol 24:1.
4. 2 *M* sodium acetate, pH 4.0.
5. Cold ethanol, 95%.
6. Ethanol, 70%.
7. Bottled distilled water (Gibco-BRL).
8. Siliconized polypropylene 1.5-mL microcentrifuge tubes.
9. Aerosol-resistant pipet tips.

2.3. Reverse Transcriptase (RT), Outer PCR, and Nested PCR Materials

1. GeneAmp RNA PCR kit (Perkin Elmer). Stock solutions include: 25 m*M* $MgCl_2$; 10X PCR buffer (500 m*M* KCl, 100 m*M* Tris-HCl, pH 8.3); 10 m*M* each dNTP (dATP, dCTP, dGTP, dTTP); 20 U/μL RNase inhibitor; 50 U/μL reverse transcriptase; and 5 U/μL Ampli*Taq* DNA polymerase.

2. Oil-free microcentrifuge tubes.
3. Thermal Cycler.

2.4. PCR Product Detection Materials

1. Submarine Gel apparatus (gel-PRO, Curtin Matheson Scientific) with power source.
2. FMS SemKem GTG agarose.
3. 1 mg/mL stock solution of ethidium bromide.
4. Tris acetate buffer (TAE): 4.84 g Tris ultrapure base, 1.14 mL glacial acetic acid, 2 mL 0.5 *M* EDTA, pH 8.0, q.s. 1 L distilled water.
5. DNA 100-bp ladder (Gibco-BRL).
6. Agarose gel sample buffer (Type IV), 6X stock solution: 0.25% bromphenol blue, 40% (w/v) sucrose in water, stored at 4°C ***(21)***.
7. UV illuminator.
8. Photography equipment.

3. Methods

3.1. Extraction Methods

1. Ten mL of whole semen is centrifuged at 600*g* for 20 min ***(22)***.
2. Five hundred µL of lysis buffer is then added to an equal volume of cell fraction and mixed by repetitive pipeting.
3. The suspension from **step 2** (500–700 µL) is added to an equal volume of phenol and chloroform isoamyl alcohol (24:1) (e.g., 250 µL ultrapure Tris-saturated phenol and 250 µL chloroform-isoamyl alcohol) in a siliconized 1.5 mL microcentrifuge tube.
4. This mixture is vortexed and centrifuged 5 min at 10,000*g* in a microcentrifuge at room temperature.
5. The upper phase is removed and **steps 3** and **4** are repeated.
6. The aqueous phase is transferred to a new tube containing 500 µL of chloroform-isoamyl alcohol (24:1) and vortexed.
7. This mixture is then centrifuged at 10,000*g* for 5 min at room temperature.
8. The volume of the aqueous phase is estimated and a 1/3 vol of 2 *M* sodium acetate (pH 4.0) is added. Then 2 vol of 95% cold ethanol is added and vortexed.
9. The RNA is precipitated at –70°C for 1 h.
10. Tubes are then centrifuged for 30 min at 16,000*g* at 4°C.
11. To wash the precipitate, the supernatant is carefully discarded and 100 µL of 70% ethanol is added to each tube.
12. Tubes are then centrifuged at 16,000*g* for 5 min at 4°C.
13. **Steps 11** and **12** are repeated.
14. The supernatant is discarded and the pellet air dried for 5–10 min.
15. The pellet is then resuspended in 30 µL of bottled distilled water.

3.2. RT, Outer PCR, and Nested PCR Procedures

Each GeneAmp PCR kit is used so that a master mix for each of 10 PCR assays is in an aliquot (**Table 1**).

Table 1
Reverse Transcriptase RT-PCR Master Mix for 10 PCR Reactions and Thermal Cycler Protocols

Reverse transcriptase	Outer PCR reaction	Nested PCR reaction
(1)[a]		
40 μL $MgCl_2$	40 μL $MgCl_2$	60 μL $MgCl_2$
20 μL 10X PCR buffer	80 μL 10X PCR buffer	80 μL 10X PCR buffer
10 μL sterile water	665 μL sterile water	195 μL sterile water
20 μL of each dNTP		
10 μL RNase inhibitor		
(2)		
10 μL outer antisense primer	20 μL outer sense primer	30 μL sense nested primer
	5 μL *Taq* polymerase	30 μL antisense nested primer
10 μL reverse transcriptase		5 μL *Taq* polymerase
18 μL of the above master mix is added to 2 μL of extracted RNA (20 μL total volume)	80 μL of the above master mix is added to each tube that contains the RT reaction (100 μL total volume)	48 μL of the above master mix is added to a new tube along with 2 μL of the outer PCR product (50 μL total volume)
Thermal cycler protocol	Thermal cycler protocol	Thermal cycler protocol
42°C for 15 min	95°C for 25 s	95°C for 25 s
99°C for 5 min	58°C for 5 s	58°C for 5 s
5°C for 5 min	74°C for 25 s	74°C for 25 s
1 cycle	40 cycles	30 cycles

[a]*See* **Note 1** for discussion of table.

3.3. PCR Product Detection

1. A 1.5% SeaKem agarose gel is made by mixing 0.45 g SeaKem GTG with 30 mL of TAE buffer and heated to boiling in a microwave until dissolved.
2. After boiling, ethidium bromide (15 μL of a 1 mg/mL ethidium bromide stock solution) is added to the agarose, mixed, and the agarose is poured into the tray of a submarine gel apparatus.
3. A 10-well comb is placed in the gel before the gel solidifies and removed after solidification.
4. TAE buffer, 400 mL, is poured into the gel apparatus so that the gel is covered prior to electrophoresis.
5. From each nested PCR reaction 9 μL is mixed with 2 μL of 1X sample buffer and loaded into a single well per sample.

6. A portion of the DNA ladder (0.5 μL) is mixed with 7 μL of 1X sample buffer and loaded into an individual well as a reference marker.
7. The power source is them connected to the gel apparatus and set at 85 V for approx 1 h 15 min.
8. The gel is then removed from the gel apparatus and viewed with UV illumination.

3.4. PCR Basepair Products

1. For samples containing the PRRSV in which ORF 1b primers were used, a 252-bp outer and 200-bp nested PCR product were obtained.
2. For samples containing the PRRSV in which ORF 7 primers were used, a 484-bp outer and 236-bp nested PCR product were obtained.

4. Notes

1. **Table 1:** All reactions are performed in oil-free microcentrifuge tubes and only sterile bottled water (Gibco-BRL) is used. Reagents listed in (1) are combined and frozen at –20°C prior to RT/PCR amplification. Reagents listed in (2) are added to reagents in (1) immediately prior to RT/PCR amplification. All reagents are kept on ice. Primers are stored as a 40 μ*M* stock solution.
2. Quality control is of utmost importance when nested PCR reaction products are obtained because false-positive reactions may easily occur.
   a. To minimize the potential for "carry-over" from one sample to another, 10 samples are used for PCR at one time. This includes eight semen samples, and a positive and a negative control sample.
   b. Four separate rooms are used for RNA extraction, outer PCR, nested PCR, and agarose gel detection.
   c. Lab coats and gloves are worn at all times and gloves are changed often.
   d. Aerosol-resistant pipet tips and dedicated pipets were used for each step of the procedure.
3. The sensitivity of the PCR can be determined with a 10-fold dilution series of the VR-2332 PRRSV isolate. As few as 10 virions (1 log unit of virus) per milliliter could be detected with the ORF 7 nested primers. Use of ORF 7 outer primers alone requires 10^5 virions (5 log units of virus) per milliliter for detection. Other arteriviruses as well as six other viruses do not react with either ORF 1b or ORF 7 primers. To date, all US PRRSV field isolates tested react with primers from both ORF 1b and 7. However, the Lelystad virus can only be detected with primers, that were derived from ORF 1b and not with ORF 7 primers that were derived from the US VR-2332 PRRSV isolate.
4. A cDNA probe is derived from the VR-2332 PRRSV isolate (*see* **Subheading 2.1.**). This probe is end-labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase as previously described ***(16)***. This probe is not routinely used for diagnostic PCR testing because the nested PCR reaction gives adequate sensitivity.

References

1. Keffaber, K. K. (1989) Reproductive failure of unknown etiology. *AASP Newsletter* **2,** 1–10.

2. Wensvoort, G., Terpstra, C., Pol, J. M. A., der Laak, E. A., Bloemrad, M., deKluyer, E. P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F., Broekhuijsen, J. M., Moonen, P. L. J. M., Zetstra, T., de Boer, E. A., Tibben, H. J., de Jong, M. F., van't Veld, P., Groenland, G. J. R., van Gennep, J. A., Voets, M. T., Verheijde, H. J. M., and Braamskamp, J. (1991) Mystery swine disease in the Netherlands: the isolation of Lelystad virus. *Vet. Q.* **13,** 21–130.
3. Ohlinger, V. F., Weiland, F., Haas, B., Visser, N., Ahl, R., Mettenleiter, T. C., Weiland, L., Rziba, H. J., Saalmuller, A., and Straub, O. C. (1991) Der "seuchenhafte Spatabort beim Schwein"—ein Beitrag zur Atiologie des "porcine reproductive and respiratory syndrome (PRRS). " *Tieraerztl. Umsch.* **46,** 703–708.
4. Ahl, R., Pensaert, M., Robertson, I. B., Terpstra, C., van der Sande, W., Walker, J. K., White, M. E., and Meredith, M. (1992) Porcine reproductive and respiratory syndrome (PRRS or blue-eared pig disease). *Vet. Rec.* **130,** 87–89.
5. Benfield, D. A., Nelson, E. A., Collins, J. E., Harris, L., Goyal, S. M., Robison, D., Christianson, W. T., Morrison, R. B., Gorcyca, D., and Chladek, D. (1992) Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). *J. Vet. Diagn. Invest.* **4,** 127–133.
6. Collins, J. E., Benfield, D. A., Christianson, W. T., Harris, L., Hennings, J. C., Shaw, D. P., Goyal, S. M., McCullough, S., Morrison, R. B., Joo, H. S., Gorcyca, D., and Chladek, D. (1992) Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. *J. Vet. Diagn. Invest.* **4,** 117–126.
7. Meulenberg, J. J. M., Hulst, M. M., de Meuer, E. J., Moonen, P. L. J. M., den Besten, A., de Kluyver, E. P., Wensvoort, G., and Moormann, R. J. M. (1993) Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. *Virology* **192,** 62–72.
8. Zimmerman, J. J. (1995) General overview of PRRS virus: a perspective from the United States. *Proc. Sec. Intl. Symp. on PRRS,* 2.
9. Yaeger, M. J., Prieve, T., Collins, J., Christopher-Hennings, J., Nelson, E. A., and Benfield, D. (1993) Evidence for the transmission of porcine reproductive and respiratory syndrome (PRRS) virus in boar semen. *Swine Health Prod.* **1,** 5–9.
10. Swenson, S. L., Zimmerman, J. J., Evans, L., Bechtol, D., Hopkins, S., Wills, R., Yoon, K. J., Schwartz, K., Hill, H., and McGinley, M. (1995) Exposure of gilts to PRRS virus by artificial insemination. *Proc. Sec. Intl. Symp. on PRRS,* **42.**
11. Swenson, S. L., Hill, H. T., Zimmerman, J. J., Evans, L. E., Landgraf, J. G., Wills, R. W., Sanderson, T. P., McGinley, M. J., Brevik, A. K., Ciszewski, D. K., and Frey, M. L. (1994) Excretion of porcine reproductive and respiratory syndrome virus in semen after experimentally induced infection in boars. *J. Am. Vet. Med. Assoc.* **204,** 1943–1948.
12. Mermin, J. H., Holodniy, M., Katzenstein, D. A., and Merigan, T. C. (1991) Detection of human immunodeficiency virus DNA and RNA in semen by the polymerase chain reaction. *J. Infect. Dis.* **164,** 769–772.
13. Van Engelenburg, F. A. C., Maes, R. K., Van Oirschot, J. T., and Rijsewijk, F. A. M. (1993) Development of a rapid and sensitive polymerase chain reaction assay

for detection of bovine herpesvirus type 1 in bovine semen. *J. Clin. Microbiol.* **31,** 3129–3135.
14. Wiedmann, M., Brandon, R., Wagner, P., Dubovi, E. J., and Batt, C. A. (1993) Detection of bovine herpesvirus-1 in bovine semen by a nested PCR assay. *J. Virol. Meth.* **44,** 129–140.
15. Chirnside, E. D. and Spaan, W. J. M. (1990) Reverse transcription and cDNA amplification by the polymerase chain reaction of equine arteritis virus (EAV). *J. Virol. Meth.* **30,** 133–140.
16. Christopher-Hennings, J., Nelson, E. A., Nelson, J. K., Hines, R. J., Swenson, S. L., Hill, H. T., Zimmerman, J. J., Katz, J. B., Yaeger, M. J., Chase, C. C. L., and Benfield, D. A. (1995) Detection of porcine reproductive and respiratory syndrome virus in boar semen by PCR. *J. Clin. Microbiol.* **33,** 1730–1734.
17. Evenson, D. P., Darzynkiewicz, Z., and Melamed, M. R. (1980) Comparison of human and mouse sperm chromatin structure by flow cytometry. *Chromosoma* **78,** 225–238.
18. Christopher-Hennings, J., Nelson, E. A., Nelson, J. K., and Benfield, D. A. (1995) Evaluation of a modified-live PRRSV vaccine in adult boars. *Conference Res. Workers Anim. Dis.* 193.
19. Christopher-Hennings, J., Nelson, E. A., Hines, R. J., Nelson, J. K., Swenson, S. L., Zimmerman, J. J., Chase, C. C. L., Yaeger, M. J., and Benfield, D. A. (1995) Persistence of porcine reproductive and respiratory syndrome virus in serum and semen of adult boars. *J. Vet. Diagn. Invest.* **7,** 456–464.
20. Murtaugh, M. P., Elam, M. R., and Kakach, L. T. (1995) Comparison of the structural protein coding sequences of the VR-2332 and Lelystad virus strains of the PRRS virus. *Arch. Virol.* **140,** 1451–1460.
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 455.
22. Gerfen, R. W., White, B. R., Cotta, M. A., and Wheeler, M. B. (1994) Comparison of the semen characteristics of FengJing, Meishan and Yorkshire boars. *Theriogenology* **41,** 461–469.

8

Quantitative and Discriminative Detection of Individual HIV-1 mRNA Subspecies by an RNase Mapping Assay

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1. Introduction

In addition to the Gag, Pol, and Env structural proteins, HIV-1 encodes at least six regulatory proteins: Tat, Rev, Nef, Vif, Vpr, and Vpu. All HIV-I proteins are encoded by overlapping reading frames and are expressed through the complex alternative splicing of a single precursor RNA leading to three major RNA classes ***(1–8)***: an unspliced class which includes both genomic RNA and *gag-pol* mRNA; a singly spliced class that includes mRNAs coding for Env, Vpu, Vif, Vpr, and a truncated form of Tat protein; and a multiply-spliced class that includes mRNAs coding for the regulatory proteins Tat, Rev, and Nef (**Fig. 1**). The function of the regulatory proteins Tat, Rev, Nef, Vif, Vpr, and Vpu has not yet been fully elucidated. Nevertheless, they seem to play specific roles during the different steps of the HIV-1 replication cycle ***(9–11)***. For this reason, the detection of mRNA species encoding these proteins at different steps of the virus replication cycle will provide important information about the function of these proteins. In order to obtain both a quantitative and qualitative detection of these mRNAs, we used a ribonuclease protection assay. Ribonuclease protection assays are commonly used for the detection and quantification of mRNAs ***(12,13)*** and are well-suited for mapping the position of internal and external junctions in mRNAs ***(14,15)***. In addition, as the hybridization takes place in liquid conditions, this technique is more sensitive than other quantitative methods of detection of RNAs such as Northern blotting ***(16)***. Our assay uses a DNA template specific of the HIV-1 strain to be investigated. The template of probe RNA synthesis was obtained by one-step RT-PCR amplification of HIV-1 mRNAs produced by the cells infected by the given HIV-1 strain. This template includes a region of the HIV-1 genome containing the alternative splice

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

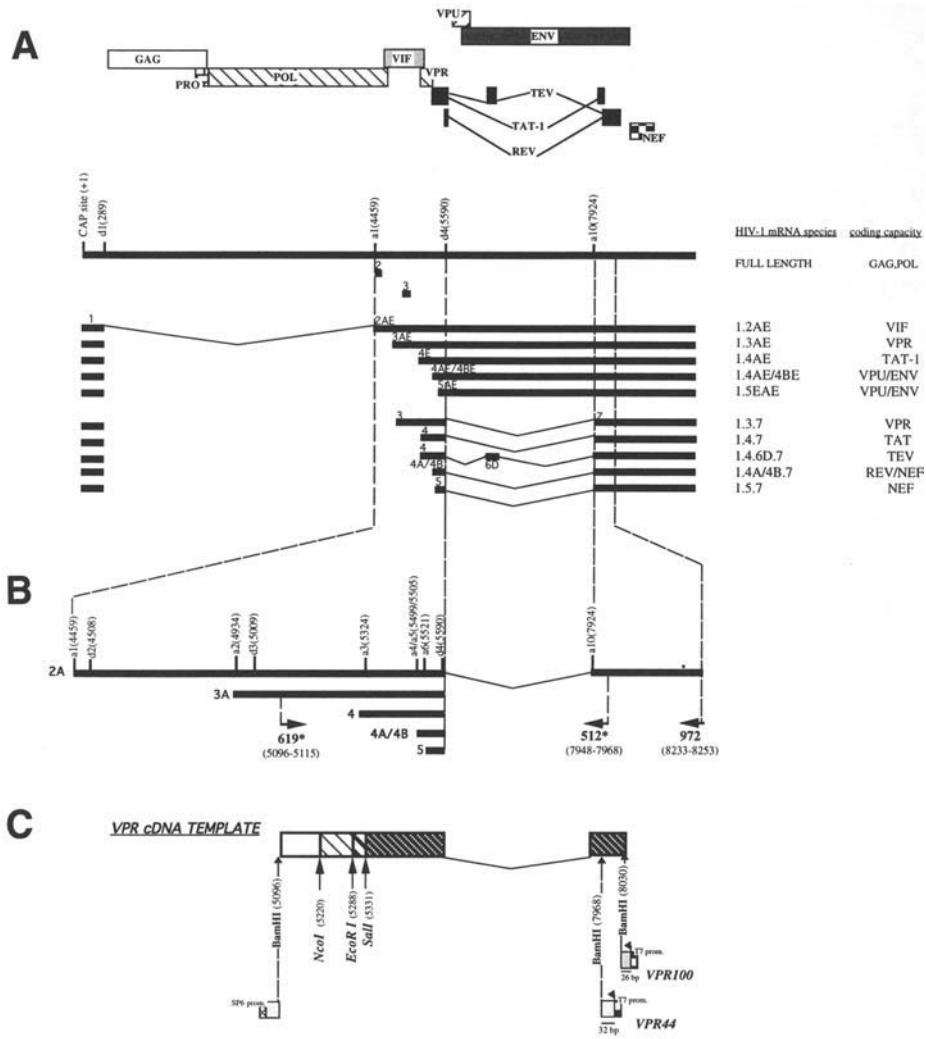

Fig. 1. **(A)** HIV-1 mRNA splicing pattern: HIV-1 genomic organization and the different exons are shown at the top. HIV-1 major splice sites are indicated and the three mRNA classes (unspliced, singly spliced, and multiply spliced) are represented. Both singly spliced and multiply spliced mRNA species contain a splice from the 5' splice site d1 to one of the 3' splice sites a1–a6. Multiply spliced mRNAs contain an additional splice from the 5' splice site d4 to the 3' splice site a10. Correspondence of each coding exon to its encoded protein(s) is given at the right of the panel referring to Schwartz's nomenclature. **(B)** Enlargement of the HIV-1 genome central region amplified for T7 polymerase template construction. Major splice acceptors used for singly spliced *vif, vpr, tat, vpu/env*, and multiply spliced *vpr, tat, rev, nef, tev* mRNA coding exons are indicated at the top. Horizontal arrows indicate RT-PCR primer pair

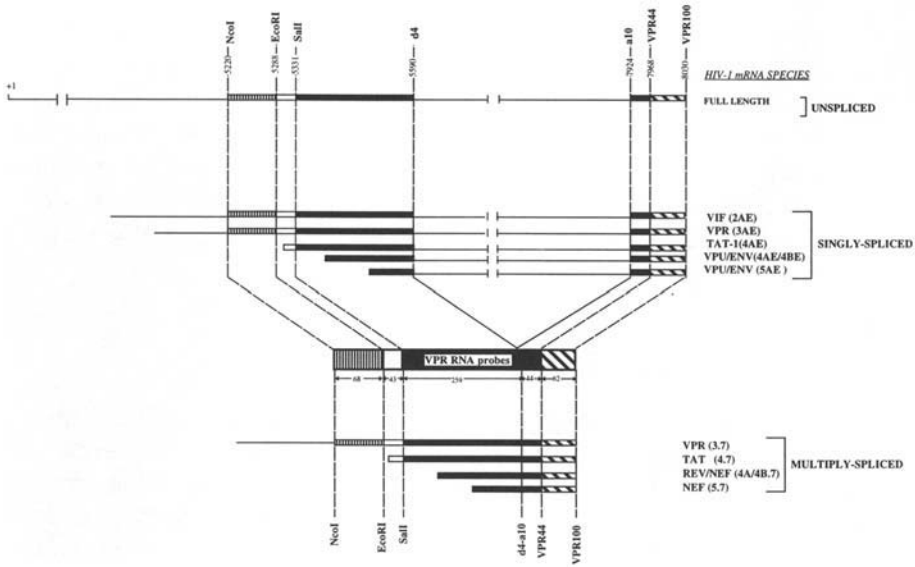

Fig. 2. HIV-1 mRNA regions protected by VPR cDNA probes. Protected regions are represented by boxes. Unspliced and singly spliced RNAs are protected on two regions: a region upstream from the 5' splice site d4 whose size is variable; and a region upstream from the 3' splice site a10 whose size is common to all species. Probes allow discrimination between the multiply spliced *vpr, tat, rev,* and *nef* mRNAs, the singly spliced *tat-1* and *vpu/env* mRNAs and the unspliced RNA. Singly spliced *vpr* and *vpr* mRNAs are protected on the same region as unspliced RNA and thus cannot be discriminated from each other. The size of the regions protected by the probes is indicated in **Table 1**. Vertical numbers indicate positions refering to the HIV-1/HXB2c CAP site. Horizontal numbers indicate the size of the VPR probes.

sites, the use of which determines the coding capacity of HIV-1 mRNAs species. Thus, the RNA probe transcribed from this template will hybridize with these RNAs species on variable sizes. These hybridized mRNAs will be protected from RNase treatment resulting in discrimination between them (RNase mapping, **Fig. 2**). In this chapter, we will describe two applications of this assay: in promyelocytic HL60 cells infected by HXB2 strain of HIV-1 (HL60/HXB2);

positions used for *vpr* cDNA amplification. **(C)** *vpr* cDNA template. This region was obtained by RT-PCR amplification of *vpr* mRNA with the primers pair 619-512. Vertical arrows indicate restriction enzyme site positions used for template cloning or vector linearization. Restriction enzyme sites provided by RT-PCR primers sequence are indicated by an asterisk (*). Other sites are internal in the HIV-1/HXB2c sequence. The box above the scheme represents sequences from the pGEM vector. Numbers in brackets indicate nucleotide positions referring to the HXB2 CAP site.

and in promonocytic U1 cells induced by PMA (U1/PMA). By this strategy, we can detect unspliced *gag-pol*, singly spliced *vpu/env* and multiply-spliced *vpr, rev,* and *nef* mRNAs in infected HL60/HXB2 and U1. In addition, *tat* mRNAs were detected in U1 cells. This application is convenient for the study of the sequential expression of HIV-1 mRNAs during virus production following cell infection or induction. Moreover, this application could be extended to all viruses with complex expression patterns such as retroviruses like HTLV-1 ***(17)***, SIV-1 ***(18,19)***, or spumaretroviruses ***(20)***.

2. Materials

2.1. Cells and RNA Extraction

1. HL60/HXB2 is a subclone of a post-infected HL60 promyelocytic cell line (V. Cheynet et al, unpublished) and exhibits strong constitutive HIV-1 expression. U1 cells are a U937 derivative promonocyte line ***(21)*** and were obtained from the AIDS Research and Reference Reagent Program (National Institue of Health repository). U1 cells can be induced to produce infectious HIV-1 viral particles (*see* **Note 1**). These cell lines can be maintained in RPMI 1640 supplemented with 10% fetal calf serum and standard antibiotics.
2. RNA extraction: RNazol (Bioprobe, Montreuil-Sous-Bois, France), chloroform, isopropanol, sodium acetate (3 *M*, pH 4.8), ethanol 70%.

2.2. Oligonucleotides and One-Step Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

1. The sequences of the HIV-1 specific primers are as follows (positions of the corresponding sequence in the HIV-1/HXB2c sequence are indicated in brackets): 619 (oligo 3'), 5' GAGGATCCATGGAACAAGCC 3' (5096-5115); 512 (oligo 5'), 5' TTTAAGGATCCCTATTCCTTCGGGCCTGTCGGG 3' (7948-7969). Point mutations were introduced to generate BamHI restriction enzyme sites.
2. Sterile water.
3. RT-PCR buffer.
4. Deoxynucleotides (dATP, dTTP, dCTP, dGTP, 0.25 m*M* each, Boehringer Mannheim).
5. Sterile 0.5 mL PCR reaction tubes.
6. AMV reverse transcriptase (Boehringer Mannheim, Meylan, France).
7. *Taq* polymerase (Perkin Elmer, Branchburg, NJ).

2.3. T7/SP6 Polymerase Expression Vectors and Synthesis of RNA Probes

1. T7 RNA polymerase kit (BRL, Cergy-Pantoise, France).
2. SP6 RNA polymerase kit (Boehringer Mannheim).
3. α^{32}P UTP (10 μCi/μL, Amersham, Buckinghamshire, UK).
4. Polyacrylamide 5%, 8 *M* urea gel: acrylamide solution (40%); *bis*-acrylamide solution (2%); urea ultrapure grade (Amresco, Solon, OH), ammonium persulfate (10%); TEMED (Sigma, St. Quentin-Fallavier, France).

5. Elution buffer: 7.5 *M* ammonium acetate, 25 mg/mL tRNA, 1% SDS.
6. Hybridization buffer: 80% deionized formamide, 40 m*M* PIPES, pH 6.4, 400 m*M* sodium acetate, pH 6.4, 1 m*M* EDTA.

2.4. RNase Protection Assay

1. RPA II kit Ambion (Austin, TX).
2. Molecular weight marker (ØX 174, *Hae*III fragments).
3. 5% polyacrylamide, 6 *M* urea gel:acrylamide solution (40%), *bis*-acrylamide solution (2%), urea ultrapure grade (Amresco), ammonium persulfate (10%), TEMED (Sigma).
4. RNA loading buffer: 80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 2 m*M* EDTA.

3. Methods

3.1. Cells and RNA Extraction

1. Collect approx 4×10^7 infected cells, and pellet the cells by centrifugation at 500*g* for 5 min.
2. Extract RNA from the cells with RNazol kit according to manufacturer's instructions (*see* **Note 2**).
3. Determine the RNA content and purity of the extracts by measuring A_{260}/A_{280} optical density ratio with a UV spectrophotometer.

3.2. One-Step RT-PCR and Templates Preparation (see *Note 3*)

1. For each sample, prepare 100 µL reaction mixture ***(22)*** containing 1 µg of RNAs derived from infected cells, 310 n*M* of specific 3' and 5' primers, 0.25 m*M* of dATP, dGTP, dCTP, and dTTP, 40 U of calf placental RNase inhibitor (Boehringer Mannheim), 1X PCR buffer (10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin), 10 U of AMV reverse transcriptase, 2.5 U of *Taq* polymerase.
2. Incubate samples for 10 min at 65°C for RNA denaturation and 8 min at 50°C for reverse transcription. Load the tubes into the thermal cycler bolck and run 35 PCR cycles (denaturation for 5 min at 95°C, primer annealing for 2 min at 55°C, and polymerization at 72°C for 2 min 30 s), with a final 8 min elongation step at 72°C.
3. The *vpr* cDNA, produced by one-step RT-PCR amplification from HL60/HXB2 infected cells or from U1/PMA cells, is cloned in the *Bam*HI polylinker site of pGEM-blue (Promega, Charbonniéres, France). The plasmid (pVPR 44) is linearized with *Nco*I (pVPR/*Nco*I) to generate a 432-bp (pVPR/*Nco*I) T7 RNA polymerase template. pGEM-GAPDH is obtained by cloning a 804-bp fragment of the cDNA of the human GAPDH (glyceraldehyde phosphate deshydrogenase) gene in a pGEM vector. pGEM-GAPDH is linearized with *Hin*dIII to obtain SP6 polymerase template.

3.3. T7/SP6 Polymerase Expression Vectors and Synthesis of RNA Probes

1. Synthetize the radiolabeled antisense RNA probes using T7 RNA polymerase or SP6 RNA polymerase in the presence of α^{32}P UTP under manufacturer's conditions (*see* **Notes 4** and **5**).

2. Ethanol precipitate the probes and load onto a 5% polyacrylamide, 8 *M* urea gel.
3. The full-length RNA probe band can be localized by autoradiography. Excise the corresponding bands from the gel, and elute in 400 µL elution buffer at 37°C for 3 h. After elution, the full length probes are recovered by ethanol precipitation and resuspended in hybridization buffer.

3.4. RNase Protection Assay

The assay is performed using the RPA II Kit from Ambion modified according to Hod *(23)*.

1. Add each RNA sample to 500 pg of full-length antisense probe in 20 µL hybridization buffer volume and incubate overnight at 45°C to allow hybridization.
2. Add 200 µL of digestion buffer containing 0.5 U RNase A and 20 U RNase T1 to each sample and incubate at 30°C for 15 min.
3. After digestion of nonhybridized RNAs, add 300 µL of inactivation/precipitation buffer.
4. Precipitate protected RNA fragments by centrifuge (10,000*g*) and resuspend in RNA loading buffer.
5. Load samples on a 5% polyacrylamide, 6 *M* urea gel and run for 3 h at 150 V. The gel is dried and autoradiographied with a film that has not been preflashed.

3.5. Analysis of Results

1. Three micrograms of RNAs from HL60/HXB2 were hybridized to the pVPR/*Eco*RI probe, and subjected to an RNAse mapping assay as described in **Subheadings 2. and 3.** (**Fig. 3**). The sizes of hybridization signals were established relative to the ØX174 DNA molecular weight marker. As previously described *(24)*, these signals respectively correspond to the common protected size of unspliced and singly spliced *vif* and *vpr* mRNAs (302 bp), *rev* mRNA (135/129 bp), *nef* mRNA (113 bp), *vpu/env* mRNA (69 bp) and the common protected size to 3' region of unspliced and singly spliced RNAs (44 bp). This pattern of expression is characteristic of cells expressing high levels of HIV-1 virus.
2. Time course of expression of HIV-1 mRNAs in U1 cells induced by the PMA. To the aim of investigating the sequential expression of individual HIV-1 mRNA species following the stimulation of U1 cells by PMA, we analyzed RNAs extracted from U1 cells at various times after the stimulation by RNase mapping (**Fig. 4**).GAPDH and VPR44/*Nco*I probe were added in the same assay. Because GAPDH is a housekeeping gene, the hybridization signal of GAPDH provides an internal control of the amount of RNAs loaded on each lane. The hybridization signal pattern of each lane was then analyzed by densitometry (**Fig. 5**). No hybridization signal was detected in unstimulated U1 cells. However, a basal level of *nef* (1.5.7.), *rev* (1.4A.7 and 1.4B.7.), and *vpu/env* (1.5E) mRNAs was detected in experiments performed with 10 µg of RNAs from unstimulated U1 cells with a probe of higher specific activity (data not shown). These mRNA species increase between 0 and 9 h postinduction (**Fig. 3A**). During the same period, mRNA species employing splice acceptor 3, i.e., multiply spliced *tat*

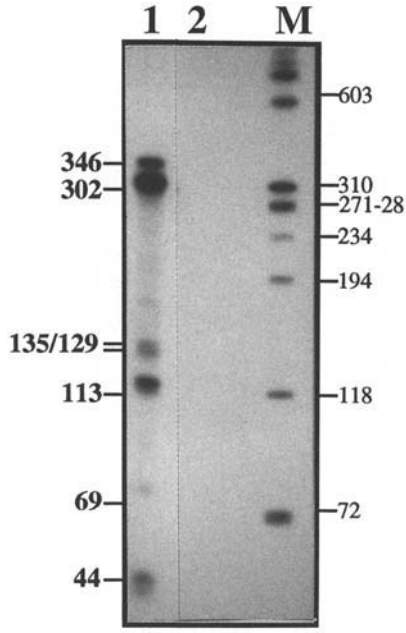

Fig. 3. RNase mapping hybridization pattern in HL60/HXB2. 4.5 μg of RNA drived from HL60/HXB2 (lane 1) and 10 μg of yeast RNAs (lane 2) were incubated with 500 pg of VPR44/*Nco*I probe (10^8 cpm/μg) to perform RNase mapping as described in **Subheading 3.** M: Φ X 174/*Hae*III size marker. Signal sizes are indicated at the left of the panel.

(1.4.7.) and singly spliced *tat* (1.4E), appear and increase to a larger extent than the other species. The signal for singly spliced *tat* is stronger than that for multiply spliced *tat*. All these signals increased between 9 and 21 h postinduction, except the *nef* signal. The intensity of all these signals increases between 21 and 48 h, the relative proportion of the hybridization signals being unchanged. Two additional hybridization signals, one corresponding to unspliced RNA and other to multiply spliced *vpr* mRNA appear and start to increase from 12 h. Their intensity is lower than that of the other signals even at 48 h post-induction. This analysis of HIV-1 mRNAs by RNAse mapping assay gives informations on the composition of each mRNA class expressed in PMA-stimulated U1 cells: the singly spliced class includes *tat* and *vpu/env* mRNAs and the multiply spliced class includes *tat, rev, nef*, and *vpr* mRNAs; the selective increase of singly spliced mRNA with regards to multiply spliced mRNA: this effect is because of the apparition and increase of two mRNA species in addition to the *vpu/env* (1.5E) mRNA, i.e., singly spliced *tat* mRNA at 9 h post-induction and *vpu/env* mRNA (1.4AE and 1.4BE) at 21 h; and the sequential use of splice acceptors: We observe sequential use of the splice acceptors 3, 4A, 4B, and 5 during the stimulation of

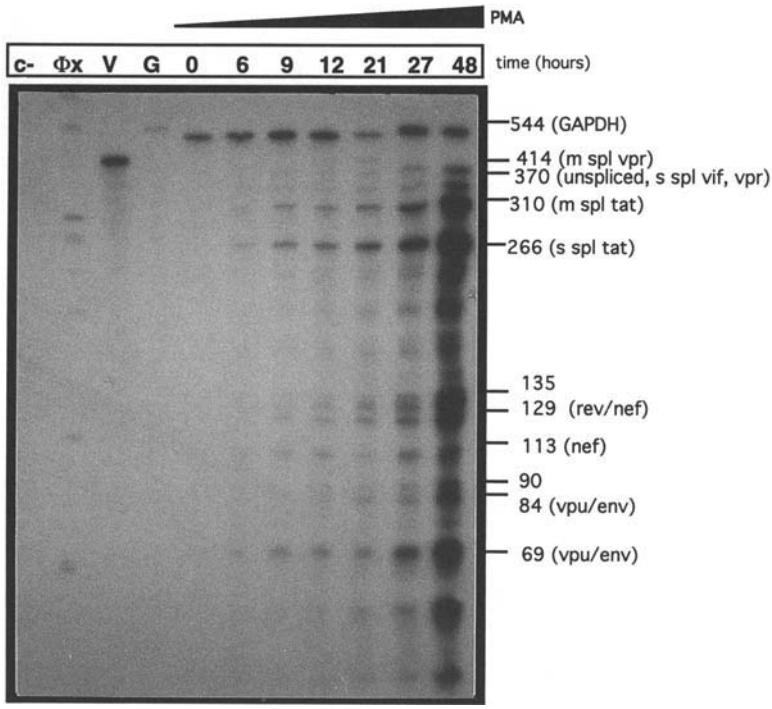

Fig. 4. Changes in the expression of HIV-1 mRNA species during the induction of U1 cells by PMA: 6 μg were analyzed by RNase mapping with the radiolabeled probes VPR44/*Nco*I (108 cpm/μg) and GAPDH (10^6 cpm/μg) as described in **Subheading 3.** The size of the hybridization signal was calculated according to the position of the bands of the size marker ΦX174. C: negative control with 6 μg of uninfected U937 cells; M: ØX174 size marker; V: unhybridized VPR probe; G: unhybridized GAPDH probe.

U1 cells by PMA; mRNA species using the splice acceptors 3 (*tat*) and 5 (*nef* and *vpu/env*) are predominant over those using the splice acceptors 4A and 4B between 0 and 12 h. The use of the splice acceptors 4A and 4B (*rev* and *vpu/env*) selectively increases between 12 and 21 h. The interest of this analysis with regard to northern blotting analysis previously used ***(25–27)*** is the discrimination between RNAs species, which allows investigation of their behavior during the stimulation. We also investigated the time course of expression of HIV-1 mRNAs after infection of peripheral blood lymphocytes by different HIV-1 strains. This application of RNase mapping provides precious tools to understand the mechanisms controlling the expression of HIV-1 mRNAs.

4. Notes

1. U1 Cells growing at a density of 10^6 cells/mL are induced in medium adjusted to 20 n*M* phorbol-12-myristate-13-acetate (PMA, Sigma). Cells are maintained in this environment until they are harvested.

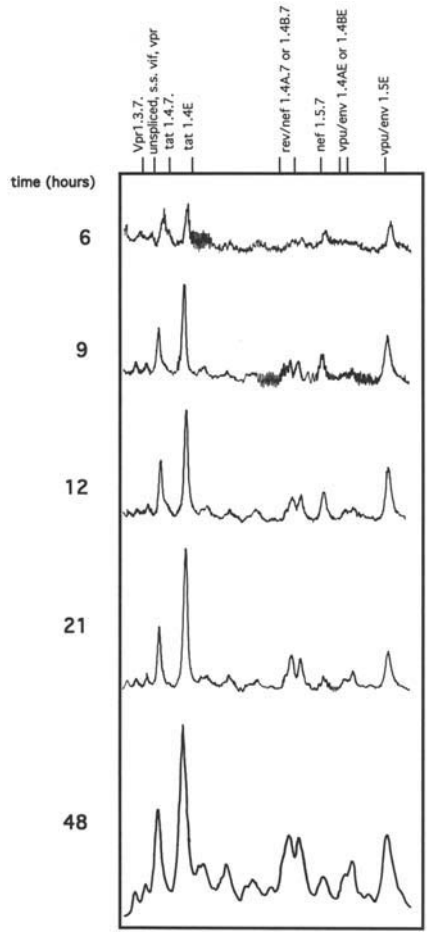

Fig. 5. Densitometric analysis of hybridization signals of HIV-1 mRNA species shown in **Fig. 4**. Autoradiograms were scanned on the Molecular Dynamic scanner (Genescan) and the image was quantitated on a Pharmacia Phosphoimager. The RNA species corresponding to each hybridization signal are indicated at the top of the panel.

2. The standard guanidinium isothiocyanate cesium chloride method ***(28)*** modified according to current protocols ***(29)*** can be also used.
3. Our detection is intended to discriminate between mRNA species of different coding capacities. In the case of HIV-1, these mRNAs differentiate by the use of the 3' splice sites a1–a6; and the d4-a10 splice junction (**Fig. 1B**). However, a region including all these splice sites would be too large for application to the RNase mapping assay. Thus, we restricted our choice to a region including: the sequence enclosed by a3 and d4; the d4-a10 splice junction; and a sequence down-

Table 1
Protection of Multiply Spliced vs Singly Spliced Species

mRNA class	Encoded protein	pVPR44/ *Nco*I	pVPR44/ *Eco*RI	pVPR100/ *Sal*I
Unspliced	GAG-POL	370	302	259
	VIF (2AE)	370	302	259
	VPR (3AE)	370	302	259
Singly spliced	TAT-1 (4AE)	266	266	259
	VPU/ENV (4A/4B)	91/85	91/85	91/85
	VPU/ENV (5E)	69	69	69
	VPR (3.7)	414	346	365
	TAT (4.7)	310	310	365
Multiply spliced	REV/NEF (4A.7/4B.7)	135/129	135/129	197/191
	NEF (5.7)	113	113	174

stream from the 3' splice site a10 (**Fig. 1C**). According to Schwartz's description *(7)*, this region is included in multiply spliced *vpr* cDNA. Such a region was obtained by RT-PCR amplification of the *vpr* mRNA with the primer pair 619-512 and cloned as described in **Subheading 3.** The probe will discriminate all mRNAs species using one of the 3' splice sites a3–a6 (**Fig. 2**). Species that do not use any of these splice sites (e.g., unspliced RNA, *vif* and *vpr* mRNAs) are protected on the complete 3' part of the probe. Alternatively, the 5' part of the probe containing the d4-a10 splice junction and a sequence downstream to a10 allows the discrimination of the multiply spliced mRNAs from singly spliced RNAs. Consequently, multiply spliced species using this splice junction are protected on a larger region (including the 3' part of the probe) than singly spliced species not using this splice junction (**Table 1**).

4. We investigated the pattern of expression of HIV-1 mRNAs in HL60/HXB2 cells and in U1 cells induced by the PMA. The specific activity of the probe was relative to the level of HIV-1 expression in these infected cells. A convenient detection of hybridization signals is obtained when the specific activity of the probe is on average 10^8 cpm/μg. This was obtained when the cold UTP to α^{32}P UTP ratio was equal to unity in the transcription mixture, i.e., a cold UTP concentration of 6.25 μ*M*. In cells expressing low levels of HIV-1, the specific activity of the probe should be increased. In this case, do not add cold UTP in the reaction mixture of probe synthesis.
5. It is important that the probe is specific to the HIV-1 strain to investigate. This will prevent mismatches during the hybridization resulting in RNases digestion inside the hybridization sequence. For this purpose, the sequence of the RT-PCR product should be checked.

References

1. Furtado, M. R., Balachandran, R., Gupta, P., and Wolinsky, S. M. (1991) Analysis of alternatively spliced human immunodeficiency virus type-1 mRNA species, one of which encodes a novel tat-env fusion protein. *Virology* **185,** 258–270.

2. Robert-Guroff, M., Popovic, M., Gartner, S., Markham, P., and Gallo, R. C. (1990) Structure and expression of tat-, rev-, and nef-specific transcripts of human immunodeficiency virus type 1 in infected lymphocytes and macrophages. *J. Virol.* **64,** 3391–3398.
3. Guatelli, J. C., Gingeras, T. R., and Richman, D. D. (1990) Alternative splice acceptor utilization during human immunodeficiency virus type 1 infection of culured cells. *J. Virol.* **64,** 4093–4098.
4. Schwartz, S., Felber, B. K., Benko, D. M., Fenyö, E. M., and Pavlakis, G. N. (1990) Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. *J. Virol.* **64,** 2519–2529.
5. Benko, D. M., Schwartz, S., Pavlakis, G. N., and Felber, B. K. (1990) A novel human immunodeficiency virus type 1 protein, tev, shares sequences with tat, env, and rev proteins. *J. Virol.* **64,** 2505–2518.
6. Schwartz, S., Felber, B. K., Fenyö, E. M., and Pavlakis, G. N. (1990) Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple biscistronic mRNAs. *J. Virol.* **64,** 5448–5456.
7. Schwartz, S., Felber, B. K., and Pavlakis, G. N. (1991) Expression of human immunodeficiency virus type 1 vif and vpr mRNAs is Rev-dependent and regulated by splicing. *Virology* **183,** 677–686.
8. Smith, J., Azad, A., and Deacon, N. (1992) Identification of two novel human immunodeficiency virus type 1 splice acceptor sites in infected T cell lines. *J. Gen. Virol.* **73,** 1825–1828.
9. Antoni, B. A., Stein, S. B., and Rabson, A. B. (1994) Regulation of human immunodeficiency virus infection: implications for pathogenesis. *Adv. Virus Res.* **43,** 53–145.
10. Cullen, B. R. (1992) Mechanism of action of regulatory proteins encoded by complex retroviruses. *Microbiol. Rev.* **56,** 375–394.
11. Steffy, K. and Wong-Staal, F. (1991) Genetic regulation of human immunodeficiency virus. *Microbiol. Rev.* **55,** 193–205.
12. Roller, R. J., Kinloch, R. A., Hiroaka, B. Y., Li, S. S.-L., and Wassarman, P. M. (1989) Gene expression during mammalian oogenesis and early embryogenesis: quantification of three messenger RNAs abundant in fully grown mouse oocytes. *Development* **106,** 251–261.
13. Anonymous (1993) HIV seroconversion after occupational exposure despite early prophylactic zidovudine therapy. *Lancet* **341,** 1077,1078.
14. Kekule, A. S., Lauer, U., Meyer, M., Caselmann, W. H., Hofschneider, P. M., and Koshy, R. (1990) The preS2/S region of integrated hepatitis B virus DNA encodes a transcriptional transactivator. *Nature* **343,** 457–461.
15. Calzone, F. J., Britten, R. S., and Davidson, E. H. (1987) Mapping of gene transcripts by nuclease protection assays and cDNA primer extention. *Meth. Enzymol.* **152,** 611–632.
16. Lee, J. J. and Costlow, N. A. (1987) A molecular titration assay to measure transcript prevalence levels. *Meth. Enzymol.* **152,** 633–648.
17. Ciminale, V., Pavlakis, G. N., Derse, D., Cunningham, C. P., and Felber, B. K. (1992) Complex splicing in the human T-cell leukemia virus (HTLV) family of retroviruses: novel mRNAs and proteins produced by HTLV type 1. *J. Virol.* **66,** 1737–1745.

18. Park, I. W., Steen, R., and Li, Y. (1991) Characterization of multiple mRNA species of simian immunodeficiency virus from macaques in a CD4+ lymphoid cell line. *J. Virol.* **65,** 2987–2992.
19. Unger, R. E., Stout, M. W., and Luciw, P. A. (1991) Simian immunodeficiency virus (SIVmac) exhibits complex splicing for tat, rev, and env mRNA. *Virology* **182,** 177–185.
20. Muranyi, W. and Flügel, M. (1991) Analysis of splicing patterns of human spumaretrovirus by polymerase chain reaction reveals complex RNA structures. *J. Virol.* **65,** 727–735.
21. Folks, T. M., Justement, J. S., Kinter, A., Schnittman, S. M., Orenstein, J., Poli, G., and Fauci, A. S. (1988) Characterization of a promonocyte clone chronically infected with HIV and inducible by 13-phorbol-12-myristate acetate. *J. Immunol.* **140,** 1117–1122.
22. Mallet, F., Oriol, G., Mary, C., Verrier, B., and Mandrand, B. (1995) Continuous RT-PCR using AMV-RT and Taq DNA polymerase: characterization and comparison to uncouples procedures. *BioTechniques* **18,** 678–687.
23. Hod, Y. (1992) A simplified ribonuclease protection assay. *BioTechniques* **13,** 852–854.
24. Mary, C., Telles, J. N., Cheynet, V., Oriol, G., Mallet, F., Mandrand, B., and Verrier, B. (1994) Quantitative and discriminative detection of individual HIV-1 mRNA subspecies by an RNAse mapping assay. *J. Virol. Meth.* **49,** 9–23.
25. Ma, X., Sakai, K., Sinangil, F., Golub, E., and Volsky, D. J. (1990) Interaction of a noncytopathic human immunodeficiency virus type 1 (HIV-1) with target cells: efficient virus entry followed by delayed expression of its RNA and protein. *Virology* **176,** 184–194.
26. Klotman, M. E., Kim, S., Buchbinder, A., DeRossi, A., Baltimore, D., and Wong-Staal, F. (1991) Kinetics of expression of multiply spliced RNA in early human immunodeficiency virus type 1 infecti on of lymphocytes and monocytes. *Proc. Natl. Acad. Sci. USA* **88,** 5011–5015.
27. Kim, S., Byrn, R., Groopman, J. E., and Baltimore, D. (1989) Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. *J. Virol.* **63,** 3708–3713.
28. Chirgwin, J. M., Przybyla, A. E., Macdonald, R. J., and Rutter, W. J. (1979) Isolation of biologically active ribonucleic acid from sources enriches in ribonuclease. *Biochemistry* **18,** 5294,5295.
29. Kingston, R. E., Chomczynski, P., and Sacchi, N. (1991) Guanidium method for total RNA preparation, in *Current Protocols in Molecular Biology,* vol. 1 (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds.), Wiley, Boston, p. 4.2.1.

9

Detection of the Heat-Labile Toxin Coding Gene (LT-Gene) of Enterotoxigenic *Escherichia coli*

Séamus Fanning, Deirdre O'Meara, Lesley Cotter, John O'Mullane, and Bartley Cryan

1. Introduction

World-wide enteric pathogens are a leading cause of potentially preventable morbidity and mortality. They are responsible for an estimated 700–1000 million episodes of diarrhea and cause four to five million deaths each year ***(1)***. Infants and young children in the developing world are particularly prone to infection and dehydration. *Escherichia coli* was identified as the causative agent with four main groups recognized as both animal and human enteropathogens; enterohemorrhagic *E. coli* (EHEC) enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC).

Enterotoxigenic *E. coli* (ETEC) is the commonest bacterial cause of diarrhea in developing countries especially among young children ***(2)***. They account for an estimated 650 million cases and 800,000 deaths each year. Furthermore there have been well documented outbreaks of ETEC-linked diarrhea in England, Ireland, and the United States (***3*** and references therein).

Acquired ETEC infection is by fecal–oral transmission, which arises through the ingestion of contaminated water or food. ETEC colonize the small intestine, elaborating one or more enterotoxins resulting in fluid loss. The predominant symptom is watery diarrhea (ranging from mild to severe cholera-like disease) associated with nausea, abdominal cramps, dehydration, and a low-grade fever. The main burden of ETEC diarrheal disease is in the developing world. The epidemiology of the organism has not been completely elucidated and therefore preventative strategies tend to be based on general principles rather than focused on ETEC. To define the epidemiology of these organisms and audit preventative strategies robust, sensitive, and relatively cheap methodologies are necessary.

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

Two types of ETEC-specific plasmid encoded enterotoxins have been described: a heat-labile (LT) toxin and a heat-stable toxin (ST-1a and ST-1b). Virulent strains may possess either or both toxin-encoding genes simultaneously. These genes have been previously cloned and characterized ***(4–6)***. It is interesting to note that the LT-toxin is structurally, antigenically, and functionally related to the *Vibrio cholerae* enterotoxin (CT). These proteins form part of a family sharing common properties ***(7)*** acting on adenylate cyclase to increase cAMP levels. A similar yet genetically distinct LT-toxin was isolated from porcine *E. coli* ***(8)***. Comparison with the human ETEC-derived toxin shows significant homology at the amino acid level.

The ST-toxin group are small 18–19 amino acid toxins and the one most closely associated with human disease is ST-1a ***(9)***. This polypeptide acts to elevate cGMP levels leading to a reduction in the chloride and sodium absorption and eventually diarrhea.

Both the LT-and ST-toxin encoding genes and/or their corresponding protein products outlined above have formed the basis for the development of ETEC organismal detection strategies. Detection methods have focused on both phenotypic and genotypic characteristics. However, further discussion here will be confined to the detection of virulent ETEC strains on the basis of the LT-toxin encoding gene.

Assays used to detect ETEC involve the isolation of *E. coli* from samples, confirming the identity of organisms and incubating the isolates in an environment conducive to the production of enterotoxins. Such strategies were adapted from those designed to identify the CT-toxin of *V. cholerae*, and involved the use of bioassays with both Chinese hamster ovarian (CHO) and the Y-1 mouse adrenal cell lines ***(10,11)***. The Y-1 mouse adrenal assay is the more sensitive and reliable detection method for the LT-toxin ***(12)***. By comparison, the CHO assay may be affected by the cytolethal distending toxin of *E. coli* and if not further investigated may lead to misinterpretation ***(13)***.

As the LT-toxin itself is immunogenic, several methods were developed based on this feature, including the latex agglutination test, a GM1 enzyme-linked immunoassay (ELISA), radioimmunoassay (RIA), and the Bicken agar diffusion test ***(14–16)***. All of these phenotypic methods depend on the isolation of live ETEC, therefore the sensitivity of the culture method and the numbers of organisms in each sample determines their lower detection limits. In addition, the requirement for toxin production, upon which these tests depend, necessitates the use of optimized media and culturing. Genotype-based methods are an attractive alternative as they are independent of the above limitations.

Nucleic acid hybridization using [^{32}P]-radiolabeled probes corresponding to defined regions within the toxin genes have been applied to differentiate ETEC from non-ETEC organisms ***(17)***. Colony dot-blots can identify the bacterial

genotype(s) without the need for the corresponding gene(s) to be fully expressed. Although it is acknowledged that radioactively labeled probes are more sensitive when compared to the immunobased assays above, there are also well recognized limitations associated with their use (e.g., short half-life, disposal problems, and biohazard), making them less attractive as an analytical detection system ***(18)***. When applied directly to stool samples these assays were found to be expensive, labor-intensive and were relatively insensitive. In vitro enzyme-mediated amplification strategies offered the promise of sensitive signal detection independent of culture limitations.

The development of the polymerase chain reaction (PCR) represents a significant advance allowing the direct detection and identification of nucleic acid (both DNA and RNA) sequences ***(19,20)***. Numerous reports describe its application in a variety of settings (for example, *see* **ref. *21***). In particular, PCR can be used to screen for a number of unrelated pathogens in a single-tube experiment, using multiplex-priming ***(22,23)***. However, owing to the practical difficulties associated with the detection of amplified DNA using gel electrophoresis or hybridization, arising from large sample numbers there is a need for a high-throughput nonisotopic PCR assay with an integrated detection system adapted for use with equipment currently available in diagnostic laboratories.

Traditional detection formats used in DNA analysis include agarose gels stained with the intercalating dye ethidium bromide followed by electrophoresis, resolving DNA bands on the basis of size, and nucleic acid probe hybridization assays based on the dot-blot or Southern blot formats ***(24)***. In light of the current demands for technology transfer, these methods are time consuming and have involved the use of radioisotopes. Although agarose gel electrophoresis is easy to perform, giving a size estimation for any DNA fragment, it lacks sensitivity. Use of PCR to screen biological samples from any origin has obvious advantages compared to the other methods outlined. However, to provide PCR-based strategies as an integral part of the routine methodology of any laboratory requires the development of a universal nonisotopic amplification detection strategy (*see* **Fig. 1** as an example of a generic model).

Increasingly nonradioactive systems have begun to replace analogous radioactive methods. Nonisotopic bioanalytical systems in current use are biotin (bio):streptavidin and digoxigenin (DIG):anti-DIG among others. The former is based on the incorporation of the vitamin biotin (as bio-11-dUTP) into a nucleic acid probe that is subsequently detected by streptavidin conjugated to alkaline phosphatase ***(25)***. This detection format has been used for a variety of applications (for references *see* ***26***), including the detection of the LT-toxin encoding gene ***(27)***. As biotin is a ubiquitous biological molecule, high background signal difficulties have been reported ***(28)***.

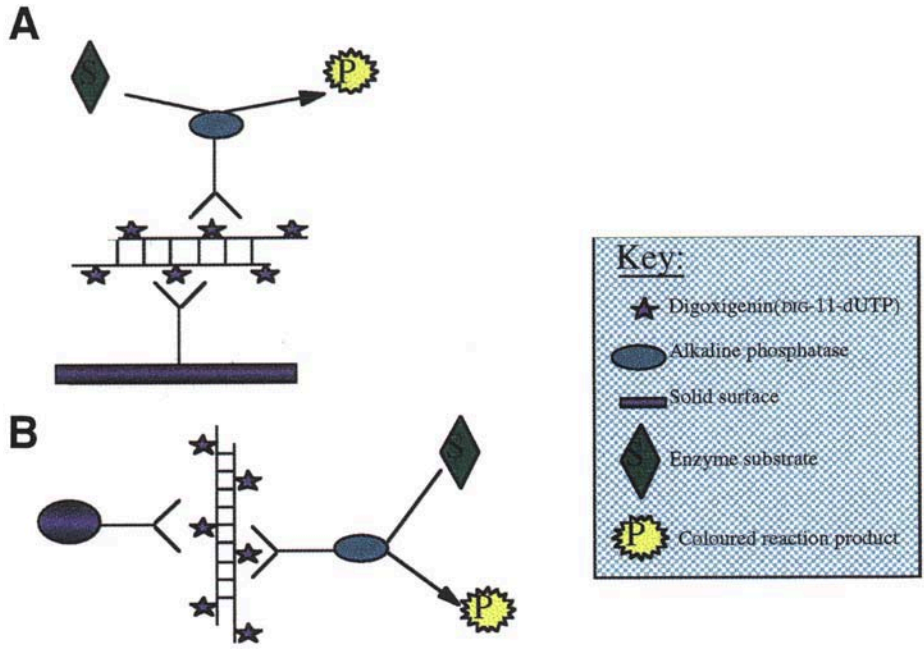

Fig. 1. Colorimetric detection of digoxigenin (DIG)-labeled DNA. Heat-labile toxin (LT) gene-specific primers are used to generate DIG-labeled amplicons that are then captured onto either a microtiter well surface (as in **A**) or on a magnetic bead (as in **B**). In both cases anti-DIG antibodies are used to coat the solid-phase surfaces. Arrested DIG-labeled-amplicons then bind alkaline phosphatase conjugated anti-DIG antibody. Introduction of a suitable chromogenic substrate produces a colored product following phosphate group removal (*see* **Fig. 2B**) by the alkaline phosphatase enzyme. (*See* color plate 1 after p. 114.)

Such technical problems arising from the use of biotin as a label prompted the development of alternative bioanalytical-detection systems, such as digoxigenin ***(29)***. Digoxigenin is a cardenolide steroid that interacts with a high-affinity DIG-specific antibody ***(30)***, in a format similar to that of an enzyme-linked immunoassay (ELISA). As DIG is unique to *Digitalis* plants, the chances of nonspecific background reactions occurring are significantly reduced. When compared to biotin and radioactive labels, DIG has been reported to be as sensitive as radioactive labels ***(31–33)***.

This chapter describes the application of a nonisotopic PCR-based detection method, using the LT-toxin gene as the DNA target. Such a model could be generally applied to any DNA target with characteristics that would lend themselves to high-throughput sampling.

Table 1
Genotype and Serotype of ETEC Strains

Strain no.	LT-toxin genotype	Serotype
473 (4)	+	O78 K80
390 (1)	+	O78 K80
33	+	O78 K80
73	+	O78 K80
3	+	O78 K80
938 (2)	+	O78 K80
285 (v)	+	O78 K80
6	+	O78 K80
851 (1)	+	O78 K80
398 (1)	+	O78 K80
46	+	O78 K80
128	+	078 K80
8	+	O78 H12
387 (v)	+	ns
450 (3)	+	ns
C600	–	ns
PHLS 6085[a]	+	H12
PHLS 8068	+	ns
PHLS 9060	–	H18
PHLS 7539	–	O78 K77

[a]Abbreviations: ns = non-serotypable; PHLS = public health laboratory strain.

2. Materials

2.1. Template DNA Preparation

1. ETEC strains used in this study are listed in **Table 1**.
2. Nutrient broth dispensed in 5 mL volumes and sterile 2 mL Eppendorf tubes.
3. 1 *M* NaCl solution.
4. Tris-HCl-EDTA (THE) buffer: 50 m*M* Tris-HCl (pH 8.0), 50 m*M* EDTA.
5. 2 mg/mL Lysozyme (Sigma, Poole, UK) in 5X TE buffer (50 m*M* Tris-HCl, pH 8.0, 5 m*M* EDTA) (*see* **Note 1**).
6. 20% (w/v) SDS (Boehringer Mannheim, Germany).
7. 10 mg/mL Proteinase K (Sigma, Poole, UK) in TE buffer (*see* **Note 1**).
8. Phenol-chloroform solution (Sigma). Store this solution at 4°C when not in use (*see* **Note 2**).
9. Chloroform-isoamyl alcohol (25:1) solution.
10. 1X TE buffer: 10 m*M* Tris-HCl (pH 8.0), 1 m*M* EDTA.
11. 3.3 *M* NH_4-acetate.
12. Cold ethanol stored at –20°C.

2.2. PCR Amplification

1. Sterile water.
2. 10X PCR buffer: 100 m*M* Tris-HCl (pH 9.0), 500 m*M* KCl, 1% Triton X-100, 3 m*M* $MgCl_2$.
3. Working nucleotide mixture: 2 m*M* each of dATP, dCTP, dGTP, 1.5 m*M* dTTP, and 0.5 m*M* DIG-11-dUTP (Boehringer Mannheim, Germany).
4. Oligonucleotide primers diluted in sterile water to 25 m*M*. LT-1 (forward direction) 5'-TTA CGG CGT TAC TAT CCT CTC TA-3' and LT-2 (reverse direction) 5'-GGT CTC GGT CAG ATA TGT GAT TC-3' ***(34)*** (*see* **Note 3**).
5. Sterile 0.5 mL PCR reaction tubes.
6. 2.5 U *Taq* DNA polymerase (Promega, Madison, WI).
7. Positive control DNA template diluted to 100 ng/mL.
8. 4 *M* LiCl.
9. QIAquick PCR purification kit (QIAgen, Surrey, UK).

2.3. Agarose Gel Electrophoresis

1. NuSieve agarose (FMC BioProducts, Rockland, ME).
2. Ethidium bromide (10 mg/mL) in sterile water (*see* **Note 4**).
3. 10X Tris-acetate-EDTA (TAE) buffer: prepare by adding 48.5 g Tris, 22.9 mL glacial acetic acid and 7.6 g EDTA to 1 L of dH_2O.
4. Molecular weight markers: Several preparations of DNA molecular weight markers are commercially available, differing in their fragment ranges. In this laboratory the fX174 *Hae*III (Promega), are routinely used. Fragments range in size from 72 bp to 1373 kb.
5. Gel loading dye: 0. 25% (w/v) bromophenol blue, 80% (v/v) glycerol in sterile dH_2O.

2.4. Colorimetric Detection

1. Nylon membrane (Millipore, France).
2. Vacuum dot blot apparatus (Millipore milliblot D system).
3. DIG Nucleic Acid Detection Kit (Boehringer Mannheim).
4. Dot blot reagents:
   Buffer 1: 100 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl.
   Buffer 2: 1.5 g blocking reagent (*see* **Note 5**).
   Buffer 3: 100 m*M* Tris-HCl (pH 9.5), 100 m*M* NaCl, 50 m*M* $MgCl_2$.
   Buffer 4: 10 m*M* Tris-HCl (pH 8.0), 1 m*M* EDTA.
5. DIG-antibody-alkaline phosphatase (AP)-conjugate: vial 3 from detection kit containing 200 µL of polyclonal sheep antidigoxigenin F_{ab} fragments, conjugated to AP (750 U/mL). Dilute the conjugate in Buffer 1 (to 150 mU/mL) prior to use.
6. Color solution: Vial 4 from the detection kit containing 1 mL of 4-nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) stock solution in 67% (v/v) dimethyl sulfoxide (DMSO). Prepare freshly before use by adding 200 µL of stock NBT/BCIP to 10 mL of Buffer 3 (*see* **Note 6**).

2.5. Fluorescent Detection

1. Applied Biosystems 310 Prism Genetic Analyzer (Applied Biosystems, Foster City, CA).
2. ABI Prism 0.5 mL sample tubes and septa.
3. ABI Prism GENESCAN capillary (61 cm × 70 mm).
4. ABI Prism GENESCAN Polymer and Genetic Analyzer Buffer.
5. ABI Prism™ GENESCAN-500-*N,N,N,N*'-tetramethyl-6-carboxyrhodamine (TAMRA) size standards (*see* **Note 7**).

3. Methods

3.1. Template DNA Isolation

1. A single colony of an ETEC strain was inoculated into 5 mL of nutrient broth and was incubated on a rotating shaker at 37°C for 18 h.
2. Cells were recovered by centrifugation and the cell pellet was washed in 2 mL of 1 *M* NaCl. After another centrifugation step, the cells were then washed in 2 mL of THE solution, and centrifuged at high speed before being resuspended in 0.7 mL of the same THE buffer. Bacterial cell wall degradation is initiated by adding 0.1 mL of the lysozyme solution to the washed cells.
3. The cell suspension was then incubated at 37°C for 30 min after which 30 µL of 20% SDS was added and incubation continued at 65°C for 10 min. Lysis was completed by the addition of 60 µL of Proteinase K with a final incubation step at 37°C for 1 h.
4. To remove any remaining protein the cell lysate was extracted twice with 1 mL of a phenol-chloroform solution.
5. Traces of phenol were then removed by extracting once with chloroform-isoamyl alcohol (25:1) and finally the DNA was precipitated from the aqueous phase with 0.1 vol of 3.3 *M* NH_4-acetate and 2.5 vol of cold ethanol overnight at –20°C.
6. After centrifugation at 14, 000*g* for 15 min, the precipitated DNA pellet was then washed twice in 70% ethanol and then dissolved in 100 µL 1X TE.
7. DNA concentration was measured spectrophotometrically at A_{260nm} with a UV-vis spectrophotometer and the required amount of DNA per reaction was calculated.

3.2. PCR Reaction Mixture and Cycling

1. Amplification reactions were performed in a total reaction volume of 50 µL consisting of 25 pmol each of LT-1 and -2 primers, 5 µL 10X PCR reaction buffer, and 8 µL dNTP.
2. Add 100 ng of template DNA (be sure to include the positive control at this point).
3. Add 0.5 µL (2.5 U) of *Taq* DNA polymerase.
4. Overlay the amplification mixture with 50 µL mineral oil (*see* **Note 8**).
5. Firmly cap the tubes and place them in the thermal cycler (*see* **Note 9**).
6. Cycle all samples using the following program:

Initial denaturation:	92°C for 4 min
Followed by 25 cycles of:	92°C for 1 min,
	55°C for 30 s,
	72°C for 1 min, and a
Final extension step of:	72°C for 5 min

7. After cycling hold all samples at 4°C until analysis.

3.3. Recovery of DIG-Labeled PCR Product

1. Following amplification, DIG-labeled amplicon(s) are recovered from unincorporated label.
2. Unincorporated DIG-11-dUTP nucleotide was removed by precipitation in 0.14 vol of 4 *M* LiCl together with 4 vol of cold ethanol at –20°C for 12 h.
3. The labeled DNA was precipitated by centrifuging at high speed for 30 min, and the recovered DNA pellet was resuspended in a final volume of 300 µL of sterile dH_2O of which 5 µL was used for agarose gel analysis and 100 µL was used for solid-phase detection.

3.4. Agarose Gel Electrophoresis

1. Prepare a 2% NuSieve agarose gel in 1X TAE buffer containing 0.5 µg/mL ethidium bromide, (by adding 1.7 g of NuSieve and 0.3 g (normal) agarose to 100 mL of 1X TAE buffer). Heat this mixture until the agarose dissolves. Allow the molten agarose to cool before casting the gel (*see* **Note 10**).
2. When cool, cast the gel and allow it to set for 20–30 min. Remove the comb and place in the gel box. Fill the box with 1X TAE buffer, completely immersing the gel.
3. Onto a strip of parafilm spot 2 µL of loading dye for each DNA marker and PCR sample to be analyzed. Add 10 µL (approx 0.5 µg) of the DNA marker to one spot and 5 µL of sample to the remaining dye spots.
4. Load the samples into the preformed wells and run the gel on constant voltage at 70 V until the dye front has reached the end of the gel.
5. Photograph the gel using a Polaroid Land Camera through an orange filter onto Polaroid Type 667 (black and white film) (*see* **Fig. 2A** and **Note 11**).

3.5. Colorimetric Detection

1. DIG-labeled control DNA (provided with the kit) together with 100 µL DIG-labeled DNA (precipitated as described in **Subheading 3.3.**), were applied directly onto a nylon or nitrocellulose membrane under vacuum using a dot blot apparatus (*see* **Note 12**).
2. The filter was washed in 10 mL of Buffer 1 for 15 min and incubated in blocking buffer (Buffer 2) for 30 min. The filter is again washed and incubated with DIG-antibody-AP-conjugate for 30 min. The unbound conjugate is removed by washing for 15 min in Buffer 1, this step was repeated once.
3. Prior to color development, the membrane is briefly equilibrated with 20 mL of Buffer 3. The membrane is then incubated with 10 mL of freshly prepared color reagent (*see* **Note 6**). An insoluble violet precipitate is formed at the site where DIG-labeled DNA contacts the anti-DIG antibody following oxidation of the BCIP and the reduction of the NBT dye supplied in the DIG Nucleic Acid Detecion Kit. Color development is complete within 30 min and the reaction is stopped by washing the membrane in 50 mL of Buffer 4 (*see* **Fig. 2B** and **Note 13**).

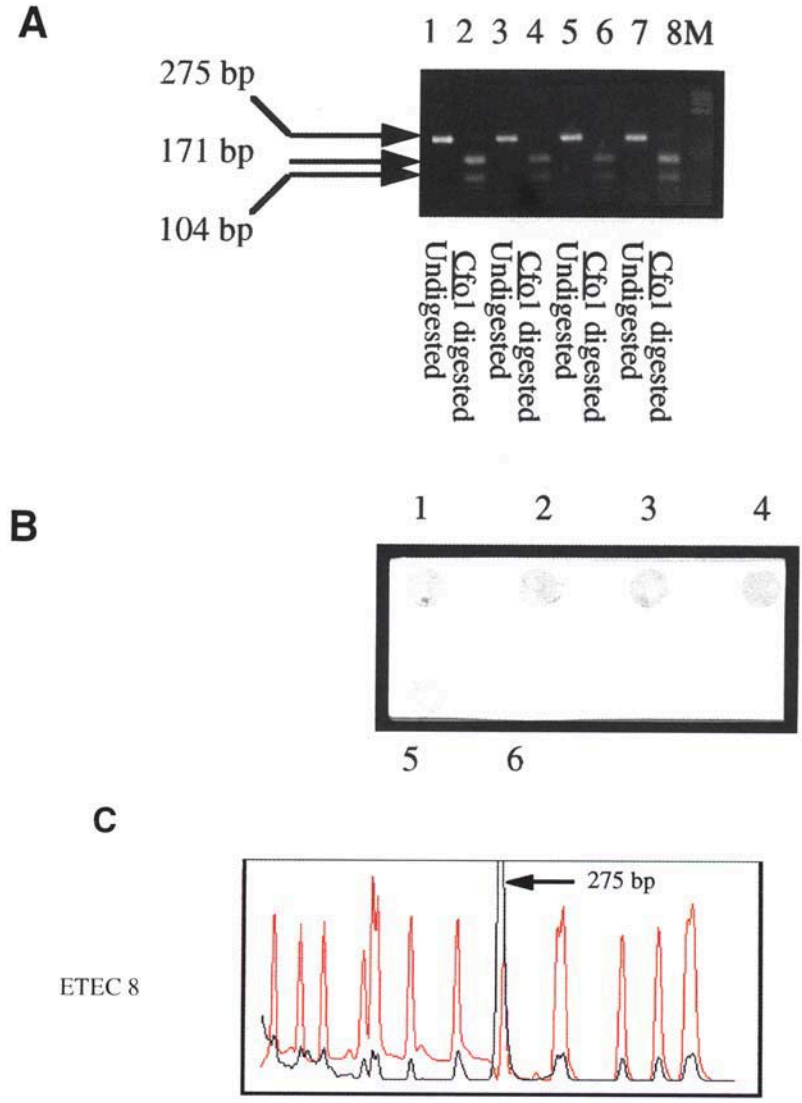

Fig. 2. **(A)** Agarose gel electrophoresis of amplified LT-specific PCR products (2% NuSieve gel in 1X TAE buffer). Lane M, molecular weight markers; lane 1, ETEC 3; lane 2, ETEC 3 LT-gene PCR product digested with *Cfo*I enzyme; lane 3, ETEC 33; lane 4, ETEC 33 LT-gene PCR product digested with *Cfo*I enzyme; lane 5, ETEC 8; lane 6, ETEC 8 LT-gene PCR product digested with *Cfo*I enzyme; lane 7, ETEC 46; lane 8, ETEC 46 LT-gene PCR product digested with *Cfo*I enzyme. **(B)** Colorimetric dot-blot detection. DIG-labeled PCR product generated as outlined in **Subheading 3.3.** Dot-1, ETEC 3; Dot-2, ETEC 33; Dot-3, ETEC 8; Dot-4, ETEC 46; Dot-5, DIG-labeled pBR328 positive kit control (10 pg/μL) and Dot-6, negative control. (**C**) Fluorescent detection of the 275-bp DNA fragment from the LT-toxin gene after labeling the forward primer LT-1 (*see* **Subheading 1.4.3.**) with the HEX-fluorescent dye. The black peak represents the LT-toxin gene derived DNA amplicon and the red peaks are from TAMRA-labeled GENESCAN 500 molecular weight standards. (*See* color plate 3 after p. 114.)

3.6. Fluorescent Detection

1. The 275-bp LT-toxin derived amplicon can also be detected using fluorescence, wherein one or both primers are labeled with a fluorescent dye.
2. Following PCR amplification of the LT product using the fluorescent 4, 7, 2', 4', 5', 7'-heaxchloro-6-carboxyfluorescein (HEX)-labeled PCR primer, the product was column purified using the QIAquick PCR purification kit following the manufacturers instructions. This clean up step is designed to remove extraneous primers, nucleotides, polymerases, and salts from the desired product using microspin technology.
3. Each sample is then prepared for GENESCAN analysis by adding 48 μL of ddH_2O, 0.5 μL of TAMRA-500 size standard and 0.5 μL of QIAquick purified PCR product into the ABI Prism sample tubes with septa attached (*see* **Note 14**). The samples are placed in the holding rack of the ABI 310 Prism Genetic Analyzer.
4. Each sample is electroinjected for 5 s at 7.0 kV and then run at 11 kV at 30°C for 25 min.
5. Results are displayed in an electropherogram on the accompanying PowerMac (Apple Computers, Cupertino, CA). The display shows the GENESCAN standard as a series of red peaks ranging in size from 35 to 500 bp (*see* **Notes 15** and **Fig. 2C**).

4. Notes

1. A stock solution can be prepared and aliquoted into smaller volumes which can then be stored at –20°C until required.
2. This solution should be of Analar quality.
3. All primers used in this laboratory are purchased from R and D Systems, Abington,UK and are purified by polyacrylamide gel electrophoresis prior to use. For fluorescent detection, the LT-1 primer was labeled with the HEX (4, 7, 2', 4', 5', 7'-heaxchloro-6-carboxyfluorescein)-dye.
4. Ethidium bromide is a carcinogenic compound and should be handled with care. Latex gloves should be worn when handling buffers or gels containing ethidium bromide.
5. Dissolve the blocking agent (supplied with the kit) by heating the solution to 60°C in Buffer 2 at least 2 h prior to use. Experiments carried out in this laboratory have shown that casein behaves in an identical fashion to the supplier's reagent.
6. As the color reagents are light-sensitive, color development should be carried out in a darkened area. Ideally the membrane plus color solution can be wrapped in aluminum foil and placed in a drawer.
7. All of the reagents and software required to run the ABI 310 Prism are supplied as specialist items by Applied Biosystems (Foster City, CA).
8. Inclusion of the oil prevents evaporation of the reaction mixture.
9. The thermocycler used in this study was a Techne Programmable Dry-Block PHC-2 (Techne, Cambridge, UK).
10. Never vigorously stir molten agarose as the flask may boil-over causing severe skin burns.
11. All ETEC strains listed in **Table 1** were tested for the presence of an LT-toxin encoding gene by PCR. A 275 bp internal amplification product delineated by primers LT-1 and LT-2 was detected in all cases as demonstrated by agarose gel electrophoresis

(*see* **Fig. 2A**, lanes 1, 3, 5, and 7). The PHLS strains 7539, 9060, and C600 do not contain the LT-toxin encoding gene and consequently failed to produce this PCR product ***(35)***. Similarly, when other enteric pathogens including *V. cholerae*, were tested no amplified product was detected. This LT-PCR assay described is specific for the ETEC-encoding toxin gene only as no crossreaction at the DNA level was detected with *V. cholerae* despite the fact that both toxins are immunologically similar.

In order to confirm the specificity of the PCR product the amplified fragments were tested for the presence of a *Cfo*I restriction site (5'-GCGC-3'). Digestion of the 275-bp amplicon with *Cfo*I produces two DNA fragments of 104 and 171 bp. All LT-toxin encoding ETEC strains tested produced these expected fragments (*see* **Fig. 2A**, lanes 2, 4, 6, and 8).

12. All of these volumes relate to a nylon membrane of 100 × 100 mm surface area. These volumes can be scaled up or down as appropriate.
13. Incorporation of DIG-11-dUTP in the nucleotide mixture allows simultaneous amplification and DIG-labeling of the resultant PCR product. **Figure 2B** shows a colorimetric detection assay for the ETEC strains 3, 8, 33, and 46. Detection of DIG-labeled amplicons following PCR increases sensitivity compared with probing methods using the same label. Fourteen bacterial colony forming units can be detected ***(33)***. Large numbers of bacteria can be rapidly analyzed for the presence of the LT-toxin gene by pooling individual isolates as already demonstrated. After PCR, labeled product is then detected in <2 h. Several reports have described the application of nonradioactive strategies for the detection of *E. coli* enterotoxin coding genes ***(35–40)***.
14. Attachment of the septa to each tube is critical to guide the the electrode for sample electroinjection. Check to ensure that each septum is open prior to use.
15. The LT-toxin coding gene in ETEC 8 (*see* **Table 1**) was amplified as previously described (***see*** **Subheading 3.2.**). However in this example the forward primer LT-1, was modified by conjugating a fluorescent dye via an aminolink to the 5'-end of the oligonucleotide. Following PCR, amplified product is then electrophoresed through a laser beam and the emitted fluorescence detected. **Figure 2C** shows the dye-labeled LT-PCR product (275-bp) as a single black peak, which can be sized by direct comparison with the internal TAMRA-labeled size standards. Detection by this method (following PCR) is possible in <20 min and again like the solid-phase method above large sample numbers are conveniently analyzed.

Amplification-mediated assays linked to colorimetric systems provide a sensitive LT-toxin gene detection method that may be used in a routine laboratory setting. Their sensitivity would be important in the investigation of ETEC disease epidemiology. Specific applications would include the monitoring of carriers (both human and animal) and the investigation of environmental sources, food, and water. These studies will be necessary if this organisms epidemiology is to be fully understood and effective control measures implemented.

Acknowledgments

The authors wish to thank John Flynn and Fergus Shanahan, Carmel Wall, Sinéad Cotter, and Liam Blake for critically evaluating this manuscript.

Research funding for some of this work was obtained from both the Health Research Board (grant numbers: 50/92 and 57/93) and Forbairt (grant number: ST/92/028) and this financial support is gratefully acknowledged. L. C. is supported by a scholarship from the Cork Regional Technical College and S. F. is a Fulbright Scholar.

References

1. Snyder, J. D. and Merson, M. H. (1982) The magnitude of the global problem of acute diarrhoeal disease: a review of active surveillance data. *Bull. WHO* **60,** 605–613.
2. Tardelli-Gomes, T. A., Rassi, V., MacDonald, K. L., Silva-Ramos, S. R. T., Trabulsi, L. R., Vieira, M. A. M., Guth, B. E. C., Candeias, J. A. N., Ivey, C., Toledo, M. R. F., and Blake, P. A. (1991) Enteropathogens associated with acute diarrhoeal disease in urban infants in Sào Paulo, Brazil. *J. Infect. Dis.* **164,** 331–337.
3. Cryan, B. (1989) A competitive enzyme-linked immunosorbent assay for detecting Escherichia coli heat-stabile enterotoxin and its application to clinical isolates. *J. Infect.* **18,** 59–66.
4. Yamamoto, T. and Yokota, T. (1989) Sequence of heat-labile enterotoxin of *Escherichia coli* pathogenic for humans. *J. Bacteriol.* **1550,** 729–733.
5. Dallas, W. S. and Falkow, S. (1980) Amino acid sequence homology between cholera toxin and *Escherichia coli* heat-labile toxin. *Nature* **288,** 499–501.
6. Moseley, S. I., Hardy, J. W., Huq, M. H., Escheverria, P., and Falkow, S. (1983) Isolation and nucleotide sequence determination of a gene encoding a heat-stable enterotoxin of *Escherichia coli. Infect. Immun.* **39,** 1167–1174.
7. Sixma, T. K., Pronk, S. E., Kalk, K. H., Wartna, E. S., van Zanten, B. A. M., Witholt, B., and Hol, W. G. J. (1991) Crystal structure of a cholera toxin-related heat-labile enterotoxin from *E. coli. Nature* **351,** 371–377.
8. Acheson, D. W. K. (1992) Enterotoxins in acute infective diarrhoea. *J. Infect.* **24,** 225–245.
9. Echeverria, P., Taylor, D. N., Seriwatana, J., Brown, J. E., and Lexomboon, U. (1989) Examination of colonies and stool blots for detection of enteropathogens by DNA hybridization with eight DNA probes. *J. Clin. Micro.* **27,** 331–334.
10. Sack, D. A. and Sack, R. B. (1975) Test for enterotoxigenic *Escherichia coli* using Y1 adrenal cells in miniculture. *Infect. Immun.* **11,** 334–336.
11. Guerrant, R. L., Brunton, L. L., Schnaitman, T. C., Rebhun, L. I., and Gilman, A. G. (1974) Cyclic adenosine monophosphate and alteration of chinese hamster ovary cell morphology; a rapid, sensitive, *in vitro* assay for the enterotoxins of *Vibrio cholerae* and *Escherichia coli. Infect. Immun.* **10,** 320–327.
12. Pedler, S. J. and Orr, K. E. (1990) Examination of faeces for bacterial pathogens. *J. Clin. Pathol.* **43,** 410–415.
13. Johnson, W. M. and Lior, H. (1987) Response of chinese hamster ovary cells to cytolethal distending toxin (CDT) of *Escherichia coli* and possible misinterpretation as heat labile enterotoxin. *FEMS. Micro. Letts.* **43,** 19–23.
14. Honda, T., Taga, S., Takeda, Y., and Miwatani, T. (1981) Modified Elek test for detection of heat-labile enterotoxin of enterotoxigenic *Escherichia coli. J. Clin. Microbiol.* **13,** 1–5.

15. Ito, T., Kuwahar, S., and Yokota, T. (1983) Automatic and manual latex agglutination tests for the measurement of cholera toxin and heat-labile enterotoxin of *Escherichia coli. J. Clin. Microbiol.* **17,** 7–12.
16. Ristaino, P. A., Levine, M. M., and Young, C. A. (1983) Improved GM1-enzyme-linked immunosorbent assay for the detection of *Escherichia coli* heat-labile toxin. *J. Clin. Microbiol.* **18,** 808–815.
17. Moseley, S. L., Echeverria, P., Seriwatana, J., Tirapat, C., Chaicumpa, W., Sakuldaipeara, T., and Falkow, S. (1982) Identification of enterotoxigenic *Escherichia coli* by colony hybridization using three enterotoxin gene probes. *J. Infect. Dis.* **145,** 863–869.
18. Landegren, U., Kaiser, R., Caskey, C. T., and Hood, L. (1988) DNA diagnostics-molecular techniques and automation. *Science* **242,** 229–237.
19. Mullis, K. B. and Faloona, F. (1987) Specific synthesis of DNA *in vitro* via a polymerase-catalysed chain reaction. *Meth. Enzymol.* **155,** 335–350.
20. Saiki, R. K., Scharf, F., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N. (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. *Science* **230,** 1350–1354.
21. Mullis, K., Ferré, F., and Gibbs, R. A. (1994) *The Polymerase Chain Reaction* Birkhäuser-Verlag AG, Basel, Switzerland.
22. Frankel, G., Giron, J. A., Valmassoi, J., and Schoolnik, G. K. (1989) Multi-gene amplification: simultaneous detection of three virulence genes in diarrhoeal stool. *Mol. Microbiol.* **3,** 1729–1734.
23. Edwards, M. and Gibbs, R. (1994) Multiplex PCR: advantages, development and applications. *PCR Meth. Applic.* **3,** S65–S75.
24. Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98,** 503–517.
25. Langer, P. R., Waldrop, A. A., and Ward, D. C. (1981) Enzymatic synthesis of biotin-labelled polynucleotides: novel acid affinity probes. *Proc. Natl. Acad. Sci. USA* **78,** 6633–6637.
26. Bayer, E. A. and Wilchek, M. (1990) *Avidin-Binding Technology. Methods in Enzymology*, vol. 184, Academic, San Diego, CA.
27. Olive, D. M. (1989) Detection of enterotoxigenic *Escherichia coli* after polymerase chain reaction amplification with a thermostable DNA polymerase. *J. Clin. Microbiol.* **27,** 261–265.
28. Strasberg, P. (1989) Evaluation of the biotinylated (Blue gene™) vs. ^{32}P-labelled cDNA probes of β-glucocerebrosidase: relative sensitivities in genomic and other systems. *Clin. Chem.* **35,** 1512–1516.
29. Kessler, C. (1992) *Non-Radioactive Labelling and Detection of Biomolecules.* Springer-Verlag, Berlin, Germany.
30. Höltke, H. J. and Kessler, C. (1990) Non-radioactive labelling of RNA transcripts in vitro with the hapten digoxigenin (DIG); hybridization and ELISA-based detection. *Nucleic Acids Res.* **18,** 5843–5851.
31. Kessler, C. (1991) The digoxigenin:anti-digoxigenin (DIG) technology—a survey on the concept and realization of a novel bioanalytical indicator system. *Mol. Cell Probes* **5,** 161–205.

32. Holmstrøm, K., Rossen, L., and Rasmussen, O. F. (1993) A highly sensitive and non-radioactive method for detection of polymerase chain reaction products. *Anal. Biochem.* **209,** 278–283.
33. Fanning, S., O'Mullane, J., O'Meara, D., Ward, A., Joyce, C., Delaney, M., and Cryan, B. (1995) Detection of the heat-stable toxin coding gene (ST-gene) in enterotoxigenic *Escherichia coli*: development of a colour amplified PCR detection system. *Br. J. Biomed. Sci.* **52,** 317–320.
34. Furrer, B., Candrian, U., and Luthy, J. (1990) Detection and identification of Escherichia coli producing heat-labile enterotoxin type 1 by enzymatic amplification of a specific DNA fragment. *Lett. Appl. Microbiol.* **10,** 31–34.
35. O'Meara, D., O'Shaughnessy, E., Fanning, S., and Cryan, B. (1995) Colourimetric detection of heat-labile toxin-encoding gene of enterotoxigenic *Escherichia coli* by PCR. *J. Clin. Microbiol.* **33,** 1957–1960.
36. Riley, L. K. and Caffrey, C. J. (1990) Identification of enterotoxigenic *Escherichia coli* by colony hybridization with non-radioactive digoxigenin-labelled DNA probes. *J. Clin. Microbiol.* **28,** 1465–1468.
37. Abe, A., Obata, H., Matsushita, S., Yamada, S., Kudoh, Y., Bankrakulnonth, A., Ratchtrachenchat, O. R., and Danbara, H. (1992) A sensitive method for the detection of enterotoxigenic *Escherichia coli* by the polymerase chain reaction using multiple primer pairs. *Zbl. Bakt.* **270,** 170–178.
38. Ølsvik, O. and Strockbine, N. (1993) PCR detection of heat-stable, heat labile, and shiga-like toxin genes in *Escherichia coli*, in *Diagnostic Molecular Microbiology.* American Society for Microbiology, Washington, DC.
39. Schultsz, C., Pool, G. J., van Ketel, R., de Wever, B., Speelman, P., and Dankert, J. (1994) Detection of enterotoxigenic *Escherichia coli* in stool samples by using non-radioactively labelled oligonucleotide DNA probes and PCR. *J. Clin. Microbiol.* **32,** 2393–2397.
40. Tamanai-Shacoori, Z., Jolivet-Gougeon, A., Pommepuy, M., Cormier, M., and Colwell, R. R. (1994) Detection of enterotoxigenic *Escherichia coli* in water by polymerase chain reaction amplification and hybridization. *Can. J. Microbiol.* **40,** 243–249.

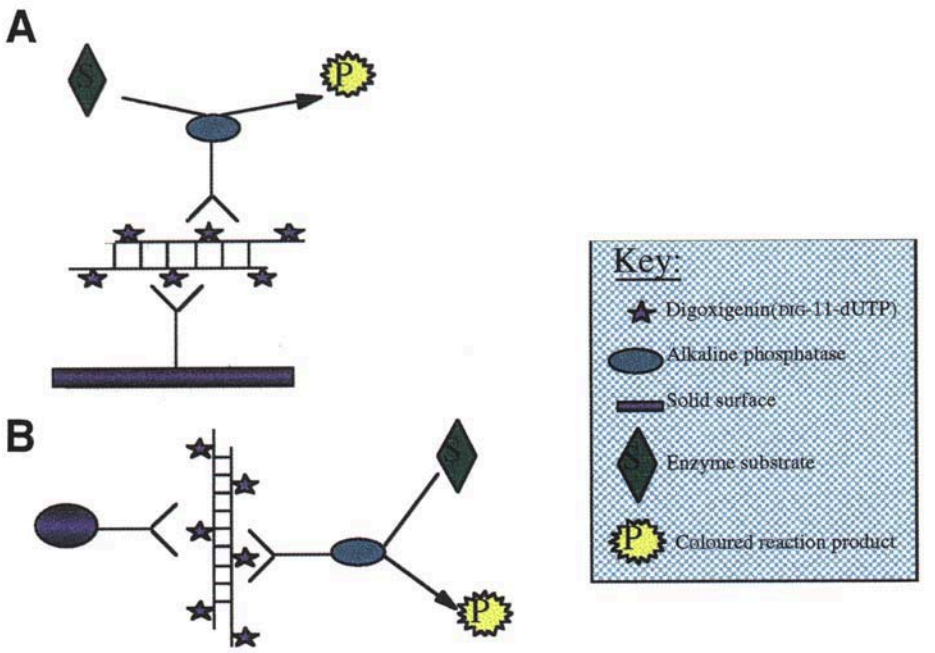

Plate 1 (Fig. 1; *see* full caption on p. 104 and discussion in Chapter 9). Colorimetric detection of digoxigenin (DIG)-labeled DNA.

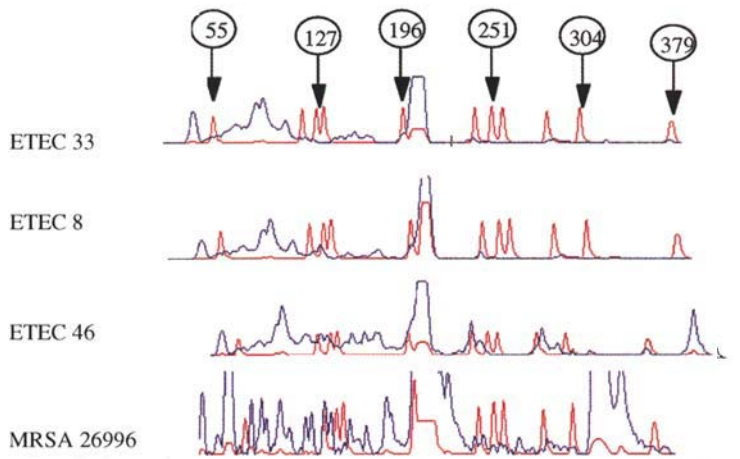

Plate 2 (Fig. 4; *see* full caption on p. 122 and discussion in Chapter 10). GeneScan analysis of ETEC isolates and a methicillin-resistant *Staphylococcus aureus* (MRSA) isolate typed with the FAM-labeled RW3A primer. The blue peaks correspond to strain-specific DNA fingerprints obtained in each case and the red peaks are internal lane molecular weight standards.

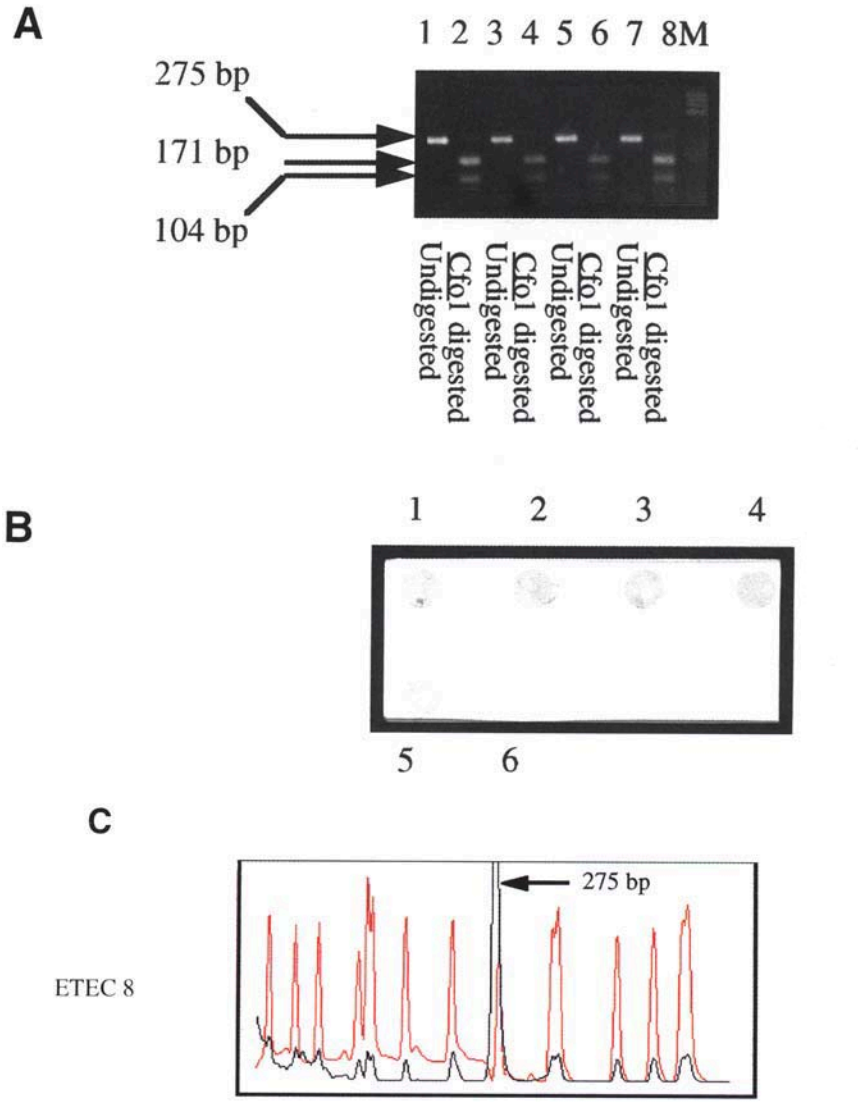

Plate 3 (Fig. 2; *see* full caption on p. 109 and discussion in Chapter 9). (A) Agarose gel electrophoresis of amplified LT-specific PCR products (2% NuSieve gel in 1X TAE buffer). **(B)** Colorimetric dot-blot detection. DIG-labeled PCR product generated as outlined in **Subheading 3.3.** Dot-1, ETEC 3; Dot-2, ETEC 33; Dot-3, ETEC 8; Dot-4, ETEC 46; Dot-5, DIG-labeled pBR328 positive kit control (10 pg/μL) and Dot-6, negative control. (**C**) Fluorescent detection of the 275-bp DNA fragment from the LT-toxin gene after labeling the forward primer LT-1 (*see* **Subheading 1.4.3.**) with the HEX-fluorescent dye. The black peak represents the LT-toxin gene derived DNA amplicon and the red peaks are from TAMRA-labeled GENESCAN 500 molecular weight standards.

10

Motif-Dependent Polymerase Chain Reaction (PCR)

DNA Fingerprinting Enterotoxigenic Escherichia coli

Séamus Fanning, Deirdre O'Meara, Lesley Cotter, Paddy Greer, and Bartley Cryan

1. Introduction

Fingerprinting techniques are essential tools in the investigation of transmissible diseases. Unless one is able to track a pathogenic organism from its reservoir, through its vectors and carriers into infected hosts, it is impossible to define an organism's epidemiology. In the absence of such understanding public health and other measures directed toward the eradication of the infection are unlikely to succeed.

Traditionally, *Escherichia coli* are identified on the basis of their biochemical and cultural properties. They can be further classified on the basis of lipopolysaccharide (O), capsular (K), and flagellar (H) antigens. At present, 170 O, 71 K, and 56 H antigens have been identified and varying combinations of these antigens have been detected among isolates from natural sources.

Serotyping has potential as a powerful typing approach method and based on this approach *E. coli*, which act as enteric pathogens can be classified into four main groups: enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), and enterohaemorrhagic (EHEC). Each group contains a restricted number of serotypes. The following O groups are associated with enterotoxin production 6, 8, 15, 25, 27, 63, 78, 115, 148, 153, and 159. Several serotypes have a world-wide distribution (e.g., O78). Others, such as O159 (Japan) and O139 (Brazil), have been restricted to specific areas ***(1)***.

As a fingerprinting approach serotyping is limited by the predominance of common types in geographic areas that reduce its discrimination. It remains useful in outbreaks associated with unusual serotypes.

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

Antibiotic sensitivities are easy to carry out and give clinically useful information. Antibiograms can be used to type strains; while locally useful this approach may be misleading because of variable expression of the resistance phenotype.

Technical considerations related to typing microorganisms include issues like reproducibility, sensitivity, power of discrimination, and typability ***(2)***. The ability to examine a microbial genotype directly using DNA-based methods has several advantages for both infectious disease diagnosis and epidemiology. Phenotypic approaches to organism typing depend(s) on the expression of host genes in artificial culture conditions. This feature contributes significantly toward the variability frequently noted with such methods as phage typing, bacteriocine production, biotyping, antibiogram profiling, and serotyping. In any population of organisms examined using these methods it would not be unusual to obtain a number of negative results (i.e., nontypable). For example this observation is frequently noted when methicillin-resistant *Staphylococcus aureus* organisms are being phage-typed (Cotter, unpublished results). By comparison genetic characteristics are relatively stable and can usually be recovered independent of culture conditions. Chromosomal typing has the potential to analyze any organism under investigation from which DNA can be isolated.

Genome-amplification typing strategies can be used to track any organism in a microbial population. An ideal strategy would type all organisms, and would be capable of discriminating and reproducing fragment arrays or fingerprints over time and between laboratories. In addition, such a method should be simple to use and fast enough to provide clinicians with informative results. A molecular method that facilitates the differentiation between microorganisms would prove to be a valuable tool in areas such as infection diagnosis and surveillence, epidemiology, quality control typing strains for industrial/medical diagnostic assays, food diagnostics wherein typing would be used to detect pathogenic organisms and in areas such as bioremediation and the monitoring of released genetically engineered organisms into the environment. Previously all of these necessitated the use of DNA hybridization strategies combined with specifically designed probes.

The polymerase chain reaction (PCR) has contributed towards the development of novel methods to rapidly and specifically distinguish between even closely related microorganisms (for reviews *see* **refs. *3–6***). Several useful strategies have been developed and applied to the epidemiological typing of microorganims ***(7–9)***. A number of these methods combine the use of PCR and restriction enzyme analysis to detect sequence polymorphisms, allowing strains to be differentiated ***(10)***. Others depend on specifically designed primers used to target genomic regions encoding genes for rRNA and tRNA synthesis. In these examples, PCR was used to detect either sequence polymorphisms or variations in intergenic spacer regions associated with rRNA or tRNA genes ***(11,12)***.

An alternative approach to the above methods, amplification from conserved repetitive DNA sequences found interspersed throughout prokaryotic genomes can be performed. These sequences vary in complexity and distribution and have formed the basis for motif-dependent PCR. Examples range from the simple short polynucleotide repeat (5'-GCTGG-3'), to polynucleotide sequences frequently encountered in the genomes of both *E. coli* and *Salmonella typhimurium* ***(13)***. More complex repeats include the 38-bp repetitive extragenic palindromic consensus-REP ***(14,15)***, the 126-bp enterobacterial repetitive extragenic consensus-ERIC sequence ***(16)***, the box-element ***(17)***, and the Mep-1 and -2 sequences of *Mycoplasma pneumoniae* ***(18)***.

Figure 1 details the essential features of motif-dependent PCR detection. Briefly, primer pairs are designed to the 5'- and 3'-extremeties of the repeat locus. They are orientated toward the inter-repeat regions. After each annealing step in the PCR reaction, these primers extend into this intervening region. Provided the distance to the next repeat is not beyond the processive range of *Taq* DNA polymerase, an amplified product is produced. Theoretically, these events occur at each repeat locus around the genome. Finally, resolution of all differently sized amplicons produced (consisting of unique sequences located between the repetitive elements), on an ethidium bromide stained agarose gel results in the characteristic DNA fingerprint pattern ***(19,20)***.

Data acquisition using agarose gels has several technical disadvantages (e.g., lack of sensitivity, lane-to-lane variations among gels, and low sample throughput). In more recent developments, computer-aided detection and data storage methods have been developed. In particular, by labeling one or both primers with a fluorescent dye all amplified DNA fragments become labeled and can be detected using a laser scanning device such as that found in an automated DNA sequencing machine ***(21–23)***. Suitable genescan software can then automate the gel analysis. Apart from automating data storage, this approach has the advantage of facilitating retrospective comparisons. In the future such systems could facilitate the development of a digitized data base that could be accessed directly for organism identification.

2. Methods

2.1. DNA Template Preparation

(*See* Chapter 9, Subheadings 2.1. and 3.1. for detailed DNA template isolation protocols. These methods were used for both direct DNA detection and fingerprinting because of the high quality nature of the template obtained).

2.2. PCR Amplification

1. Sterile water.
2. 10X PCR buffer: 100 m*M* Tris-HCl (pH 9.0), 500 m*M* KCl, 1% Triton X-100, 3 m*M* $MgCl_2$.

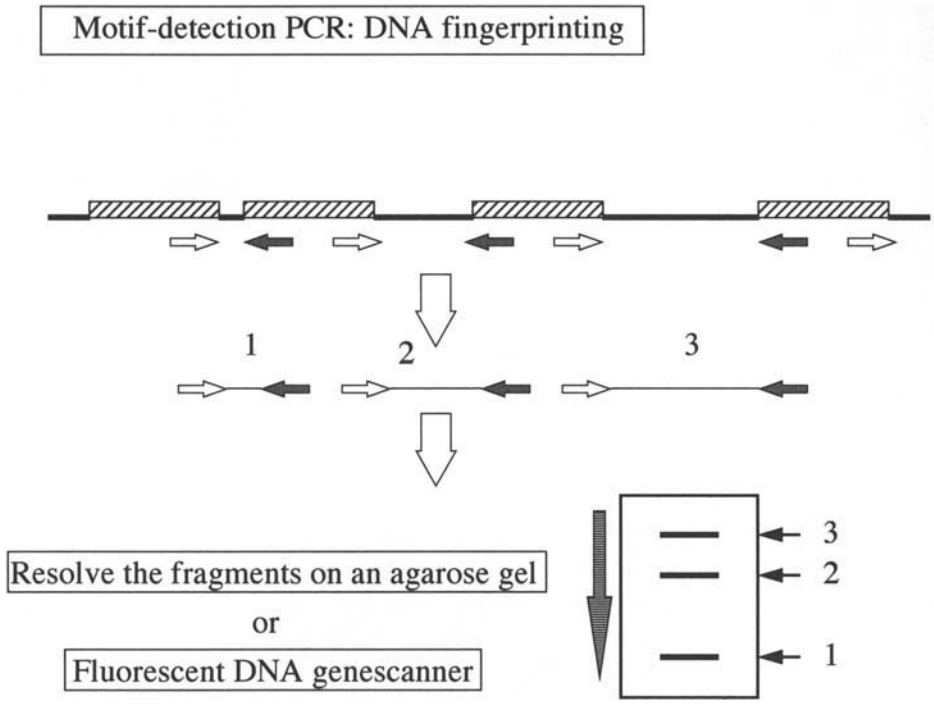

Fig. 1. Motif-dependent PCR DNA fingerprinting. Primers (represented here by the open and shaded colored arrowheads) are designed to conserved regions (hatched regions) located at the 5'- and 3'-ends of the repeat element. Importantly these primers are directed toward the inter-repeat DNA regions. PCR amplification produces a number of DNA fragments differing in size that can be resolved by conventional gel electrophoresis (*see* **Fig. 2A–C**) or by gene scanning (*see* **Fig. 4**). The DNA pattern displayed is the strain or genome specific DNA fingerprint.

3. 100 m*M* stocks of each deoxyribonucleoside triphosphates dATP, dCTP, dGTP, and dTTP (Promega, Madison, WI).
4. dNTP working stocks contain 1.25 m*M* of each dNTP. This is prepared by diluting 2.5 µL of each dNTP to 190 µL of sterile H_2O.
5. Oligonucleotide primers ***(9)*** diluted to 300 p*M* in sterile water.
   REP-1 (forward direction) 5'-III ICG ICG ICA TCI GGC-3'
   REP-2 (reverse direction) 5'-ICG ICT TAT CIG GCC TAC-3'
   (All primers were purchased from R and D Systems, Abington, UK and were purified by polyacrylamide gel electrophoresis prior to use).
6. Sterile 0.5 mL PCR tubes.
7. *Taq* DNA polymerase (5 U/mL) (Promega).
8. Mineral oil (Sigma, Poole, UK).

2.3. Agarose Gel Electrophoresis

1. Agarose (Sigma, Poole, UK).
2. Ethidium bromide (10 mg/mL) in sterile H_2O.
3. 1X Tris-acetate-EDTA (TAE) buffer (prepared as described in Chapter 9, Subheading 2.3.).
4. Gel loading dye: 0.25% (w/v) bromophenol blue, 80% (v/v) glycerol prepared in sterile H_2O.
5. DNA molecular weight markers: A range of DNA markers with various fragment molecular weight ranges are commercially available. In this case, DNA Markers III (Boehringer Mannheim, Germany) are used providing fragments from 125-bp to 21.2-kb.

2.4. Fluorescent (F) DNA Fingerprinting

1. Applied Biosystems 310 Prism Genetic Analyzer (Applied Biosystems, Foster City, CA).
2. Sterile packs of 0.5 mL sample tubes and sample tube septa (*see* **Note 1**).
3. ABI 310 Prism GENESCAN capillary (61 cm × 71 mm) (*see* **Note 2**).
4. GENESCAN polymer, electrophoresis buffer and 6-carboxy-X-rhodamine (ROX)-labeled 2500 size standards (*see* **Note 3**).
5. RW3A primer: 5'-TCG CTC AAA ACA ACG ACA CC-3' ***(18,22)***. This primer was labeled with the fluorescent dye, 6-carboxy-fluorescine (FAM), via an amino link at the 5'-end (Genosys Biotechnologies, Cambridge, UK).

3. Methods

3.1. REP-Motif Amplification

1. Amplifications were performed in 25 µL reaction volumes in 0.5 µL PCR tubes containing 27.1 pmol of REP-1 and 32.5 pmol of REP-2 primers, 200 µ*M* of each dNTP and 2 m*M* $MgCl_2$.
2. 2.5 µL 10X *Taq* DNA polymerase buffer and 2.5 U *Taq* DNA polymerase was then added.
3. Approximately 300–500 ng of DNA template, corresponding to 1.8 µL (when prepared according to the method outlined in Chapter 9, Subheading 3.1.).
4. All reaction mixes were overlaid with 100 µL mineral oil to prevent evaporation during cycling.
5. Thermocycling was carried out in a dedicated thermocycler (*see* **Note 4**), using the following cycling conditions; 90°C for 4 min (1 cycle), 90°C for 1 min, 40°C for 1 min, 65°C for 8 min (30 cycles), and 65°C for 16 min (1 cycle).
6. A negative control containing all reagents except template DNA was included in every experiment.

3.2. Agarose Gel Electrophoresis and Analysis of DNA Fingerprints

1. After amplification each reaction was analyzed on a 1% agarose gel in 1X TAE buffer.
2. Onto a strip of parafilm paper spot 2 µL of the loading dye and to this add 10 µL from each reaction sample. Include the molecular weight markers at this time.

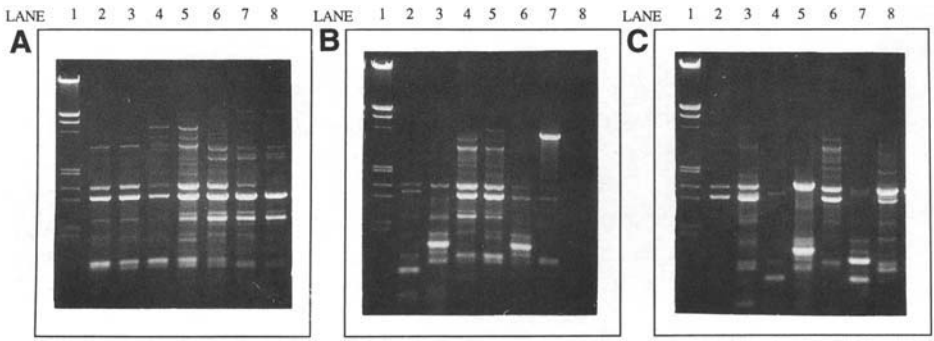

Fig. 2. Agarose gel (1%) electrophoresis of inter-repetitive extragenic palindromic (REP) amplification products. **(A)** Lane 1, molecular weight markers; Lane 2, ETEC 3; Lane 3, ETEC 938 (2); Lane 4, 851 (1); Lane 5, ETEC 473 (4); Lane 6, ETEC 390 (1); Lane 7, ETEC PHLS 6085; Lane 8, ETEC 387 (v). **(B)** Lane 1, molecular weight markers; Lane 2, ETEC 398 (1); Lane 3, ETEC 6; Lane 4, ETEC 73; Lane 5, ETEC 33; Lane 6, ETEC 450 (3); Lane 7, ETEC 46; Lane 8, negative control. **(C)** Lane 1, molecular weight markers; Lane 2, ETEC PHLS 9060; Lane 3, ETEC 285 (v); Lane 4, ETEC 128; Lane 5, ETEC PHLS 8086; Lane 6, ETEC PHLS 7539; Lane 7, C600; Lane 8, ETEC 8.

3. Load each sample into the preformed wells of the 1% agarose gel and run on constant voltage at 80 V for 1.5 h.
4. All amplification products were visualized by ethidium bromide (0.5 μg/mL) staining under UV light and photographed for a permanent record (*see* **Figs. 2A–C** and **Note 5**).
5. Quantitative analysis of all DNA fingerprint patterns was performed. A binary matrix (wherein presence of a band = 1; absence of a band = 0),was then constructed, which allowed similarity coefficients to be calculated following pairwise comparisons. Hierarchical clustering was performed using the Centroid Linkage method (***20*** and references therein). The corresponding dendogram is shown in **Fig. 3**.

3.3. (F)-Based DNA Fingerprinting

1. As an alternative to REP-DNA fingerprinting motif-dependent PCR was performed using a single primer RW3A derived from the Mep-2 repeat in *M. pneumoniae **(18)***.
2. Amplification reactions were performed in a total volume of 50 μL containing 75 pmol FAM-labeled RW3A primer ***(22)*** with the remainder of the amplification mixture identical to that outlined for REP-PCR above (*see* **Subheading 3.1.**).
3. 200 ng of template DNA was used for each reaction.
4. Thermal cycling was performed using the following temperatures and times; 94°C for 3 min (1 cycle), 94°C for 1 min, 54°C for 1 min, 72°C for 2 min (30 cycles), and finally 72°C for 5 min (1 cycle) to complete extension (*see* **Note 4**).

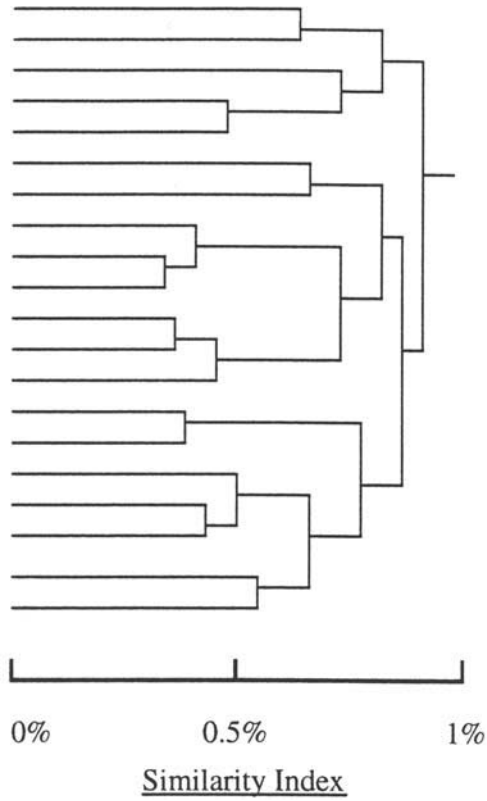

Fig. 3. Dendrogram illustrating the similarity between the ETEC isolates in this study using the Centroid Linkage method.

3.4. Analysis of (F)-Labeled Amplicons

1. To 12 μL sterile ddH_2O, an aliquot (0.5 μL) of the amplification mix and 0.5 μL of ROX-labeled GeneScan 2500 internal size standards were added.
2. This mixture was then electroinjected for 30 s at 7 kV from a 0.5 mL sample tube onto a 50 cm (*see* **Note 2**), 2.5% polyacrylamide capillary gel in the ABI 310 Prism Genetic Analyzer.
3. Both the FAM-labeled PCR fragments and the ROX-labeled size standards were resolved by running at 11 kV at for 30 min at 30°C.
4. Using the system GeneScan Software (ver. 2. 0. 2) each PCR product was automatically sized and quantified with reference to the internal lane standards (*see* **Note 6**). This approach minimizes any potential errors which may arise between runs (*see* **Note 7**).

4. Notes

1. All tubes used in the ABI 310 Prism Genetic Analyzer must have septa attached. This is necessary to properly guide the instrument electrode into the sample for electroinjection. Also ensure that all septa are open prior to use.

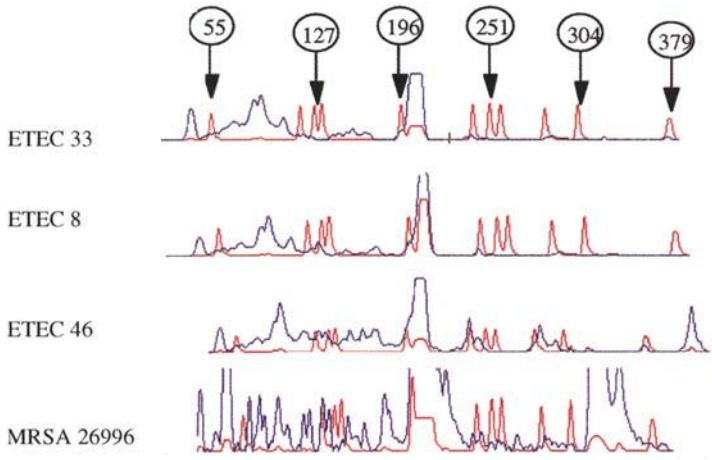

Fig. 4. GeneScan analysis of ETEC isolates and a methicillin-resistant *Staphylococcus aureus* (MRSA) isolate typed with the FAM-labeled RW3A primer described in the text. All fragments were separated in 30 min. The blue peaks (derived from the FAM-label) correspond to strain-specific DNA fingerprints obtained in each case and the red peaks (derived from a ROX-label) are internal lane molecular weight standards. Molecular weights in bp of some of the standard peaks are given in the arrowed circles. (*See* color plate 2 after p. 114.)

2. Capillaries can be shortened to provide the fragment resolution required. In this study, a 50 cm length (i.e., from sample injection to detector) was used.
3. All reagents and software for the ABI 310 Prism were purchased as specialist items from Applied Biosystems.
4. The thermocycler used in this study was a Pharmacia GeneATAQ Controller (Pharmacia LKB Biotechnology, Uppsala, Sweden).
5. The number of DNA fragments generated for each REP-fingerprint varied from four to sixteen and ranged in size from 250 bp to 4.8 kb (**Fig. 2**). **Figure 3** shows that all ETEC strains could be divided into three main groups designated REP-groups I–III. Each grouping consists of a number of closely related strains. The results of serotyping, and REP-fingerprinting are given (*see* **Table 1**)
6. All scans (*see* **Fig. 4**) were confined to a narrow size range (55–400 bp) and the scan data obtained was stored on a Macintosh Computer (Apple Computer, Cupertino, CA) for analysis. The electropherograms generated by this method are shown in **Fig. 4** for three of the strains listed in **Table 1**, each representing a different REP-fingerprint group. Clearly all ETEC strains appear to be closely related within this scan region. However, some minor peak differences were noted. Comparing the ETEC fingerprints with the unrelated methicillin-resistant *Staphylococcus aureus* (MRSA) shows significant differences,with a greater number of FAM-labeled peaks being generated in the latter case. This result reflects not only the strain difference that

Table 1
Serotype and REP-Grouping Patterns of ETEC Strains

Strain no.	Serotype	REP-grouping pattern
473(4)	O78 K80	I
390(1)	O78 K80	I
33	O78 K80	I
73	O78 K80	I
PHLS[a] 6085	H12	I
3	O78 K80	II
938(2)	O78 K80	II
285(v)	O78 K80	II
8	O78 K80	II
6	O78 K80	II
PHLS 7539	O18 K77	II
PHLS 9060	H18	II
PHLS 8068	ns	II
387(v)	ns	III
450(3)	ns	III
C600	ns	III
851(1)	O78 K80	III
398(1)	O78 K80	III
46	O78 K80	III
128	O78 K80	III

[a]Abbreviations: ns = nonserotypable; PHLS = public health laboratory strain; REP = repetitive extragenic palindromic.

exists between the ETEC and MRSA organisms, but also the increased frequency with which the Mep-2 derived repeat element occurs in the Gram-positive cell.

7. GeneScan files generated in this way can easily be stored. These archived traces may then be useful to analyse future outbreaks or to assess bacterial strain evolution. The current limitation with this approach however is the lack of a suitable fingerprinting algorithm(s) that would automate pattern comparison. It is anticipated that in the future this data output could be digitised thereby facilitating the construction of a data base for all known pathogens. Simply searching this resource would enable the rapid identification of any organism.

Acknowledgments

The authors wish to thank John Flynn and Fergus Shanahan, Carmel Wall, Sinéad Cotter, Liam Blake, and John O'Mullane for critically evaluating this manuscript. Research funding for some of this work was obtained from the Health Research Board (grant numbers: 50/92 and 57/93) and this financial support is gratefully acknowledged. L. C. is supported by a College Scholarship from the Cork Regional Technical College and S. F. is a Fulbright Scholar.

References

1. Sack, B. R. (1980) Enterotoxigenic Escherichia coli, identification and characterisation. *J. Infect. Dis.* **142,** 279–286.
2. Versalovic, J., Woods, C. R., Georghiou, P. R., Hamill, R. J., and Lupski, J. R. (1993) DNA-based identification and epidemiologic typing of bacterial strains. *Arch. Pathol. Lab. Med.* **117,** 1088–1098.
3. Caetano-Anollés, G. (1993) Amplifying DNA with arbitrary oligonucleotide primers. *PCR Meth. Applic.* **3,** 85–94.
4. van Belkum, A. (1994) DNA fingerprinting of medically important microorganisms by use of PCR. *Clin. Microbiol. Rev.* **7,** 174–184.
5. Versalovic, J., Schneider, M., de Bruijn, F., and Lupski, J. (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Meth. Cell Biol.* **5,** 25–40.
6. Towner, K. J. and Cockayne, A. (1993) *Molecular Methods for Microbial Identification and Typing.* Chapman and Hall, London, UK.
7. Welsh, J. and McClelland, M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18,** 7213–7218.
8. Williams, J. G. K., Kubelik, A. R., Livak, K. J., Antoni Rafalski, J., and Tingey, S. V. (1990) DNA polymorphisms amplified by arbitrary primers are useful genetic markers. *Nucleic Acids Res.* **18,** 6531–6535.
9. Veraslovic, J., Koeuth, T., and Lupski, J. R. (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res.* **24,** 6823–6831.
10. Regnery, R. L., Spruill, C. L., and Plikaytis, B. D. (1991) Genotypic identification of rickettsiae and estimation of interspecies sequence divergence for portions of two rickettsial genes. *J. Bacteriol.* **173,** 1576–1589.
11. McClelland, M., Petersen, C., and Welsh, J. (1992) Length polymorphisms in tRNA intergenic spacers detected by using the polymerase chain reaction can distinguish streptococcal strains and species. *J. Clin. Microbiol.* **30,** 1499–1504.
12. Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D., and Schleifer, K. H. (1994) Development of an rRNA-targetted oligonucleotide probe for the genus Acinetobacter and its application for in situ monitoring in activated sludge. *Appl. Environ. Microbiol.* **60,** 792–800.
13. Doll, L., Moshitch, S., and Frankel, G. (1993) Poly (GTG)5-associated profiles of *Salmonella* and *Shigella* genomic DNA. *Res. Microbiol.* **144,** 17–24.
14. Higgins, C. F., Ames, G. F. L., Barnes, W. M., Clement, J. M., and Hofnung, M. (1982) A novel intercistronic regulatory element of prokaryotic operons. *Nature* **298,** 760–762.
15. Stern, M. J., Ferro-Luzzi Ames, G., Smith, N. H., Robinson, E. C., and Higgins, C. F. (1984) Repetitive extragenic palindromic sequences: a major component of the bacterial genome. *Cell* **37,** 1015–1026.
16. Hulton,C. S. J., Higgins, C. F., and Sharp, P. M. (1991) ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli, Salmonella typhimurium*, and other enterobacteria. *Mol. Microbiol.* **5,** 825–834.

17. Martin, B., Humbert, O., Camara, M., Guenzi, E., Walker, J., Mitchell, T., Andrew, P., Prudhomme, M., Alloing, G., Hakenbeck, R., Morrison, D. A., Boulnois, G. J., and Claverys, J.-P. (1992) A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae. Nucleic Acids Res.* **20,** 3479–3483.
18. Wenzel, R. and Herrmann, R. (1988) Repetitive DNA in *Mycoplasma pneumoniae. Nucleic Acids Res.* **16,** 8337–8350.
19. Sheehan, C., Lynch, M., Cullen, C., Cryan, B., Greer, P., and Fanning, S. (1995) Genomic fingerprinting *Acinetobacter baumanii:* amplification of multiple inter-repetitive extragenic palindromic sequences. *J. Hosp. Infect.* **31,** 33–40.
20. Cotter, L., Lynch, M., Cryan, B., Greer, P., and Fanning, S. (1997) Investigation of a methicillin-resistant *Staphylococcus aureus* (MRSA) outbreak in an Irish hospital: triplex-PCR and DNA amplification fingerprinting. *J. Hosp. Infect.* **36,** 37–47.
21. Versalovic, J., Kapur, V., Koeuth, T., Mazurek, G. H., Whittman, T. S., Musser, J. M., and Lupski, J. R. (1995) DNA fingerprinting of pathogenic bacteria by fluorophore-enhanced repetitive sequence-based polymerase chain reaction. *Arch. Pathol. Lab. Med.* **119,** 23–29.
22. Del Vecchio, V., Petroziello, J. M., Gress, M. J., McCleskey, F. K., Melcher, G. P., Crouch, H. K., and Lupski, J. R. (1995) Molecular genotyping of methicillin-resistant *Staphylococcus aureus via* fluorophore-enhanced repetitive-sequence PCR. *J. Clin. Microbiol.* **33,** 2141–2144.

11

In Vitro Transcription/Translation Analysis for the Identification of Translation-Terminating Mutations

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1. Introduction

The identification of mutations is very important in such aspects of molecular biology as medical diagnostics, ascertaining structure/function relationships, population genetic studies, and in confirming the authenticity of new candidate genes. Presently there are a variety of different techniques used to identify somatic and germline mutations. In some instances, gross genetic alterations are best characterized by cytogenetic analysis, FISH, or by Southern blot. However, in most cases the underlying mutations are too subtle to be revealed by these techniques and are best characterized by examination of PCR products generated from putative disease alleles. While there are over a dozen different methods to screen for mutant alleles in PCR products, no technique is absolutely sensitive and each has specific advantages and disadvantages in its ability to detect unknown mutations.

Commonly used methods such as single-stranded conformation polymorphism (SSCP) ***(1)*** and heteroduplex analysis ***(2)***, while simple to perform, are relatively insensitive in their ability to identify many mutations. Moreover, multiple electrophoretic conditions may have to be applied in order to detect a mutation, and the sensitivity of these techniques falls rapidly as the size of the PCR product increases. Thus, in large genes, multiple small segments of genetic material must be analyzed to screen for an unknown mutation. Techniques such as chemical mismatch cleavage ***(3)*** and denaturing gradient gel electrophoresis (DGGE) ***(4)*** are quite sensitive in their ability to detect mutations. However, with DGGE, special equipment is needed and often the attach-

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

ment of a G-C clamp to a primer is necessary in order to detect a heteroduplex between the wild type and mutant PCR fragments ***(5)***. Chemical mismatch cleavage has the ability to screen relatively large segments of genetic material ***(6)***, however, it requires the use of toxic reagents and can be difficult to perform. A derivative of chemical mismatch cleavage has recently been described in which heteroduplexes are cleaved with bacteriophage endonucleases ***(7,8)***. This technique appears to be quite sensitive and has the added advantage of allowing for the analysis of relatively long segments of DNA. Unfortunately, these bacteriophage resolvases do not recognize all mismatches, and to date the technique has only been applied in a limited number of laboratories. Direct DNA sequence analysis, while absolutely specific in identifying a genetic alteration, is presently too laborious to screen for mutations in large segments of genetic material. Recently, a novel technique that employs a coupled in vitro transcription/translation (IVTT) reaction was developed to reveal various mutations that result in a premature termination of an open reading frame ***(9,10)***. IVTT is proving to be remarkably useful in identifying unknown mutations and has many unique advantages over other mutational screening techniques.

The principle behind IVTT is the incorporation of a bacteriophage RNA polymerase start signal (e.g., bacteriophage T7: GGATCCTAATACGAC TCACTATAG) and a eukaryotic translation initiation sequence (CCACCA TGG) into the 5' end of an upstream PCR primer ***(11)***. Consequently, the resultant PCR product can function as a complete coding unit; from the double stranded PCR product RNA is synthesized and the resultant RNA is translated into protein. The in vitro transcription and translation reactions are typically performed in a coupled reaction using commercially available rabbit reticulocyte lysates. To detect the final protein product the translation reaction is performed in the presence of ^{35}S-labeled amino acids and in vitro synthesized polypeptides are separated by polyacrylamide gel electrophoresis. The gel is then fixed, dried and exposed to X-ray film for 2–16 h. Mutations are revealed by the generation of a novel polypeptide band which generally migrates faster than the full-length protein product. Thus, an individual heterozygous for a translation terminating mutation will produce two distinct bands, a full-length product generated from the wild type allele, and a truncated band generated from the mutant allele.

This technique was initially applied to detect a nonsense mutation in the dystrophin gene ***(9)*** and various mutations in the APC gene ***(10)***. We have applied this type of analysis to screen for unknown mutations in patients with neurofibromatosis type 1 and have shown that translation terminating mutations are common and are distributed throughout the very large NF1 gene ***(12,13)***. Recently, IVTT has been used to detect mutations in the BRCA1 gene in patients with familial breast cancer ***(14)*** and preliminary data on the muta-

tional spectrum in BRCA1 suggests that this technique will prove to be useful in identifying unknown mutations in this gene ***(15,16)***. One area in which IVTT has had immediate clinical utility is in screening for unknown mutations in familial colorectal cancer syndrome genes. Because of the genetic heterogeneity, and large size of some of the genes associated with familial colorectal cancer, IVTT is proving to be an invaluable tool in revealing pathogenic mutations and identifying presymptomatic patients.

Colorectal cancer is one of the most common malignancies in humans. While the majority of colorectal cancers appear to be sporadic, and can be attributed to the accumulation of somatically acquired genetic insults in various growth regulatory genes, 15–20% of cases of colorectal cancer show familial clustering ***(17)***. Moreover, it is estimated that as many as 4% of cases of colorectal cancer result from the transmission of an autosomal dominant gene ***(18)***. Consequently, identification of mutations in familial colorectal cancer genes is of clinical significance since the identification of presymptomatic patients will allow for the implementation of colorectal cancer screening procedures (i.e., endoscopy) for the individual and will alert the physician to the possibility of other family members carrying the same mutation with the consequence that the morbidity and mortality associated with colorectal cancer can be reduced.

Phenotypically, the most distinctive hereditary colorectal cancer syndrome is familial adenomatous polyposis (FAP). FAP is an autosomal dominant disease characterized by a predisposition to develop hundreds of adenomatous polyps in the large bowel. Although the risk of malignant tranformation for any one of these polyps is no greater than the risk for sporadic colon polyps, the sheer number of these premalignant lesions puts affected individuals at extreme risk of developing colorectal cancer during their lifetimes. The primary gene responsible for FAP is the APC gene, located on the long arm of chromosome 5. Numerous studies of germline and somatic mutations of the APC gene have shown that the vast majority of the genetic alterations are either nonsense or frameshift mutations ***(19)***. These types of mutations invariably result in the generation of a truncated protein, making IVTT analysis a highly effective technique for identifying APC mutations ***(10)***.

Genetic testing of individuals at risk of developing FAP has several advantages over previously used screening methods. Historically, most individuals at risk for FAP were monitored by colonoscopy or sigmoidoscopy beginning early in the second decade of life. Endoscopy is effective in diagnosing symptomatic patients and effectively identifies patients for surgical intervention to reduce their risk for developing colon cancer. However, since there may be considerable variability in the time of presentation of polyposis, many years of endoscopy are necessary before FAP can be ruled out. Moreover, endoscopy is totally unnecessary in half of these at risk patients–those who did not inherit a

mutant APC allele. Genetic testing effectively eliminates unneeded screening by endoscopy in these individuals, and is proving to be an attractive alternative for identifying those individuals at risk for FAP who do not inherit a mutant allele. To date, we have screened over 600 patients either diagnosed with FAP or at risk of developing FAP. In 82% of those cases in which FAP was clinically confirmed, a germline mutation was revealed by IVTT. Consistent with previously published reports ***(19)***, we have found that greater than 95% of the mutations are clustered within the 5' half of the APC open reading frame.

A far more common familial colorectal cancer syndrome is hereditary nonpolyposis colorectal cancer (HNPCC). The conditions for identifying this syndrome were established by the International Collaborative Group (ICG) in HNPCC ***(20)*** and requires that three criteria be met in order for a diagnosis to be made:

1. Three or more cases of colorectal cancer must be confirmed within a kindred, with at least one of the affected patients being a first degree relative of the other two;
2. The cases of colorectal cancer occur in a minimum of two successive generations; and
3. At least one of the cases was clinically diagnosed before the age of 50.

Although numerous kindreds have been identified based upon the ICG criteria, it has been suggested that many cases of HNPCC occur that do not fulfill the rigid requirements established by the ICG ***(21)***. The primary genetic alterations responsible for HNPCC have been shown to result from germline mutations in genes involved in the DNA mismatch repair pathway. At present germline mutations have been identified in four genes involved in this pathway: hMSH2 ***(22)***, hMLH1 ***(23,24)***, hPMS1 and hPMS2 ***(25)***. Germline mutaions in these genes and the likely inactivation of the wildtype allele ***(26)*** result in a hyper mutable phenotype. An attenuated mismatch repair pathway can result in an increased somatic mutation rate and likely leads to tranformation through the acquisition of mutations in growth regulatory genes ***(27)***.

The majority of germline mutations in the four mismatch repair genes that have been reported cause premature translation termination, so that the resulting protein is truncated and presumably inactive. This suggests that IVTT may be an attractive method of screening individuals for mutations. Recently, we have shown that in at least half of the cases of HNPCC, germline mutations in either the hMSH2 or hMLH1 genes can be identified using this technique ***(28)***. Here we describe our methodologies to identify mutations in kindreds with familial colorectal cancer syndromes.

2. Materials

2.1. RNA Extraction

1. Lymphocyte Separation Medium (Organon Teknika, Durham, NC).
2. 15 mL polypropylene conical tubes.

3. Phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8 m*M* Na_2HPO_4.
4. Lysis solution: 4 *M* guanidinium thiocyanate, 25 m*M* sodium citrate, pH 7.0, 0.5% sarcosyl, 100 m*M* 2-mercaptoethanol.
5. 20 mg/mL glycogen (Boehringer Mannheim, Indianapolis, IN).
6. 1.5 *M* sodium acetate, pH 4.0.
7. Phenol (Gibco-BRL, Gaithersburg, MD).
8. Chloroform:isoamyl alcohol (24:1) (Gibco-BRL).
9. Phenol:chloroform:isoamyl alcohol (25:24:1) (Gibco-BRL).
10. Isopropanol.
11. 70% ethanol.
12. Sterile water.
13. Syringe and 23-gage needle.

2.2. DNA Extraction

1. Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN).
2. TE: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.

2.3. cDNA Synthesis

1. 1 mg/ml random hexamers (Pharmacia Biotech, Piscataway, NJ).
2. Superscript II reverse transcriptase (Gibco-BRL).
3. 5X First strand buffer (Gibco-BRL).
4. 0.1 *M* DTT (Gibco-BRL).
5. 100 m*M* dNTPs (25 m*M* of dATP, dCTP, dGTP and dTTP, Pharmacia Biotech).
6. RNasin (Promega, Madison, WI).
7. Bind-Aid amplification enhancer (Amersham, Arlington Heights, IL).

2.4. PCR Amplification

1. 10X PCR buffer: 100 m*M* Tris-HCl, pH 8.3, 500 m*M* KCl, 20 m*M* $MgCl_2$.
2. 100 m*M* dNTPs.
3. Oligomers in sterile water. Oligonucleotide concentration is 10 μ*M* for the APC gene analysis and 20 μ*M* for HNPCC gene analysis.
4. Bind-Aid.
5. Amplitaq DNA polymerase (Perkin Elmer).
6. GeneAmp and microAmp PCR tubes (Perkin Elmer).
7. Ampliwax gem wax beads (Perkin Elmer).
8. Sterile water.

2.5. In Vitro Transcription/Translation Reaction

1. ^{35}S-Express protein labeling mix (DuPont NEN, Boston, MA).
2. TNT Coupled T7 transcription/translation system (Promega).
3. RNasin (Promega).
4. Reducing buffer: 62.5 m*M* Tris-HCl, pH 6.8, 2% SDS (w/v), 5% 2-mercaptoethanol (v/v), 10% glycerol (v/v), and 0.002% bromophenol blue (w/v).

2.6. Polyacrylamide Gel Electrophoresis

1. 30% acrylamide (w/v), 0.8% bisacrylamide (w/v) prepared in sterile water.
2. Stacking gel buffer stock: 500 m*M* Tris-HCl, pH 6.8.
3. Resolving gel stock: 3 *M* Tris-HCl, pH 8.8.
4. Electrophoresis buffer: 25 m*M* Tris-HCl, 0.192 *M* glycine, 0.1% sodium dodecyl sulfate (SDS) (w/v).
5. Freshly made 1.5% ammonium persulfate in sterile water (Bio-Rad, Hercules, CA).
6. TEMED (Bio-Rad).
7. 10% SDS (w/v) (Gibco-BRL).
8. Fixing solution: 30% methanol (v/v), 10% acetic acid (v/v).
9. Entensify solutions A and B (Dupont-NEN).
10. Kodak X-Omat film.

3. Methods

3.1. RNA Extraction

1. Layer 5 mL of EDTA anticoagulated whole blood onto 5 mL of lymphocyte separation solution in a 15 mL polypropylene centrifuge tube.
2. Centrifuge at 400*g* for 30 min at room temperature.
3. Transfer the lymphocyte layer to a fresh tube.
4. Add 10 mL of PBS, cap the tube and invert several times.
5. Centrifuge at 400*g* for 5 min at room temperature. Pour off the supernatant.
6. Add 2 mL of lysis solution and aspirate the solution through a 23-gage needle until the pellet has fully dissolved. Transfer 0.5 mL to a 1.5 mL Eppendorf tube and store the remainder at –20°C.
7. Add 1 μL of glycogen, 75 μL of sodium acetate, 0.5 mL of phenol, and 0.2 mL of chloroform:isoamyl alcohol.
8. Vortex the tube and place on ice for 15 min.
9. Centrifuge at 13,000*g* for 10 min at room temperature.
10. Transfer the aqueous layer to a fresh tube.
11. Repeat the extraction with phenol/chloroform/isoamyl alcohol and transfer the aqueous layer to a fresh tube.
12. Add 0.5 mL of isopropanol and invert the tube 30 times.
13. Place the tube in the freezer at –20°C for 1 h to overnight.
14. Centrifuge at 13,000*g* for 15 min to pellet the RNA. Pour off the supernatant.
15. Wash the pellet by adding 0.1 mL of 70% ethanol and then gently pour it off.
16. Centrifuge briefly to sediment any residual ethanol and then remove this with a pipet tip. Air-dry the tube for 15 min on ice.
17. Resuspend the RNA in 0.1 mL of sterile water.

3.2. DNA Extraction

1. Extract the DNA from EDTA-anticoagulated whole blood using a Puregene kit according to the manufacturer's instructions.
2. Resuspend the DNA (approx 30 μg from 1 mL of blood) in 200 μL of TE.

3.3. cDNA Synthesis

1. Transfer 10 µL of RNA (2–3 µg) to a 0.5-mL GeneAmp tube.
2. Add 1 µL of random hexamers and 3 µL of water.
3. Cap the tube and heat at 70°C for 10 min. Place the tube on ice for 2 min.
4. Prepare a mastermix of reagents containing (per tube) 5 µL of 5X first strand buffer, 2 µL of DTT, 1 µL of dNTPs, 0.5 µL of RNasin, 1 µL of Bind-aid, and 1.5 µL of reverse transcriptase. Make enough for one extra reaction to ensure there is enough for all samples.
5. Add 11 µL of mastermix to each tube, vortex briefly, microfuge the tube briefly and then place in a thermal cycler at 37°C for 1 h.
6. Inactivate the reverse transcriptase by heating to 65°C for 10 min and then cool to 4°C. If not used immediately, store the cDNA at –20°C.

3.4. Polymerase Chain Reaction

3.4.1. APC Amplification Segment 1 (RT-PCR)

1. Prepare a mastermix of reagents containing (per tube) 2 µL of 10X PCR buffer, 0.075 µL of dNTPs, 0.2 µL of external oligo A, 0.2 µL of external oligo B, and 14.5 µL of sterile water (*see* **Notes 1** and **2**, **Table 1**).
2. Pipet 17 µL of mastermix into each tube.
3. Each specimen is run in triplicate with 1 µL of cDNA and 2 µL of water in the first tube, 2 µL of cDNA and 1 µL of water in the second tube, and 3 µL of cDNA in the third tube.
4. Add a wax bead to each tube, cap and heat to 80°C for 5 min. Cool to 15°C.
5. Prepare a separate mastermix containing (per tube) 0.5 µL of 10X PCR buffer, 0.5 µL of Bind-aid, 0.5 µL of *Taq* polymerase and 3.5 µL of water. Remove the tubes from the thermal cycler and pipet 5 µL of the *Taq* mastermix onto the wax layer.
6. Recap and amplify for 15 cycles of: 95°C, 30 s
58°C, 30 s
72°C 120 s with a final hold at 15°C
7. Prepare an internal mastermix which contains (per tube) 2.5 µL of 10X PCR buffer, 0.075 µL of dNTPs, 1.5 µL of internal oligo A, 1.5 µL of internal oligo B, 0.5 µL of Bind-aid, 0.775 µL of *Taq* polymerase, and 18.15 µL water. Add 25 µL of internal mastermix to each tube, layering on top of the wax layer.
8. Recap and amplify for 35 cycles of: 95°C, 30 s
62.5°C, 30 s
72°C, 90 s with a final hold at 72°C for 10 min.

3.4.2. APC Segments 2–5 (PCR)

1. Into a microAmp tube, pipet 4 µL of 10X PCR buffer, 0.4 µL of dNTPs, 2 µL of oligo A, 2 µL of oligo B, 100 ng of DNA, and make the volume up to 40 µL with sterile water.
2. Add a wax bead to each tube, cap and heat to 80°C for 5 min. Cool to 15°C.
3. Prepare a mastermix containing (per tube) 1 µL of 10X PCR buffer, 0.5 µL of Bind-

Table 1
Oligonucleotide Primers Used for the APC, hMSH2, and MLH1 PCR Amplifications

Primers	Sequence (5' to 3')
APC-S1external A	CAA GGG TAG CCA AGG ATG GC
APC-S1external B	TTG CTA GAC CAA TTC CGC G
APC-S1internal A	GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGC TGC AGC TTC ATA TGA TC
APC-S1internal B	CTG ACC TAT TAT CAT CAT GTC G
APC-S2A	GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGA TGC ATG TGG AAC TTT GTG G
APC-S2B	GAG GAT CCA TTA GAT GAA GGT GTG GAC G
APC-3A	GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGT TTC TCC ATA CAG GTC ACG G
APC-3B	GGA GGA TCC TGT AGG AAT GGT ATC TCG
APC-4A	GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGA AAA CCA AGA GAA AGA GGC AG
APC-4B	TTC ACT AGG GCT TTT GGA GGC
APC-5A	GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGG TTT ATC TAG ACA AGC TTC G
APC-5B	GGA GTG GAT CCC AAA ATA AGA CC
MSH2-S1A	GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGC GGT GCA GCC GA
MSH2-S1B	CAT CCT GGG CTT CTT CAT ATT CTG TTT TAT
MSH2-S2A	GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGT TGG CTT GGA CCC TGG CAA AC
MSH2-S2B	TCA ATA TTA CCT TCA TTC CAT TAC TGG GAT TT
MLH1-S1A	GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGC ATC TAG ACG TTT CCT TGG C
MLH1-S1B	CAT CCA AGC TTC TGT TCC CG
MLH1-S2A	GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGG GGT GCA GCA GCA CAT CG
MLH1-S2B	GGA GGC AGA ATG TGT GAG CG

Aid, 0.5 μL of *Taq* polymerase, and 8 μL of sterile water. Pipet 10 μL of mastermix onto the wax layer.

4. Recap and amplify using the same conditions for the second amplification of segment 1 above.

3.4.3. HNPCC Amplification (RT-PCR)

1. Prepare a mastermix of reagents containing (per tube) 4 μL of 10X PCR buffer, 0.4 μL of dNTPs, 1 μL of oligo A, 1 μL of oligo B, and 30.6 μL of sterile water (*see* **Note 3**).
2. Pipet 37 μL of mastermix into microAmp tubes.
3. Each specimen is run in duplicate with 1 μL of cDNA in the first tube and 3 μL in the second tube. Add 1 μL of cDNA reaction and 2 μL of sterile water to the first tube. Add 3 μL of cDNA reaction to the second tube.
4. Add a wax bead to each tube, cap and heat to 80°C for 5 min. Cool to 15°C.
5. Prepare a mastermix of reagents containing (per tube) 1 μL of 10X PCR buffer, 0.5 μL of *Taq* polymerase, 0.5 μL of Bind-Aid, and 8 μL of sterile water.
6. Pipet 10 μL of this mastermix onto the wax layer, cap the tube, and cycle as follows:

15 cyles of:	95°C, 30 s
	67.7°C, 30 s
	72°C, 30 s
then 28 cycles of:	95°C, 30 s
	70°C 90 s, then a hold at 72°C for 10 min.

3.5. In Vitro Transcription/Translation

1. Thaw out components of the TNT kit and place on ice.
2. Prepare a TNT mastermix containing (per tube) 3.5 μL of protein labeling mix, 0.5 μL of TNT buffer, 0.25 μL of translation mix minus methionine, 0.25 μL of RNasin, 6.25 μL of lysate, and 0.35 μL of T7 RNA polymerase.
3. Pipet 11.1 μL of TNT mastermix into 0.5-mL tubes.
4. Add 3 μL of PCR reaction to each tube and mix by pumping with a pipet tip.
5. Incubate the tubes for 60–90 min at 30°C.
6. Pipet 20 μL of reducing buffer into 0.5-μL tubes.
7. Pipet 3 μL of IVTT product into each tube and mix. Boil for 5 min at 100°C.
8. Briefly centrifuge the tubes to sediment any condensation and cool to room temperature.

3.6. Polyacrylamide Gel Electrophoresis

1. Place the glass plates in the gel casting stand using 0.75 mm spacers (*see* **Note 4**).
2. For each gel mix: 12.5 mL of acrylamide, 3.75 mL of resolving buffer, 0.3 mL of 10% SDS, 1.5 mL of 1.5% ammonium persulfate, and 11.95 mL of sterile water.
3. Add 25 μL of TEMED, swirl to mix and pour into the gel cast. Layer on 0.5 mL of water and leave for 20 min to polymerize.
4. Pour off the water.
5. Mix 2.5 mL of acrylamide, 5 mL of stacking buffer, 0.2 mL of 10% SDS, and 1 mL of ammonium persulfate, and 11.3 mL of water.
6. Add 30 μL of TEMED, swirl to mix, and pipet into the gel apparatus. Insert the comb and wait for 20 min for the acrylamide to polymerize.
7. Remove the comb and construct the gel apparatus, pouring electrophoresis buffer into upper and lower chambers.
8. Pipet the samples into the wells using gel loading tips.
9. Electrophorese at 30 mA constant current per gel until the dye front reaches the edge of the gel (approx 3 h).

3.7. Development

1. Remove the gel from the apparatus and immerse it in fix solution in a glass tray and gently shake for 25 min.
2. Repeat the fixing step.
3. Remove the fix solution and replace with Entensify solution A. Agitate for 25 min.
4. Replace the fix solution with Entensify solution B and agitate for 25 min.
5. Place the gel on Whatman paper and dry on a gel dryer.
6. Place the dried gel next to film overnight in a film cassette.

3.8. Interpretation

Under ideal circumstances, IVTT will yield a single full-length polypeptide from a wild-type gene segment, or a truncated polypeptide from a mutant allele in addition to the full-length product in individuals heterozygous for a translation

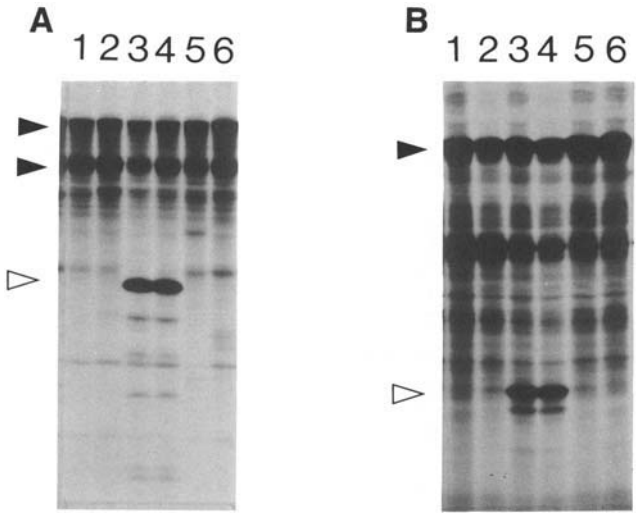

Fig. 1. In vitro transcription/translation of the APC and hMSH2 genes. IVTT reactions were performed in duplicate from the RT-PCR products from three individuals. Solid arrows indicate the full-length polypeptides from the wild-type allele. Open arrows indicate the truncated polypeptides. **(A)** Segment 1 of the APC gene. Individual 2 (lanes 3, 4) contains a truncated polypeptide. **(B)** Segment 2 of the hMSH2 gene. Individual 2 (lanes 3, 4) contains a truncated polypeptide.

terminating mutation. However, our experience has shown that in all gene transcripts analyzed in vitro synthesized polypeptides other than those generated from wild-type or mutant alleles are inevitably encountered (**Fig. 1**). These bands result from residual transcripts present in the reticulocyte lysate, alternately spliced transcripts, mispriming from the T7-containing PCR primer, and internal initiation by the reticulocyte ribosomes. In order to properly interpret an IVTT assay, a thorough understanding of the sources of these spurious bands is essential.

The most easily recognized nonallelic bands are those generated from endogenous transcripts present in the reticulocyte lysate. In all assays performed, two distinct bands with an apparent molecular mass of 42 and 22 kDa are seen. These bands are most likely generated from endogenous rabbit globin transcripts that escaped micrococcal nuclease hydrolysis, perhaps by being protected in polysomes. Although these bands are generally much less intense than those generated from the PCR template, they can pose interpretation problems when a low amount of the PCR amplification product is added to the IVTT reaction. In this situation, more ^{35}S-containing amino acids are incorporated into the products from the endogenous transcripts and may be confused as a band generated from an allele possessing a truncating mutation. Differen-

tiating between these two possibilities necessitates careful inspection of the size of the truncated product to determine if it has the same molecular mass as the products products by the endogenous reticulocute mRNAs. The simplest way to identify bands generated from endogenous reticulocyte transcripts is to perform a translation reaction in the absence of exogenous PCR product.

A second source of extra bands occurs as a result of natural alternatively spliced transcripts. Products generated from this source are also easily recognized as they are generally present in all samples analyzed from the same segment of the gene being analyzed. However as a complication we have observed that alternatively spliced transcripts can be present in vastly different amounts in RNAs isolated from different tissues, and in similar tissues with different proliferative states (i.e., cultured leukocytes vs leukocytes from fresh whole blood). Differentiating products generated from different mRNA isoforms requires the use of an appropriate negative control from a similar tissue source, and an understanding that an extra lower molecular weight band could be the result of an alternatively spliced transcript, even if its intensity is different from that of the control reaction. Fortunately, products generated from alternatively spliced transcripts will generate a polypeptide with a characteristic molecular weight, like the situation of products from endogenous transcripts. Differentiating these bands from bands generated from a mutant allele requires careful comparison to the negative control, and/or altering the electrophoretic conditions to increase the resolution in the size range in question.

A more difficult source of spurious banding to interpret occurs when the T7-containing oligonucleotide misprimes during the PCR reaction. If mispriming occurs with the T7 primer and it occurs in frame with the natural open reading frame of the gene segment of interest, a final protein product of lower molecular weight will be produced. This is of particular concern since this type of mispriming may take place in the early stages of PCR, resulting in a relatively large amount of spurious PCR product. This will in turn result in the production of a low molecular weight polypeptide with a signal intensity similar to the wild-type band. In this situation, without proper controls, it is impossible to ascertain whether the extra band is produced from mispriming or is generated from a mutant allele containing a truncating mutation. Our experience has shown that the best way to differentiate between these two possibilities is to always analyze the PCR product by agarose gel electroporesis prior to IVTT. The presence of a spurious PCR product, and an extra band revealed by IVTT, alerts one to the possibility that the IVTT band may be artifactual. Fortunately, mispriming by the T7 primers is generally a stochastic event and all of our IVTT reactions are performed in duplicate using the product from separate PCR reactions. Typically we perform either two or three different PCR reactions using varying amounts of the cDNA reaction and select the PCR products

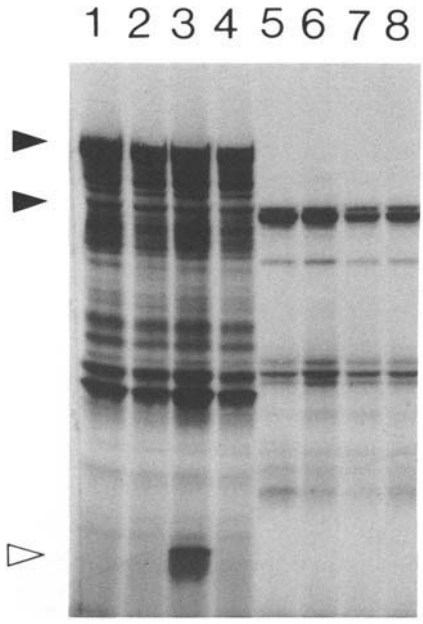

Fig. 2. Mispriming artifact in IVTT. In vitro transcription/translation analysis of hMSH2 was performed in two overlapping segments, segment 1 (lanes 1–4) and segment 2 (lanes 5–8). IVTT reactions were performed on two different RT-PCR products from two individuals. Solid arrows indicate the full-length polypeptides. An open arrow indicates the position of the spurious band seen in lane 3 but not lane 4.

with the least spurious banding for subsequent IVTT analysis. **Figure 2** shows an IVTT analysis of the hMSH2 gene for two individuals. Lane 3 shows a reaction demonstrating a protein product from mispriming. The truncated polypeptide produced by the misprimed PCR product would not be recognized as artifactual if it were not compared to a second reaction which did not have mispriming. Thus, it is imperative that multiple PCR reactions be performed, and at least two IVTT reactions from separate PCR products be performed for the proper identification of an allele containing a truncating mutation.

The final source of major erroneous banding results from internal translation initiation. In vivo, eucaryotic ribosomes generally initiate translation at the first methionine which conforms to a consensus sequence described by Kozak ***(29)***. All T7 primers used in our analyses contain the sequence CCACCATGG with the underlined nucleotides positioned to be in frame with the natural open reading frame of the transcript of interest. While this sequence is in complete agreement with the Kozak consensus sequence, we have often observed that this site is skipped, and translation is initiated at a site down-

stream. Interestingly, we have observed that different genes analyzed by IVTT show different degrees of internal initiation, even though they all contain the identical translation initiation start sequence shown above. We can only conclude that this difference is because secondary structural differences between the various in vitro generated transcripts analyzed by IVTT. Although it is difficult to differentiate between internal translation initiation and products generated from alternatively spliced transcripts, we are confident that internal initiation is a common event during IVTT. We make this conclusion based on our analysis of products produced from genomic DNA and from cloned sequences with large open reading frames. For example, the 15th exon of the APC gene contains a continuous open reading frame of over 6.5 kb. IVTT analysis of this exon (analyzed in 4 overlapping segments) shows numerous additional bands. Since these bands cannot be the result of alternative splicing and do not correspond to products generated from endogenous mRNAs or from spurious PCR products, they must therefore result from internal translation initiation. Additionally, analysis of cloned wild type cDNA sequences also show extra banding, which is identical to the pattern observed in reactions performed directly from cDNA and the heterozygous products from the cDNA reaction is a composite of both the wild-type and mutant patterns (**Fig. 3**). The most likely explanation for this observation is that the mutant allele creates an altered secondary structure in the in vitro produced transcripts, and this changes the preference for selection of internal initiation sites. Fortunately, internal translation initiation is a consistent event, that is, it appears to occur at identical sites within each same segment analyzed, and is therefore not easily confused with products generated from mutant alleles.

3.9. Advantages

IVTT has many advantages over other techniques used for screening for unknown mutations. Perhaps the most significant of these is its ability to analyze large segments of cDNA or genomic DNA in a single reaction. We typically analyze approx 1.5–2.5 kb of DNA or cDNA per reaction which is considerably more than most other screening techniques. Although it is theoretically possible to analyze even longer sequences, we have found that the amount of sequence that can be analyzed is limited by a reduction in the PCR product generated when very long segments are amplified, and by the ability to resolve the in vitro synthesized polypeptides by conventional polyacrylamide gel electrophoresis conditions. Nevertheless, the ability of IVTT to rapidly screen greater than 2 kb of sequence information in a single analysis (which combine in the case of NF1 to represent approx 8.5 kb of continuous coding sequence) is a significant advantage over most other techniques used to screen for unknown mutations.

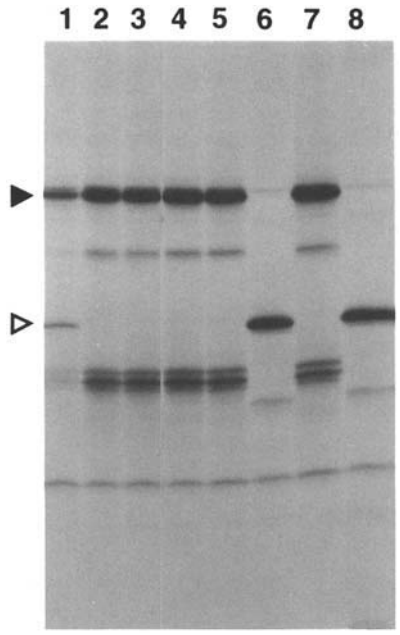

Fig. 3. The IVTT pattern of a heterozygote is a composite of normal and mutant bands. The RT-PCR product from an individual identified as a hMSH2 segment 2 heterozygote was cloned into TA vector and screened by a second round of PCR followed by IVTT. A heterozygous band pattern from the original RT-PCR product (lane 1) is clearly a composite of wild-type (lanes 2, 3, 4, 5, 7) and mutant (lanes 6, 8) banding patterns. The wild-type band is marked by a solid arrow. The mutant band is marked by an open arrow.

A second, often under appreciated advantage of IVTT, is the fact that this technique only identifies functionally significant genetic alterations. Other mutational screening techniques generally identify genetic differences based on analysis of heteroduplex formation between sequences from different alleles (e.g., heteroduplex analysis, chemical cleavage, RNase protection assays, and others). Thus, if a heteroduplex complex is detected, it is generally impossible to absolutely identify it as a deleterious mutation. Since IVTT analysis only reveals alterations which grossly alter an open reading frame, alterations detected by IVTT are undoubtedly pathologic, and allows one to readily differentiate between deleterious mutations and benign polymorphisms.

Finally, since mutations detected by IVTT most commonly result from the generation of a premature stop codon, the position of the truncated protein allows for the mapping of the approximate postion of the mutation which caused the premature stop codon. By plotting the size of the truncated protein

from its migration, one can readily estimate where in the open reading frame the premature stop occurred. This allows one to design sequencing primers to identify the mutation at the nucleotide level. Our experience has shown that mapping mutations this way is only an estimation, and the sequencing primers should be designed to anneal approx 100 nucleotides downstream of the mapped site when sequencing in the upstream direction and approx 200 nucleotides upstream when sequencing in the downstream direction.

The ability to differentiate pathologic mutations from benign polymorphisms, the ability to analyze large segments of genetic material, and the relative ease to perform this technique makes IVTT remarkably effective in identifying unknown mutations. Although at present IVTT is only useful in revealing chain terminating mutations (or relatively large in-frame insertions) it may be possible to modify the analysis to detect more subtle genetic alterations. The ability to synthesize polypeptides only from the alleles of interest (i.e., minimizing spurious peptide products) will undoubtedly lead to new ways of characterizing the polypeptide products with more subtle genetic alterations. How important missense mutations are as a source of disease-causing mutations in familial colon cancer genes is not yet established. If future studies show that missense mutations represent a substantial source of pathologic mutations then it may be possible to modify IVTT in order to detect them. For example, missense mutations which change the charge of an in vitro synthesized polypeptide could be detected by two dimensional gel electrophoresis or by HPLC. Alternatively, missense mutations which change amino acids (e.g., sulfhydryl groups of cysteine residues) could be revealed by simple chemical modification (e.g., alkylation with labeled iodoacetic acid).

4. Notes

1. All PCR reactions have been adapted to the Perkin Elmer 9600 thermal cycler using microAmp tubes.
2. The APC gene is amplified in five overlapping segments using the oligonucleotide primers listed in **Table 1**. The first segment is a nested RT-PCR, segments 2–5 are PCR from genomic DNA.
3. The hMSH2 and hMLH1 genes are both amplified by RT-PCR in two overlapping segments. The oligonucleotide primers are listed in **Table 1**.
4. Gels in our laboratory are run on a Bio-Rad Protean II gel apparatus with 16 × 18 cm gel dimensions and either 20 or 25 well combs.

References

1. Orita, M., Suzuki, Y., Sekiya, T., and Hayashi, K. (1989) Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* **5,** 874–879.

2. White, M. B., Carvalho, M., Derse, D., O'Brien, S. J., and Dean, M. (1992) Detecting single base substitutions as heteroduplex polymorphisms. *Genomics* **12,** 301–306.
3. Cotton, R. G. H., Rodrigues, N. R., and Campbell, R. D. (1988) Reactivity of cytosine and thymine in single-base-pair mismatches with hydroxylamide and osmium tetroxide and its application to the study of mutations. *Proc. Natl. Acad. Sci. USA* **85,** 4397–4401.
4. Myers, R. M., Maniatis, T., and Lerman, L. S. (1987) Detection and localization of single base changes by denaturing gradient gel electrophoresis. *Meth. Enzymol.* **155,** 501–527.
5. Sheffield, V. C., Cox, D. R., Lerman, L. S., and Myers, R. M. (1989) Attachment of a 40-base-pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc. Natl. Acad. Sci. USA* **86,** 232–236.
6. Forrest, S. M., Dahl, H. H., Howells, D. W., Dianzani, I., and Cotton, R. G. H. (1991) Mutation detection in phenylketonuria by using chemical cleavage of mismatch: importance of using probes from both normal and patient samples. *Am. J. Hum. Genet.* **49,** 175–183.
7. Youil, R., Kemper, B. W., and Cotton, R. G. H. (1995) Screening for mutations by enzyme mismatch cleavage with T4 endonuclease VII. *Proc. Natl. Acad. Sci. USA* **92,** 87–91.
8. Mashal, R. D., Koontz, J., and Sklar, J. (1995) Detection of mutations by cleavage of DNA heteroduplexes with bacteriophage resolvases. *Nat. Genet.* **9,** 177–183.
9. Roest, P. A. M., Roberts, R. G., Sugino, S., Ommen, G. J. B., and Dunnen, J. T. (1993) Protein truncation test (PTT) for rapid detection of translation-terminating mutations. *Hum. Mol. Genet.* **2,** 1719–1721.
10. Powell, S. M., Petersen., G. M., Krush, A. J., Booker, S., Jen, J., Giardiello, F. M., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. (1993) Molecular diagnosis of familial adenomatous polyposis. *N. Engl. J. Med.* **329,** 1982–1987.
11. Sarkar, G. and Sommer, S. S. (1989) Access to a messenger RNA sequence or its protein product is not limited by tissue or species specificity. *Science* **244,** 331–334.
12. Heim, R. A., Silverman, L. M., Farber, R. A., Kam-Morgan, L. N. W., and Luce, M. C. (1994) Screening for truncated NF1 proteins. *Nature Genet.* **8,** 218,219.
13. Heim, R. A., Kam-Morgan, L. N. W., Binnie, C. G., Corns, D. D., Cayouette, M. C., Farber, R. A., Aylsworth, A. S., Silverman, L. M., and Luce, M. C. (1995) Distribution of 13 truncating mutations in the neurofibromatosis 1 gene. *Hum. Mol. Genet.* **4,** 975–981.
14. Hogervorst, F. B. L., Cornelis, R. S., Bout, M., van Vliet, M., Oosterwijk, J. C., Olmer, R., Bakker, B., Klijn, J. G. M., Vasen, H. F. A., Meijers-Heijboer, H., Menko, F. H., Cornelisse, C. J., den Dunnen, J. T., Devilee, P., and van Ommen, G. J. B. (1995) Rapid detection of BRCA1 mutations by the protein truncation test. *Nat. Genet.* **10,** 208–212.
15. Struewing, J. P., Brody, L. C., Erdos, M. R., Kase, R. G., Giambarresi, T. R., Smith, S. A., Collins, F. S., and Tucker, M. A. (1995) Detection of eight BRCA1

mutations in 10 breast/ovarian cancer families, including 1 family with male breast cancer. *Am. J. Hum. Genet.* **57,** 1–7.
16. Szabo, C. I. and King, M. C. (1995) Inherited breast and ovarian cancer. *Hum. Mol. Genet.* **4,** 1811–1817.
17. St. John, D. J. B., McDermott, F. T., Hopper, J. L., Debney, E. A., Johnson, W. R., and Hughes, E. S. R. (1993) Cancer risk in relatives of patients with common colorectal cancer. *Ann. Intern. Med.* **118,** 785–790.
18. Bailey-Wilson, J. E., Elston, R. C., Schuelke, G. S., Kimberling, W., Albano, W., Lynch, J. F., and Lynch, H. T. (1986) Segregation analysis of hereditary nonpolyposis colorectal cancer. *Genet. Epidemiol.* **3,** 27–38.
19. Nagase, H. and Nakamura, Y. (1993) Mutations of the APC (adenomatous polyposis coli) gene. *Hum. Mut.* **2,** 425–434.
20. Vasen, H. F. A., Mecklin, J. P., Meera Khan, P., and Lynch, H. T. (1991) The international collaborative group on hereditary non-polyposis colorectal cancer (ICG-HNPCC). *Dis. Colon Rect.* **34,** 424–425.
21. Lynch, H. T., Smyrk, T. C., Watson, P., Lanspa, S. J., Lynch, J. F., Lynch, P. M., Cavalieri, R. J., and Boland, C. R. (1993) Genetics, natural history, tumor spectrum and pathology of hereditary nonpolyposis colorectal cancer: an updated review. *Gastroenerology* **104,** 1535–1549.
22. Leach, F. S., Nicolaides, N. C., Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomaki, P., Sistonen, P., Aaltonen, L. A., Nystrom-Lahti, M., Guan, X. Y., Zhang, J., Meltzer, P. S., Yu, J. W., Kao, F. T., Chen, D. J., Cerosaletti, K. M., Fournier, R. E. K., Todd, S., Lewis, T., Leach, R. J., Naylor, S. L., Weissenbach, J., Mecklin, J. P., Jarvinen, H., Petersen, G. M., Hamilton, S. R., Green, J., Jass, J., Watson, P., Lynch, H. T., Trent, J. M., de la Chapelle, A., Kinzler, K. W., and Vogelstein, B. (1993) Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. *Cell* **75,** 1215–1225.
23. Bronner, C. E., Baker, S. M., Morrison, P. T., Warren, G., Smith, L. G., Lescoe, M. K., Kane, M., Earabino, C., Lipford, J., Lindblom, A., Tannergard, P., Bollag, R. J., Godwin, A. R., Ward, D. C., Nordenskjold, M., Fishel, R., Kolodner, R., and Liskay, R. M. (1994) Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. *Nature* **368,** 258–261.
24. Papadopoulos, N., Nicolaides, N. C., Wei, Y. F., Rubin, S. M., Carter, K. C., Rosen, C. A., Haseltine, W. A., Fleischmann, R. D., Fraser, C. M., Adams, M. D., Venter, J. C., Hamilton, S. R., Petersen, G. M., Watson, P., Lynch, H. T., Peltomaki, P., Mecklin, J. P., de la Chapelle, A., Kinzler, K. W., and Vogelstein, B. (1994) Mutation of a mutL homolog in hereditary colon cancer. *Science* **263,** 1625–1629.
25. Nicolaides, N. C., Papadopoulos, N., Liu, B., Wei, Y. F., Carter, K. C., Ruben, S. M., Rosen, C. A., Haseltine, W. A., Fleischmann, R. D., Fraser, C. M., Adams, M. D., Venter, J. C., Dunlop, M. G., Hamilton, S. R., Petersen, G. M., de la Chapelle, A., Vogelstein, B., and Kinzler, K. W. (1994) Mutations of two PMS homologues in hereditary nonpolyposis colon cancer. *Nature* **371,** 75–80.
26. Hemminki, A., Peltomaki, P., Mecklin, J. P., Jarvinen, H., Salovaara, R., Nystrom-Lahti, M., de la Chapelle, A., and Aaltonen, L. A. (1994) Loss of the wild type

MLH1 gene is a feature of hereditary nonpolyposis colorectal cancer. *Nat. Genet.* **8,** 405–410.

27. Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R. S., Zborowska, E., Kinzler, K. W., Vogelstein, B., Brattain, M., and Willson, J. K. V. (1995) Inactivation of type II TGF-b receptor in colon cancer cells with microsatellite instability. *Science* **268,** 1336–1338.
28. Luce, M. C., Marra, G., Chauhan, D. P., Laghi, L., Carethers, J. M., Cherian, S. P., Hawn, M., Binnie, C. G., Kam-Morgan, L. N. W., Cayouette, M. C., Koi, M., and Boland, C. R. (1995) In vitro transcription/translation assay for the screening of hMLH1 and hMSH2 mutations in familial colon cancer. *Gastroenterology* **109,** 1368–1374.
29. Kozak, M. (1987) An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15,** 8125–8133.

12

A Single-Tube Nested RT-PCR for the Detection of Ross River Virus

Loryn Sellner

1. Introduction

The polymerase chain reaction (PCR) has proven to be an extremely powerful tool in the field of virus detection. It has been employed in a variety of situations where previous techniques were too slow, too cumbersome, or simply nonexistent. Some of these are as follows.

1. Diagnosis of human infection with viruses that
   a. are difficult or impossible to isolate by culture, e.g., Hepatitis C ***(1)***, or
   b. are highly infectious and hence laboratory culture is undesirable, e.g., Lassa virus ***(2,3)***, or
   c. reliable diagnosis cannot be made on serological grounds, or
   d. existing techniques for diagnosis are too slow.
2. Monitoring of environmental samples either directly for pathogenic viruses, or indirectly by monitoring for other viruses as indicators of possible pathogenic contamination, e.g., detection of enteroviruses in water supplies ***(4,5)***.
3. Surveillance of viral vectors or hosts to monitor levels of virus activity.

Ross River virus (RR virus) is a mosquito-borne alphavirus that has been found in Australia, Papua New Guinea, and the Pacific Islands. It is the etiological agent of epidemic polyarthritis, a debilitating disease with symptoms of arthritis, arthralgia, lethargy, rash, and fever lasting from weeks to months ***(6,7)***. The normal transmission cycle of RR virus in Australia is between kangaroos or other macropods (the major vertebrate hosts) and mosquitoes, with humans being only incidentally infected ***(8)***. The virus has become endemic in some areas of Australia, but in other areas it has an epidemic occurrence, with outbreaks occurring after heavy summer rains or high summer tides in coastal regions providing ideal mosquito breeding conditions ***(9)***. Surveillance of RR

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

virus activity is performed in order to predict when outbreaks are likely to occur, so control measures may be instituted. These may consist of reducing mosquito population numbers by use of pesticides, and/or informing the public of the potential threat so they can reduce their risk of infection by minimizing possible exposure to mosquitoes.

The method of choice for surveillance of RR virus activity involves monitoring the mosquito vectors. Information regarding increased vector abundance and distribution can be used as an indicator of possible impending epidemics, particularly in areas where mosquito population densities are normally quite low. The most accurate predictor of a possible epidemic is the rate of isolation of virus from field-caught mosquitoes ***(10)***. Until recently, this has been achieved using the laborious and time-consuming method of isolating virus from mosquito pools using tissue culture systems. This requires 2–3 passages of mosquito homogenate in tissue culture, followed by enzyme immunoassays, hemagglutination inhibition, or neutralization assays to identify the isolated virus. This process requires 2–4 wk, and hence does not provide a very early indicator of epidemic potential. In this chapter, a one-tube nested RT-PCR is described that can detect RR virus in mosquitoes or other samples in 1–2 d.

PCR has several factors in its favor in such a surveillance setting. First and foremost it is fast, allowing detection of virus early in an epidemic so that effective control measures can be taken and the number of human infections limited. Second, it is sensitive, rivaling tissue culture, which is the current gold standard. It is also a powerful tool in epidemiological analyses. Viruses can be typed without any further manipulation than the PCR by use of a nested PCR that uses group-specific primers in the first round of amplification, then type-specific primers in the nested PCR. This procedure has been used for detection and typing of dengue and hepatitis C viruses ***(11,12)***. Alternatively, PCR product can be hybridized with probes designed to differentiate between virus groups. This procedure has also been used for detection and typing of dengue virus serotypes ***(13,14)***. Sequence data can be obtained using PCR without prior isolation of the virus, which can provide information on evolution and spread of virus strains.

In this chapter methods are described for extraction of viral RNA from mosquitoes or serum, amplification of viral sequences using a single-tube RT-PCR or nested RT-PCR, and typing of viral isolates using oligonucleotide probe hybridization. We have found that using the combined method of guanidine thiocyanate extraction of RNA and nested RT-PCR, it is possible to detect RR virus in laboratory-infected mosquitoes 2–28 d postinfection (time points earlier than 2 d and later than 28 d were not tested). It was also possible to consistently detect virus in a single infected mosquito mixed with up to 500 uninfected mosquitoes. A pilot sample of 60 pools of field-caught mosquitoes that were tested for virus presence gave the same results when tested by nested

RT- PCR and tissue culture. These examples demonstrate the utility of this technique for surveillance of virus activity by monitoring mosquito vectors.

This method has also been used for detection of RR virus in serum from patients suffering from epidemic polyarthritis. We have found that virus can be detected in approximately one-third of serum samples collected early in infection, i.e., when RR virus-specific antibody titer is low or negative. As the antibody titer rises, the proportion of samples with detectable virus decreases, as would be expected since virus would be cleared from the circulation. Hence, this method may be used as an aid to disease diagnosis, particularly in sera that have low amounts of or no RR virus specific antibody, since these samples have the highest probability of being positive by nested RT-PCR, and diagnosis can be made on a serological basis if high levels of specific antibody are detectable, whereas no serologically based diagnosis can be made in the absence of virus-specific antibody.

2. Materials

2.1. Sample Preparation

1. RNase-free water.
2. Proteinase K.
3. Guanidine thiocyanate lysis buffer: 8 *M* guanidine thiocyanate, 50 m*M* sodium citrate, 100 m*M* β-mercaptoethanol, 1% n-lauroylsarcosine, 1 μg/mL yeast tRNA.
4. 2 *M* sodium acetate, pH 4.0.
5. Tris-buffered phenol.
6. Chloroform.
7. Isopropanol.

2.2. RT-PCR

1. PCR primers at 5 pmol/μL (outer primers) and 50 pmol/μL (inner primers).
2. Yeast tRNA at 2 μg/μL.
3. 5X reaction buffer: 10 m*M* $MgCl_2$, 500 μ*M* dNTPs, 335 m*M* Tris-HCl, pH 8.9, 250 m*M* KCl, 30 μ*M* EDTA, 0.5% Triton X-100, 2.5 m*M* dithiothreitol.
4. *Taq* DNA polymerase.
5. Avian myoblastosis virus reverse transcriptase (AMV-RT).
6. Mineral oil.
7. Agarose.
8. TBE electophoresis buffer: 45 m*M* Tris, 45 m*M* boric acid, 1 m*M* EDTA.
9. Ethidium bromide at 0.5 μg/mL.
10. *See* **Table 1** for a description of Ross River virus primers.

2.3. Hybridization

1. Oligonucleotide probe.
2. [γ-^{32}P]ATP (specific activity 5000 Ci/mmol, 10 mCi/mL).

Table 1
Primers for Detection of Ross River Virus

Primer	Sequence	Product size
Outer sense	TCC GCC CAA ATA GGT CTG GA	550
Outer antisense	TGT CAT GGC TGG TAA CGG CA	
Inner sense	ACG ACC CAT TGC CG	193
Inner antisense	CTG CCG CCT GCT GT	

3. T4 polynucleotide kinase.
4. 5X kinase buffer: 2 *M* Tris-HCl, pH 9.0, 50 m*M* magnesium acetate, 50 m*M* dithiothreitol.
5. Sephadex G50 slurry.
6. TE: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.4.
7. 20X SSC: 3 *M* NaCl, 0.3 *M* sodium citrate.
8. Denaturing solution: 1.5 *M* NaCl, 0.5 *M* NaOH.
9. Neutralizing solution: 1.5 *M* NaCl, 1 m*M* EDTA, 0.5 *M* Tris-HCl, pH 7.2.
10. 0.4 *M* NaOH.
11. Hybridization solution (7% [w/v] SDS, 0.5% [w/v] low fat skim milk powder, 1% [w/v] polyethylene glycol [PEG] 20,000, 0.5 mg/mL tRNA, 0.9 *M* NaCl, 0.05 *M* $Na_2HPO_4 \cdot 7H_2O$, 5 m*M* EDTA).
12. 5X SSC (diluted from 20X SSC stocks).
13. 3X SSC (diluted from 20X SSC stocks).
14. Wash solution 1: 0.1% SDS, 3X SSC (diluted from 20X SSC stocks).
15. Wash solution 2: 1% SDS, 1X SSC (diluted from 20X SSC stocks).
16. Wash solution 3: 0.1% SDS, 1X SSC (diluted from 20X SSC stocks).
17. X-ray film.

3. Methods

3.1. Sample Preparation

1. Grind mosquitoes in RNase-free water using tissue homogenizers at concentrations of up to 50 mosquitoes/mL.
2. For rapid preparation of mosquito homogenate for PCR analysis or preparation of tissue culture grown virus, digest 0.5 µL of sample for 30 min at 37°C with 0.5 µg Proteinase K in 15 µL water, followed by heat inactivation of the Proteinase K for 5 min at 95°C.
3. For more thorough RNA extraction (*see* **Note 1**), mix 100 µL of sample (mosquito homogenate, or serum) with 100 µL of guanidine thiocyanate lysis buffer (heat to dissolve crystals if necessary) in a 1.5 mL microcentrifuge tube.
4. Add 20 µL of 2 *M* sodium acetate per tube and mix by inversion.
5. Add 200 µL of Tris-buffered phenol and mix.
6. Add 40 µL of chloroform and mix by shaking.
7. Incubate tubes on ice for 15 min, then centrifuge for 15 min at 13,000*g* at 4°C.

8. Transfer the upper aqueous layer to a fresh tube and mix with an equal volume of isopropanol.
9. Cool the tubes for 1 h at –20°C, then centrifuge for 15 min at 13,000*g* at 4°C.
10. Carefully decant the isopropanol from the tube and drain the tubes briefly upside down on absorbent tissue. Carefully wipe excess isopropanol from inside the tube using a cotton bud.
11. Redissolve the RNA pellet was in 20 μL of RNase-free water.

3.2. RT-PCR

1. Make up a master mix containing (per reaction) 1 μL of each external primer, 1 μL tRNA, 5 μL 5X reaction mix, the optimum magnesium concentration (to be determined for each primer set, usually between 0.5 and 3 m*M*, in this case 2 m*M*), 1 U *Taq* polymerase and 0.5 U AMV-RT per tube (enzymes added last) (*see* **Note 2**). Add sufficient water to bring the total reaction volume to 25 μL, allowing for addition of sample RNA. Aliquot into 0.5-mL microcentrifuge tubes.
2. Add 2 μL of the RNA extracted from mosquitoes, or 10 μL of the RNA extracted from serum per tube (*see* **Note 3**).
3. Overlay the reaction mix with two drops of mineral oil.
4. Incubate the tubes according to the following thermal cycling protocol. Incubate at 42°C for 30 min, then heat to 94°C for 5 min, then carry out 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, followed by an incubation at 72°C for 7 min (*see* **Note 4**).
5. Electrophorese 5 μL of PCR products on a 1% agarose gel in TBE, stain with ethidium bromide, and photograph under UV transillumination.

3.3. Nested PCR

1. Prepare a nested PCR master mix containing 1 μL of each inner primer, 15 μL 5X reaction mix, an appropriate concentration of $MgCl_2$ (in this case 2 m*M*), 1 U *Taq* polymerase, and water to a final volume of 75 μL per tube.
2. Add 75 μL of nested PCR master mix to each RT-PCR tube after the initial RT-PCR has been performed.
3. Centrifuge the tubes briefly so that the nested PCR reaction components penetrate the oil barrier and mix with the first round products.
4. Perform a further 25 cycles of PCR using conditions suitable for second primer set, in this case 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min (*see* **Note 5**).
5. Electrophorese 10 μL of each reaction on a 1% agarose gel, stain with ethidium bromide solution, and photograph under UV transillumination.

3.4. Oligonucleotide Hybridization

1. To phosphorylate the oligonucleotides at their 5' end, prepare a labeling mix containing 0.5 μg oligonucleotide, 10 μL [γ-^{32}P]ATP, 30 U T4 polynucleotide kinase (T4 PNK), and 20 μL of 5X kinase buffer in a total volume of 100 μL and incubate overnight at 37°C.
2. Pour Sephadex G50 slurry into a 10 mL column and equilibrate by running through with 10 mL of TE. Add the labeled probe to the top of the column, and

wash through by gradually adding 5 mL of TE, collecting 10 0.5-mL fractions. Dilute 2 µL of each fraction into 5 mL of water and count in a scintillation counter to identify the fraction(s) containing labeled probe. Dilute the fraction containing labeled probe to 10^7 dpm/mL in hybridization solution.

3. Heat the RT-PCR products to 95°C, chill on ice, mix with an equal volume of 20X SSC, then spot onto a nitrocellulose membrane in 2-µL aliquots. Allow to dry.
4. Place the membrane on a filter paper wad soaked in denaturing solution for 5 min, then transfer to a filter paper wad soaked in neutralizing solution for 1 min. Transfer the membrane to a wad soaked in 0.4 *M* NaOH and fix for 20 min, then briefly wash by immersion in 5X SSC.
5. Prewet the membrane with hybridization buffer, then place between two pieces of filter paper. Seal the membrane sandwich inside a plastic bag with 2-mL labeled probe per 10 cm^2 membrane and incubate overnight at room temperature.
6. Rinse the membrane in 3X SSC, then wash vigorously in wash solution 1. Wash for 15 min at 37°C in wash solution 2, then rinse briefly in wash solution 3.
7. Expose the membrane to X-ray film at –70°C for 24 h (*see* **Note 6**).

4. Notes

1. The mosquito homogenate contains PCR inhibitors, which are not totally inactivated by the Proteinase K and heat treatment and can lead to false negative results if the mosquito concentration is high or the virus titer is low. For this reason, it is recommended that RNA generally be extracted from samples prior to PCR using the method originally described by Chomczynski and Sacchi ***(15)***, reproduced here.
2. We have previously found that reverse transcriptase can inhibit *Taq* polymerase activity, particularly when the total RNA concentration is low ***(16)***. To circumvent this, only 0.5 U of reverse transcriptase is added with 1 U of *Taq* DNA polymerase, and surplus yeast tRNA is added to each reaction. We have also investigated the use of Tth DNA polymerase in a single enzyme RT-PCR because it also has reverse transcriptase activity ***(17)***. However, the use of Tth polymerase in a single buffer RT-PCR was found to be 100-fold less sensitive than the AMVRT/*Taq* polymerase method for detection of RNA described here.
3. The difference in volume of RNA that can be added is because components that inhibit the RT-PCR are present in the mosquito RNA extract, but not the serum RNA extract.
4. The annealing temperature will vary with different primer sets. These incubation times were sufficient for amplification of this 550-bp product, but for larger PCR products (e.g., >1000 bp) longer extension times may be required.
5. Nested PCR was performed as a method of increasing sensitivity of the RT-PCR. We have found it increases sensitivity 1000–10,000-fold over a single round of RT-PCR. In the method described here, the nested PCR is performed in the same reaction tube as the RT-PCR, and requires only addition of further reagents to the tube. Using this approach rather than the transfer of first round RT-PCR product to a second reaction vessel for nested PCR, the risk of contamination that can cause false-positive results is substantially decreased.

6. Hybridization of RT-PCR products with labelled oligonucleotides can be used as an alternative method to nested PCR for increasing sensitivity, although we have found that a 100 fold increase in sensitivity can be obtained using hybridization instead of agarose gel electrophoresis, whereas a 1000–10,000-fold increase can be obtained with nested PCR. Nested PCR also has advantages over hybridization in that it is quicker and easier to perform, adding only 2–3 h to the total assay time, compared to 2–3 d for hybridization. Oligonucleotide hybridization has other advantages in its favor, however; for example, it can be used simultaneously as a typing tool if oligonucleotide probes are designed in such a way as to differentiate between different virus strains. We have designed oligonucleotide probes to differentiate between the three described RR virus genotypes ***(18)***, which has permitted rapid typing of RR virus isolates to acquire epidemiological data.

References

1. Weiner, A. J., Kuo, G., Bradley, D. W., Bonino, F., Saracco, G., Lee, C., Rosenblatt, J., Choo, Q.-L., and Houghton, M. (1990) Detection of hepatitis C viral sequences in non-A, non-B hepatitis. *Lancet* **335,** 1–3.
2. Lunkenheimer, K., Hufert, F. T., and Schmitz, H. (1990) Detection of Lassa virus RNA in specimens from patients with Lassa fever by using the polymerase chain reaction. *J. Clin. Micro.* **28,** 2689–2692.
3. Trappier, S. G, Conaty, A. L., Farrar, B. B., Auperin, D. D., McCormick, J. B., and Fisher-Hoch, S. P. (1993) Evaluation of the polymerase chain reaction for diagnosis of Lassa virus infection. *Am. J. Trop. Med. Hyg.* **49,** 214–221.
4. Kopecka, H., Dubrou, S., Prevot, J., Marechal, J., and Lopez-Pila, J. M. (1993) Detection of naturally occuring enteroviruses in waters by reverse transcription, polymerase chain reaction, and hybridization. *App. Env. Micro.* **59,** 1213–1219.
5. Abbaszadegan, M., Huber, M. S., Gerba, C. P., and Pepper, I. L. (1993) Detection of enteroviruses in groundwater with the polymerase chain reaction. *App. Env. Micro.* **59,** 1318–1324.
6. Hawkes, R. A., Boughton, C. R., Naim, H. M., and Stallman, N. D. (1985) A major outbreak of epidemic polyarthritis in New South Wales during the summer of 1983/1984. *Med. J. Aust.* **143,** 32–35.
7. Mudge, P. R. and Aaskov, J. G. (1983) Epidemic polyarthritis in Australia, 1980–1981. *Med. J. Aust.* **2,** 269–273.
8. Marshall, I. D. and Miles, J. A. R. (1984) Ross River virus and epidemic polyarthritis. *Curr. Topics Vector Res.* **2,** 31.
9. Lindsay, M., Condon, R., Mackenzie, J., Johansen, C., D'Ercole, M., and Smith, D. (1992) A major outbreak of Ross River virus infections in the south-west of Western Australia and the Perth metropolitan area. *Comm. Dis. Intell.* **16,** 290–294.
10. Mackenzie, J. S., Coelen, R. J., Sellner, L., Broom, A. K., Lindsay, M. D., Hall, R. A., and Oliveira, N. (1994) Surveillance of mosquito-borne viral diseases: a brief overview and experiences in Western Australia, in *Rapid Methods and Automation in Microbiology and Immunology* (Spencer, R. C., Wright, E. P., and Newsom, S. W. B., eds.), Intercept, Andover, UK, pp. 191–202.

11. Lanciotti, R. S., Calisher, C. H., Gubler, D. J., Chang, G.-J., and Vorndam, V. A. (1992) Detecting and typing dengue viruses by genomic amplification in a reverse transcriptase-polymerase chain reaction. *J. Clin. Micro.* **30,** 545–551.
12. Okamoto, H., Sugiyama, Y., Okada, S., Kurai, K., Akahane, Y., Sugai, Y., Tanaka, T., Sato, K., Tsuda, F., Miyakawa, Y., and Mayumi, M. (1992) Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. *J. Gen. Virol.* **73,** 673–679.
13. Deubel, V., Laille, M., Hugnot, J.-P., Chungue, E., Guesdon, J.-L., Drouet, M. T., Bassot, S., and Chevrier, D. (1990) Identification of dengue sequences by genomic amplification: rapid diagnosis of dengue virus serotypes in peripheral blood. *J. Virol. Meth.* **30,** 41–54.
14. Henchal, E. A., Polo, S. L., Vorndarn, V., Yaemsiri, C., Innis, B., and Hoke, C. H. (1991) Sensitivity and specificity of a universal primer set for the rapid diagnosis of dengue virus infections by polymerase chain reaction and nucleic acid hybridisation. *Am. J. Med. Hyg.* **45,** 418–428.
15. Chomczynski, P. and Sacchi, N. (1987) Single step method of RNA isolation by acid cuanidinium thiocyanate-phenol-chloroform extraction. *Analyt. Biochem.* **162,** 156–159.
16. Sellner, L. N., Coelen, R. J., and Mackenzie, J. S. (1992) Reverse transcriptase inhibits Taq polymerase activity. *Nucleic Acids Res.* **20,** 1487–1490.
17. Myers, T. W. and Gelfand, D. H. (1991) Reverse transcription and DNA amplification by a *Thermus thermophilus* DNA polymerase. *Biochemistry* **30,** 7661–7666.
18. Sammels, L. M., Coelen, R. J., Lindsay, M. D., and Mackenzie, J. S. (1995) Geographic distribution and evolution of Ross River virus in Australia and the Pacific Islands. *Virology* **212,** 20–29.

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Application of PCR to Transgenic Plants

Michael Wassenegger

1. Introduction

Genetic transformation of plants has enormously matured within the last few years. It began with the introduction of genes that imparted resistance to cultivated plants against herbicides and pathogenes. More recently, there are approaches to modify the quality of plants, such that useful traits are amplified, and/or unwanted traits are eliminated. Of particular interest in medical research are experiments in which genes are introduced into the plant genome that allow expression of herbal drugs and antibodies.

To produce transgenic plants, a number of molecular biological techniques had to be developed. For instance, genes of interest had to be screened, isolated, cloned, and fused to regulatory sequences that allow their expression in plants. Subsequently, appropriate gene constructs have had to be introduced into the plant genome via direct DNA transfer or with the aid of plant transforming vectors. Because the efficiency and frequency of transformation depends on the plant species and the gene-transfer technique, the analysis of a stable integration of transgenes is indispensable. Finally, a detailed analysis is necessary to verify the function of introduced genes. Particularly for higher plants, examination of transgene expression is important, because it was found that introduction of multiple copies of functional gene constructs can lead to the complete loss of the corresponding products. The phenomenon called cosuppression is based on gene silencing by DNA methylation or by RNA-RNA interactions. As yet, the number of copies that will be integrated into the plant genome cannot be controlled with any known transformation technique.

The polymerase chain reaction (PCR) has been applied to the abovementioned analytical procedures, with an enormous time-saving benefit. For example, Southern and Northern analysis, genomic sequencing, chemical

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

mutagenesis, nucleotide insertions, and exonuclease digestion can all be largely substituted for by the PCR technique. Nevertheless, PCR can also be problematical and for some experiments other techniques are more suitable than PCR. In the following section, advantages and disadvantages of PCR will be discussed in special consideration of plant systems.

Regardless of how plants had been transformed, the presence of foreign DNA and its correct integration into the plant genome should be confirmed before further examinations are carried out. Before the PCR technique became available, Southern analysis were performed for this purpose. Large-scale preparations of pure genomic DNA, high amounts of expensive restriction endonucleases and the use of labeled probes were required. Thus, PCR seems to be an efficient alternative to Southern analyses. The most convincing advantage of using PCR is the observation that integration of foreign DNA can be examined from as little as 200 pg of genomic DNA ***(1)***. This amount of DNA corresponds to fewer than 100 cells, which means that 2–3 mo after plant transformation, analysis of their genome can be performed.

However, this sensitivity of the PCR can lead to problems in that the slightest DNA contaminations can also serve as PCR templates. For example, contamination may come from *Agrobacteria,* which can survive for more than a year in plants previously transformed via agro-infection. Because the T-DNA of the bacteria and of the transgenic plants are identical, PCR with primer pairs that had been designed to detect the plant genome integrated T-DNA will result in products that can not be clearly assigned to the integrate or to *Agrobacterium* itself.

Application of PCR to copy number control can also cause problems because the sequences to which the foreign DNA had been anchored is usually unknown. However, to analyze different loci of foreign DNA integration by PCR, it is necessary to design primers that are complementary to the introduced DNA and to the flanking plant DNA. Although this problem can be overcome by performing inverse PCR, it is recommended to apply Southern analysis, which is confirmed in experiments describing genomic analysis of transgenic plants.

Successful agro-transformation of *Chrysanthemum* could be detected by PCR amplification of a 703 bp fragment of the β-Glucuronidase (GUS) reporter gene, but the number of integrated T-DNA copies had to be determined by Southern analysis ***(2)***. Virtually the same experiments were performed to show that a GUS gene construct was integrated into the turfgrass genome after microprojectile bombardment of embryogenic callus. Regenerated plants were analyzed for presence of the transgene by PCR using GUS-specific primers but genomic integration and transgene copy number had to be determined by Southern analysis ***(3)***. The integration of T-DNA into the genome of sunflower was verified by Southern hybridization of GUS-specific PCR products against

labeled GUS DNA fragments. And, as a control, the possibility of *Agrobacterium* contamination was investigated by amplifying sunflower leaf DNA with primers specific for the *Agrobacterium* chromosome. The copy number of T-DNA insertions was not analyzed ***(4)***. In another experiment, primary screening for presence of foreign DNA of biolistically transformed *Chrysanthemum* was achieved by PCR amplification of the NPT II and the nucleocapsid (N) gene of tomato spotted wilt virus (TSWV). Also in these experiments Southern hybridization against a ^{32}P-labeled TSWV-(N) gene as probe was necessary to corroborate the integration of the gene construct ***(5)***.

All these results may lead to the conclusion that application of PCR to transgene analysis had no remarkable advantages when compared to Southern hybridization. However, whenever the sequence of an integrated gene construct has to be determined, Southern analysis is unsuitable. The importance of sequencing an integrated transgene is well illustrated by our studies on viroid replication in which cDNAs of the 359 nt long potato spindle tuber viroid (PSTVd) were integrated into the tobacco genome via agro-infection ***(6,7)***. Viroids are autonomously replicating small (240–480 nt long) circular, single-stranded, and nonencapsulated plant pathogenic RNA molecules. Their host enzyme-dependent replication proceeds via the RNA-RNA pathway, and although viroids do not code for any protein, they can cause severe phenotypic symptoms of disease.

The first experiments were carried out to show that PSTVd RNA–RNA replication can be initiated in tobacco by integration of PSTVd cDNAs ***(6)***. Full-length oligomeric PSTVd cDNA units and deletion mutants of the same cDNAs were introduced into the tobacco genome, respectively. As expected viroid replication was only found in plants transformed with complete cDNAs, and it was not detectable in plants that had been transformed with the truncated cDNAs. To substantiate these results it was necessary to examine whether all plants are containing the PSTVd cDNAs. Therefore the correct integration of the transgenes was analyzed by Southern experiments. A surprising phenomenon was found when the T-DNAs were cut with methylation-sensitive restriction enzymes. The PSTVd cDNAs were found to be fully methylated whenever PSTVd RNA-RNA replication has taken place in these plants. In plants in which no PSTVd replication was detectable, the genome-integrated cDNAs remained unmethylated.

To exclude the possibility that viroid replication had caused mutations within the genome-integrated PSTVd-specific cDNA, the sequence of them had to be determined. This was realized by PCR amplification of the genomic plant DNA using a cauliflower mosaic virus 35S promoter-specific (35S P1) and a pA_{nos}-specific (pA P2) primer. Both primers were complementary to the sequences flanking the PSTVd-specific cDNA at the 5'- and the 3'-end, respec-

tively. The cloned and sequenced PCR products were found to correspond with the original viroid sequence in that the recognition sites of the methylation-sensitive restriction enzymes were not altered.

To verify that the integrated PSTVd cDNA was really methylated in viroid-replicating plants, a most recently developed technique was used to determine the precise distribution of 5-methylcytosine residues (^{5m}C). The method is based on a sodium bisulfite-mediated conversion of cytosine residues (C) (and not ^{5m}C) to uracil residues (U) in single-stranded DNA. PCR amplification of completely converted DNA subsequently allows detection of each ^{5m}C in genomic DNA ***(8)***. The conversion of C to U results in a splitting of the originally complementary upper and lower DNA strands into two noncomplementary strands. Therefore, primers which are complementary to the converted sequences are strand-specific. In **Fig. 1**, the principle of PCR amplification of bisulfite-treated DNA is depicted. To simplify matters, it is assumed that there are no ^{5m}C residues present in the primer binding sites. Because DNA methylation can be distributed heterogeneously in different cell types, it is useful to clone the PCR products. The distribution of ^{5m}C within individual clones can be detected by the appearance of signals in the C lane of a sequencing gel, unmethylated Cs are found in the T lane.

By performing PCR with bisulfite treated genomic DNA isolated from the transgenic plants and using the primers 35S18-H and PV320-R/2 the methylation pattern of the genome-integrated PSTVd cDNAs were analyzed in viroid-replicating and viroid-free tobacco plants. It was demonstrated that the introduced sequences had been *de novo* methylated in viroid replicating plants and moreover, it was clearly shown that methylation had also occurred at nonsymmetrical sequences.

In a second set of experiments on viroid replication we were interested in in vitro generating new infectious PSTVd variants with the help of transgenic plants. Although it was known that a single nucleotide substitution or deletion within the PSTVd sequence could lead to a loss of PSTVd infectivity, we found that a tobacco plant containing a nine bp deletion mutant of the PSTVd cDNA was viroid-infected ***(7)***. Northern analysis of the replicating PSTVd revealed that the RNA had become smaller by additional nine nucleotides when compared to the 350 bp long cDNA that was introduced into the tobacco plant. Because the PSTVd cDNA was controlled by the cauliflower mosaic virus (CaMV) 35S promoter (P_{35S}) and the nopaline synthetase gene termination signal (pA_{nos}) it was essential to verify whether the PSTVd-specific cDNA was mutated to the sequence represented by the replicating RNA. Southern hybridization revealed that one copy of the T-DNA was integrated without rearrangements but it was not possible to detect nine bp deletions by this method. Therefore genomic DNA of the transgenic plant was PCR amplified with prim-

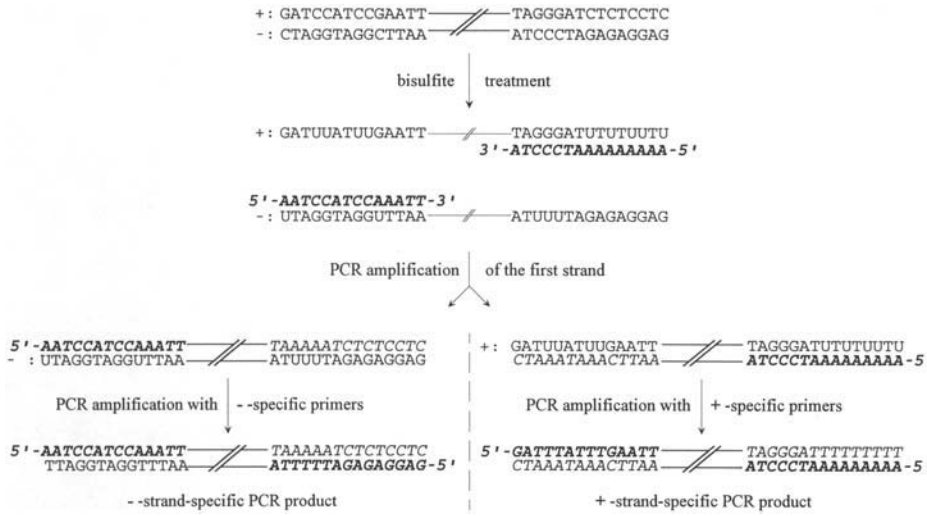

Fig. 1. The principle of PCR amplification of bisulfite-treated DNA. After bisulfite treatment of 200 pg to 3 μg of genomic DNA all cytosines should be converted to uridines. To each strand of a known genomic DNA PCR primers are designed that are complementary to the converted sequence in that an A is incorporated at all positions of the primer binding sites where the original sequence contained a G. For both strands a separated PCR is performed and after cloning of the products individual clones have to be sequenced.

ers specific for PSTVd (325-H, 326-R, 175-H, 176-R), the P_{35S} (35S P1), and the pA_{nos} (pA P2). Sequence analysis of the cloned PCR products demonstrated that the plant genome-integrated PSTVd-specific cDNA had not been altered.

Our most recent studies on viroid replication answered the question why mechanical inoculation with PSTVd KF440-2, an isolate that is known to be highly infectious for various plant species, results in a low rate of infection in tobacco plants ***(9)***. Mechanical inoculation of plants is generally used to propagate and/or to transmit viroids. For experimental reasons we had been dependent on transmitting PSTVd which was isolated from tomato to tobacco plants ***(6)***. Because it was known that genome-integrated PSTVd cDNAs could initiate viroid infection it was surprising that only in one out of ten mechanically inoculated tobacco plants PSTVd replication was detectable. Thus the PSTVd RNA replicating in transgenic and in mechanically inoculated tobacco plants was characterized by reverse-transcribing the RNA and subsequently amplifying the resulting cDNA by PCR using the PSTVd-specific primer pairs 325-H/326-R and 175-H/176-R, respectively (*see* **Note 1**). Sequence analysis of the cloned PCR products and comparison with the original sequence revealed that

a single nt substitution was necessary to convert PSTVd KF 440-2 from a non-infectious to an infectious RNA for *Nicotiana tabacum*.

2. Materials

2.1. Plant Material, Cloning Vectors, and Bacterial Strains

1. *Nicotiana tabacum* plants (cv. Petit Havana SRI) maintained under sterile conditions or grown in soil.
2. PCR cloning vector pTPCR *(7)* (*see* **Note 2**).

2.2. DNA/RNA Extraction

1. Mortar and pestle, 1.5 mL microcentrifuge tube glass pestle, sea sand.
2. Miracloth (Calbiochem) quick filter material.
3. Beckman centrifuges J2-21M and L8-70 or similar centrifuges and Eppendorf microcentrifuge. Beckman rotors JS-7.5, VTi 50, VTi 80, and corresponding centrifuge tubes or similar rotors and tubes.
4. QIAprep Spin Miniprep Kit (Qiagen).
5. HB- and HB^+-buffer: 50 m*M* Tris-HCl (pH 8.0), 5 m*M* $MgCl_2$, 0.3 *M* sucrose. For HB^+-buffer add 40 mL of Triton X-100/L.
6. RB-buffer: 30 m*M* Tris-HCl (pH 8.0), 10 m*M* EDTA, 1% Sarkosyl.
7. Protinase K (Boehringer Mannheim).
8. Z6-buffer: 8 *M* guanidinium chloride, 20 m*M* 2-morpholinoethanesulfonic acid (MES), 20 m*M* EDTA, 50 m*M* 2-mercaptoethanol, pH 7.0.
9. Additional material and chemicals (Merck, Sigma): CsCl, CsCl stock solution (1 g/mL), ethidium bromide stock solution (10 mg/mL), ethanol, propanol, propanol equilibrated with CsCl-saturated water (S-propanol), 1-mL and 5-mL syringes, 19G × 1 1/4 in. Luer-Lock needles, phenol-mix: mix 1 vol of phenol equilibrated with TE-buffer (pH 7.0) 1 vol of $CHCl_3$, TE-buffer: 10 m*M* Tris-HCl (pH 8.0), 1 m*M* EDTA.

2.3. cDNA Synthesis

1. cDNA Synthesis Kit (Boehringer Mannheim).
2. Oligonucleotide primers diluted in sterile water to 100 μ*M* (*see* **Table 1**).

2.4. Bisulfite-Treatment of Genomic DNA

1. 3.6 *M* sodium bisulfite (Sigma) solution, pH 5.0 (pH adjustment with 10 *M* NaOH), 10 m*M* hydroquinone, 3 *M* NaOH solution, 5 *M* NH_4OAc, pH 7.0, 3 *M* NaOAc, pH 5.0.
2. Wizard DNA Clean-Up System (Promega).
3. 1 μg of plasmid DNA dissolved in 10 μL of H_2O and the primer pair C1/C2 (*see* **Note 3**).

2.5. PCR Amplification

1. Goldstar DNA Polymerase (5 U/μL) and Goldstar Dilution Buffer (Eurogentec)
2. 10X PCR reaction buffer: 750 m*M* Tris-HCl, pH 9.0 (at 25°C), 200 m*M* $(NH_4)_2SO_4$, 0.1% (w/v) Tween-20, and a 25 m*M* stock of $MgCl_2$ (Eurogentec).

Table 1
Oligonucleotide Primers Used for the PCR Amplification of Viroid-, ^{35}S Promoter-, and pA_{nos}-Specific Sequences

Primers	Sequence (5' to 3')
CaMV-promoter	
35S P1	CCTTCCTCTATATAAGGAAG
35S18-H	CACAATCCCACT.ATCCTTCACAAAACCCTT
Poly (A) sequence of NOS-gene	
pA P2	GCCAAATGTTTGAACGATCGGG
Potato spindle tuber viroid (PSTVd)	
PV320-R/2	TTYAGTTGTTTYYAYYGGGTAGTAGTTGAA
325-H	TCGCCCCGGAACAAGTTA
326-R	GGGTGTTTAGCCCTTGGAA
175-H	TCCTGTCGGCCGCTGGGCACTCCC
176-R	GTAATTCCCGCCGAAACAGGG

3. PCR nucleotide mix, premixed 10 m*M* deoxynucleotide solution (Boehringer Mannheim).
4. Sterile 0.5 mL PCR reaction tubes and mineral oil, light (Sigma).
5. Oligonucleotide primers diluted in sterile water to 100 μ*M* (*see* **Table 1**).

2.6. Agarose Gel Electrophoresis and DNA Extraction from Agarose Gels

1. Agarose DNA (Biozym), QIAquick Gel Extraction Kit (Qiagen).
2. 10X TBE buffer: 1 *M* Tris-HCl, 830 m*M* boric acid, 10 m*M* EDTA (pH 8.0), add 300 μL/L of a ethidium bromide stock solution (10 mg/mL).
3 5X Gel loading dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 5 m*M* EDTA, 40% sucrose (w/v).
4. DNA marker: Any commercially available DNA marker (fragment-size: 100–8000 bp). Here λ-DNA cut with the restriction endonuclease *Pst*I was used.

2.7. Cloning of PCR Products

1. Restriction endonucleases: *Eco*RI (10 U/μL), *Bam*HI (10 U/μL), *Hin*dIII (10 U/μL), and *Dra*I (40 U/μL), supplied with 10X reaction buffer (Boehringer Mannheim).
2. TA Cloning™ Kit (Invitrogen) (*see* **Note 2**).
3. LB-Medium: Dissolve 10 g Bacto tryptone, 5 g Bacto yeast extract, 10 g NaCl, 1 g glucose in 800 mL deionized water, adjust pH to 7.4 with a 1 *M* NaOH stock solution (for LB-plates add 15 g Gibco agar), add deionized water to a final volume of 1 L, pour medium into a 1-L Duran glass flask (Schott) and sterilize by autoclaving.

4. LB-plates: The following work should be done under sterile conditions in a hood. Cool down LB-Medium to 50°C, dissolve 100 mg Ampicillin trihydrate powder (Sigma), pour medium into 90 mm Petri dishes. After the medium has become solid close Petri dishes and store the plates at 4°C. Before use distribute 200 µL of a 2% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (Boehringer Mannheim) (dissolved in dimethyl-formamide) stock solution onto each plate and let them dry under the hood.

3. Methods

3.1. DNA and RNA Extraction

1. DNA isolation: Until first centrifugation all works should be done at 4°C; 5 g of young tobacco leaves are ground with 3 mL of HB-buffer in a mortar and pestle. The slurry volume is brought to 50 mL with HB-buffer and filtered through four layers of miracloth filter. Add 50 mL of HB^+-buffer and incubate on ice for 10 min. Centrifuge at 2000 rpm for 10 min in JS-7.5 rotor at 4°C . Carefully clean the pellet of excess liquid with a paper towel. Resuspend the pellet in 30 mL of RB-buffer, add 5–10 mg of Proteinase K and incubate 12–48 h at 37°C. Dissolve 32 g of CsCl, and add 1 mL ethidium bromide stock solution (*see* **Note 4**). Transfer mixture to Vti 50 Quick-Seal centrifuge tubes and fill up tubes with CsCl stock solution. Centrifuge at 45,000 rpm for 16 h at 25°C and collect the highly viscous purple DNA band by piercing the side of the tube with a needle just below the band. Before suction of the DNA band aerate the tube by piercing the top of the tube with a second needle. Recentrifuge the DNA in a Vti 80 rotor (Quick-Seal tubes should be filled up with the CsCl stock solution) and collect the DNA as described. Extract the ethidium bromide with S-propanol (three to five times) and precipitate the DNA by adding 3 vol of propanol, gentle shaking, and centrifugation in a microcentrifuge at full speed for 15 min. Wash the pellet with 75% ethanol, recentrifuge, and resuspend the DNA in 200–500 µL TE-buffer (*see* **Note 5**).
2. RNA isolation: Cut off a 1 cm^2 disk from young tobacco leaves, push the disk into a 1.5 mL microcentrifuge tube, spread a small amount of sea sand into the tube, add 300 µL of Z6-buffer and ground the leaf tissue for 1 min with a glass pestle. Mix the slurry with another 300 µL of Z6-buffer and 600 µL of the phenol-mix, centrifugation in a microcentrifuge at full speed for 15 min, and precipitate the RNA by adding 0.7 vol of ethanol to the supernatant. After centrifugation in a microcentrifuge at full speed for 15 min, wash the pellet with 75% ethanol, recentrifuge, and resuspend the DNA in 50 µL TE-buffer.

3.2. Bisulfite-Treatment of Genomic Tobacco DNA

1. Ten micrograms of genomic tobacco DNA are cut with *Dra*I (*see* **Subheading 2.7., step 1**) in a total volume of 100 µL at 37°C for 2 h (*see* **Note 6**). The DNA is then phenol extracted by shaking out the restriction sample with 100 µL of the phenol-mix (*see* **Subheading 2.2., step 9**), and precipitated by adding 10 µL of 3 *M* NaOAc and 250 µL of ethanol. After centrifugation and wash (*see* **Subheading 3.1.**) resuspend the pellet in 50 µL H_2O and add 1 µL of the plasmid DNA.

2. Denature the DNA by adding freshly prepared NaOH to a final concentration of 0.3 *M* and incubation at 37°C for 15 min. Add the freshly prepared hydroquinone and sodium bisulfate solution to final concentrations of 0.5 m*M* and 3.1 *M*, respectively, and incubate at 55°C for 16 h.
3. Desalt the reaction mix with the Wizard DNA Clean-Up System according to the manufacturer's instructions and elute the DNA with 50 µL of TE-buffer.
4. Store the DNA at 4°C until ready for PCR amplification.

3.3. cDNA Synthesis

1. The cDNA is produced with the cDNA Synthesis Kit according to the manufacturer's instructions with 10 µL of total RNA (*see* **Subheading 3.1., step 2**, and using the primer-pairs 325-H1326-R and 175-H/176-R, respectively. After the final step (T4-DNA-Polymerase) the cDNA is stored at –20°C without further manipulations.

3.4. PCR Amplification of cDNA

1. Dilute 10 µL of the cDNA (1:10) and use 1 µL of this dilution for each PCR amplification (*see* **Note 7**).
2. Dilute 0.5 µL of the Goldstar DNA Polymerase (1:10) in Goldstar Dilution Buffer and keep on ice.
3. Fill into each of three 0.5 mL PCR-reaction tubes 66 µL H_2O, 10 µL of the dNTP mix, 10 µL of the 10X PCR reaction buffer, 10 µL of the 25 m*M* the $MgCl_2$ stock solution, and 1 µL of the cDNA and mix.
4. Pipet 1 µL of the following primers: tube A: 325-H and 326-R, tube B: 175-H and 176-R, tube C: 35S P1 and 325-H (*see* **Note 8**), add 1 µL of the diluted polymerase to each reaction mix, and protected against drying up with 100 µL of mineral oil.
5. Transfer the tubes into the thermal cycler block and start 30 cycles of: 95°C, 60 s; 55°C, 30 s; 72°C, 60 s.
6. Store samples at –20°C.

3.5. PCR Amplification of Genomic DNA

1. Proceed as described (**Subheading 3.4.**) but use 3–5 µg of genomic DNA instead of the cDNA and for each reaction one of the primer pairs 35S P1/pA P2, 35S P1/325-H, and 326-R/pA P2, respectively.

3.6. PCR Amplification of Bisulfite-Treated Genomic DNA

1. Proceed as described in **Subheading 3.4., steps 2** and **3**, but fill 55 µL of H_2O instead of 66 µL into each tube.
2. Add the following DNA into tube A and B: 10 µL of bisufite-treated genomic DNA and tube C: 10 µL of untreated genomic DNA (1 µg).
3. Add 1 µL of each of the following primers to: tube A and C: 35S18-H and PV320-R/2 and tube B: C1 and C2.
4. Add 1 µL of the diluted polymerase to each reaction mix, protected against drying up with 100 µL of mineral oil, transfer the tubes into the thermal cycler block,

and run the cycler under the following conditions: 94°C/120 s × 1 cycle; 94°C/60 s, 50°C/120 s, 72°C/180 s × 5 cycles; 94°C/30 s, 50°C/120 s, 72°C/150 s × 25 cycles; 72°C/6 min × 1 cycle.
5. Store samples at –20°C.

3.7. Cloning of PCR Products

1. Prepare a 1.5% agarose gel on a 8 × 7 cm gel tray, place it into the gel box, fill the box with 1X TBE buffer, and remove the well comb (13 wells [0.4 × 0.2 mm]).
2. Remove the mineral oil from each PCR sample as long as the reaction mix is frozen.
3. Let the reaction mix thaw, mix 10 µL of each with 2 µL of the gel loading dye, and load the samples and the DNA marker onto the agarose gel.
4. Run the gel on constant current at 60 mA for 45 min and photograph the gel on a UV transilluminator (302 nm).
5. Each of the PCR products banding at the appropriate size for the primer pair that was used is excised from the gel and transferred into a 1.5-mL microcentrifuge tube.
6. The DNA is extracted from the agarose using the QIAquick Gel Extraction Kit according to the manufacturer's instructions.
7. Precipitate the DNA by adding 5 µL of 3 *M* NaOAc and 125 µL of ethanol. After centrifugation and wash (*see* **Subheading 3.1.**) resuspend the pellet in 5 µL H_2O.
8. Ligation of the PCR products, transformation of the *E. coli* strain INVαF'; and plating of the bacteria is performed with the TA Cloning Kit according to the manufacturer's instructions.

3.8. Extraction and Purification of Plasmid DNA

1. Each, but not more than 64/d, of the white bacteria colonies are picked from the LB plate with a toothpick, transferred into 2 mL of liquid LB-medium containing ampicillin, and grown at 37°C for 16 h.
2. The plasmid DNA is extracted using the QIAprep Spin Miniprep Kit according to the manufacturer's instructions.

3.9. Restriction and Sequence Analyses of Plasmid DNA

1. Three microliters of each of the purified plasmid DNAs are mixed with 10 µL H_2O, 1.5 µL of the corresponding 10X restriction endonuclease reaction buffer, and 0.5 µL of *Eco*RI.
2. Incubate samples at 37°C for 1–2 h.
3. Prepare a 1.5% agarose gel as described (**Subheading 3.7.**), add 3 µL of the gel loading dye to each sample and proceed as described (**Subheading 3.7.**).
4. Each clone of which the *Eco*RI-released insert is banding at the appropriate size for the primer pair that was used is stored at 4°C until ready for sequencing.
5. Sequence analyses of plasmid DNA (*see* **Note 9**).

4. Notes

1. Although in our hands RT-PCR was successfully performed to amplify numerous viroids, including PSTVd, we prefer to reverse-transcribe the PSTVd RNA

before application of PCR. To verify the sequence of a certain PSTVd isolate the viroid RNA has to be reverse transcribed twice to ensure that there are no alterations within the primer-binding site. Because of the stable secondary structure of PSTVd RNA and depending on the region to which the PCR primers were complementary, RT-PCR amplification sometimes failed, whereas cDNA synthesis with the Boehringer cDNA Kit was always successful.

2. Although cloning of our PCR products was performed with pTPCR, which is not commercially available, the use of the TA Cloning Kit (Invitrogen) is described here. The vector pTPCR was constructed in our laboratory to save money *(7)*. It is very similar to the pCRII that is delivered with the Invitrogen cloning kit.
3. In centrifugation gradients the position of the banding DNA is dependent on the amount of CsCl. Therefore the precise amount of salt should be added.
4. Because the plasmid DNA serves as a control for complete bisulfite-conversion of cytosine residues, any plasmid DNA can be used. The PCR primer pair C1/C2 have to be complementary to the plasmid DNA and should be designed according to **Fig. 1**. No C residues should be present in the primer binding sites and the resulting PCR product should be in a size range of 100–200 bp.
5. Never dry the DNA pellet of genomic DNA, because it may take months to dissolve it again.
6. Before the bisulfite-treatment the genomic DNA has to be cut with a restriction endonuclease to destruct the viscosity of the DNA and to obtain linear fragments smaller than 20 kb.
7. When undiluted cDNA is used as template, several unspecific PCR products are visible on an agarose gel and the amount of the specific product decreases.
8. The PCR amplification with the primer pair 35S P1 and 325-H serves as a control for contamination of the cDNA with genomic DNA and should not give a specific PCR product.
9. The plasmid DNA can be used for any sequencing technique. In our laboratory, sequence analysis is performed with an ALFexpress DNA Sequencer (Pharmacia Biotech) according to the manufacturer's instructions, and using the chemicals and Sequencing Kits that are recommended by Pharmacia.

Acknowledgments

I thank Heinz L. Sänger and Robert Gillies for critical revision of the manuscript. The work was supported by the Deutsche Forschungsgemeinschaft (Habilitandenstipendium Wa 1019/1-1).

References

1. Clark, S. J., Harrison, J., Paul, C. L., and Frommer, M. (1994) High sensitivity mapping of methylated cytosines. *Nucleic Acids Res.* **22,** 2990–2997.
2. De Jong, J., Mertens, M. M. J., and Rademaker, W. (1994) Stable expression of the GUS reporter gene in *chrysanthemum* depends on binary plasmid T-DNA. *Plant Cell Rep.* **14,** 59–64.

3. Zhong, H., Bolyard, M. G., Srinivasan, C., and Sticklen, M. B. (1993) Transgenic plants of turfgrass *(Agrostis palustris* Huds.) from microprojectile bombardment of embryogenic callus. *Plant Cell Rep.* **13,** 1–6.
4. Grayburn, S. W. and Vick, B. A. (1995) Transformation of sunflower *(Helianthus annuus* L.) following wounding with glass beads. *Plant Cell Rep.* **14,** 285–289.
5. Yepes, M. L., Mittak, V., Pang, S.-Z., Gonsalves, C., Slightom, J. L., and Gonsalves, D. (1995) Biolistic transformation of *chrysanthemum* with the nucleocapsid gene of tomato spotted wilt virus. *Plant Cell Rep.* **14,** 694–698.
6. Wassenegger, M., Heimes, S., Riedel, I., and Sanger, H. L. (1994) RNA-directed *de novo* methylation of genomic sequences in plants. *Cell* **76,** 567–576.
7. Wassenegger, M., Heimes, S., and Sänger, H. L. (1994) An infectious viroid RNA replicon evolved from an *in vitro*-generated non-infectious viroid deletion mutant via a complementary deletion *in vivo. EMBO J.* **13,** 6172–6177.
8. Frommer, M., McDonald, L. E., Millar, D. S., Collis, C. M., Watt, F., Grigg, G. W., Molloy, P. L., and Paul, C. L. (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci. USA* **89,** 1827–1831.
9. Wassenegger, M., Spieker, R. L., Thalmeir, S., Gast, F.-U., Riedel, L., and Sänger, H. L. (1996) A single nucleotide substitution converts potato spindle tuber viroid (PSTVd) from a non-infectious to an infectious RNA for *Nicotiana tabacum. Virology* **226,** 191–197.

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Detection of Hepatitis C Virus RNA by Semiquantitative Reverse-Transcription PCR

Anderson S. Gaweco and David H. Van Thiel

1. Introduction

The human hepatitis C virus (HCV) is an RNA virus discovered in 1989 that accounts for the majority of posttransfusion and sporadic non-A non-B hepatitis. More than half of patients with acute HCV infection develop a chronic course that is associated with a high risk of developing liver cirrhosis and hepatocellular carcinoma.

The detection of HCV RNA in tissue and blood with the reverse-transcription polymerase chain reaction (RT-PCR) technique is currently the diagnostic standard for HCV infection. The highly sensitive HCV RNA PCR is more reliable than anti-HCV based (ELISA, RIBA) assays that are subject to false-positive results. The accurate diagnosis of HCV infection in patients with impaired immune responses (e.g., HIV, immunosuppressed patients), ambiguous HCV serology (e.g., alcoholics, autoimmune disease, paraproteinemia, hemodialysis) and those in the early phase of acute infection prior to seroconversion can only be reliably confirmed by PCR-based testing as opposed to serological assays. More important than the initial detection of HCV infection is the assessment of interferon-treatment efficacy by monitoring viral HCV titers using PCR during the course of therapy.

We utilize an in-house semiquantitative PCR-based method that can reliably detect HCV positive-strand RNA in human liver tissue and serum. The HCV genome consists of a positive single-stranded RNA approx 10,000 nucleotides long with a single open reading frame (ORF) encoding different structural and nonstructural viral proteins. The ORF is bounded by noncoding regions at its 5' and 3' termini. The noncoding region (NCR) at the 5'-end is highly conserved among different strains and is the preferred target sequence

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

of PCR-based analyses. The sense HCV101 and antisense HCV102 primers used in the given protocol are specific for the highly conserved 5' NCR of the viral HCV genome ***(1,2)***.

2. Materials

2.1. Sampling

1. Sterile cryo tubes (Nunc).
2. Red-top tubes.

2.2. RNA Extraction

1. Nitrogen weighing paper that does not absorb moisture (Fisher).
2. Sterile disposable scalpel blades.
3. 2 mL Eppendorf reaction tubes.
4. Diethylpyrocarbonate (DEPC)-treated water: Add 1 mL of DEPC (Sigma) to 500 mL of distilled water and shake vigorously. Autoclave and cool at room temperature under a fume hood. Always work with gloves and under a fume hood, as DEPC is carcinogenic. All solutions for RNA extraction should be prepared with DEPC-treated water.
5. Extraction solution: Water-saturated nucleic acid-grade phenol (Gibco), 4 *M* guanidinium thiocyanate [GTC] (Fluka)/25 m*M* sodium citrate (pH 7.0) solution and 2 *M* NaOAc (pH 4.0) in a ratio of 1:1:0.1 supplemented with β-mercaptoethanol per 100 mL of working solution. The extraction solution can be stored for 1 mo at 4°C.
6. 4 *M* GTC/25 m*M* sodium citrate (pH 7.0): Stock solution can be prepared and stored for several months at 4°C.
7. Chloroform/isoamylalcohol (24:1). Store in brown bottles at 4°C.
8. Isopropanol.

2.3. Reverse-Transcription

1. 5X RT buffer (Gibco).
2. 12.5 m*M* dNTP Mix stock solution: Add 12.5 µL each of 100 m*M* Ultra Pure dATP, dCTP, dGTP, and dTTP (Pharmacia) to 50 µL sterile distilled water.
3. Moloney murine leukemia virus (MMLV) reverse transcriptase (200 U/µL, Gibco).
4. 100 m*M* dithiothreitol (DTT) (Gibco).

2.4. Polymerase Chain Reaction

1. Synthesize HCV and human β-actin primers using a DNA synthesizer (Applied Biosystems). If a DNA synthesizer is not available, oligonucleotide primers could be ordered commercially synthesized (Gibco). Determine concentration and purity using a spectrophotometer with an OD of 1 corresponding to approx 20 µg/mL of oligonucleotides. Adjust concentration to 20 µ*M* with sterile distilled water.
2. 10X PCR-buffer: 15 m*M* $MgCl_2$, 500 m*M* KCl, 100 m*M* Tris-HCl, pH 8.3 (Perkin-Elmer).

3. 1.25 m*M* dNTP Mix stock solution: Add 12.5 µL each of 100 m*M* Ultra Pure dATP, dCTP, dGTP, and dTTP (Pharmacia) to 950 µL sterile distilled water.
4. 5 U/µL *Taq*-Polymerase (Perkin-Elmer).

2.5. Agarose Gel Electrophoresis

1. 1X TAE Electrophoresis buffer: Mix 60 mL of 50X TAE buffer and 150 µL of 10 mg/mL ethidium bromide. Fill up to 3 L with distilled water.
2. 5X Loading buffer: 50 m*M* Tris, pH 7.6, 50 m*M* Na-EDTA, 0.5% SDS, 0.1% bromphenol blue.
3. 1.5% agarose gel: Add 0.75 g low melting Ultra Pure agarose (Gibco-BRL) to a 50 mL 1X TAE solution. Heat the mixture on a stirring plate until the solution becomes clear. Cool down to 65°C and add 2.5 µL of 10 mg/mL ethidium bromide (Sigma) into the mix and polymerize in an electrophoresis tray at room temperature.
4. 10 mg/mL ethidium bromide (Gibco). Always wear gloves when handling ethidium brromide as it is carcinogenic.

3. Methods

3.1. Sampling

1. Place each core liver biopsy specimen in a sterile cryo tube, snap-freeze in liquid nitrogen and store at –80°C until further processing (*see* **Notes 1** and **2**).
2. Collect freshly drawn peripheral blood in red-top tubes and allow blood to clot at 37°C in a water bath. Separate serum in a centrifuge at room temperature. Aliquot serum samples in 2 mL cryo tubes, snap-freeze in liquid nitrogen, and store at –80°C (*see* **Notes 1** and **2**).

3.2. RNA Extraction

Several RNA extraction protocols are available including commercial RNA isolation kits. We have employed the guanidinium method modified from previously described protocols ***(3,4)***.

1. Add 1 mL of cold extraction solution and 100 µL of chloroform/isoamylalcohol (24:1) in 2 mL Eppendorf reaction tubes and keep on ice prior to sample processing.
2. Homogenize snap-frozen core liver biopsy tissue by allowing tissue to thaw briefly on a Nitrogen weighing paper (Fisher) and mince using a sterile disposable scalpel. Change scalpel blades and weighing paper for every sample to be processed to prevent crosscontamination.
3. Place the tissue homogenate or 100 µL of serum/plasma sample in the 2 mL reaction tube containing extraction solution and chloroform/isoamyl alcohol (*see* **Note 3**).
4. Mix the reaction by vortexing for 1 min and incubate on ice for 30 min.
5. Centrifuge thereafter at 12,000*g* for 20 min at 4°C.
6. Transfer the upper aqueous phase (approx 500 µL) into a 1.5 mL reaction tube and mix with an equal volume of ice-cold isopropanol.
7. Incubate the reaction for 1 h or overnight for convenience at –20°C.

8. Centrifuge as described in **step 5**.
9. Wash pelleted RNA twice in ice-cold 70% ethanol and collect the RNA by centrifugation at 12,000*g* for 10 min after each wash.
10. Air-dry under a bench laminar flow for 5–10 min and resuspend the RNA pellet in an appropriate volume (approx 10–20 µL) of DEPC-water. RNA solution stored at –80°C remain intact for almost 1 yr.
11. Measure RNA concentration and purity at wavelengths of 260 and 280 nm using a spectrophotometer (GeneQuant, Pharmacia).

3.3. Reverse-Transcription

1. Mix 500 ng total RNA of each sample with 1 µL of 20 m*M* antisense HCV 102 primer (*see* **Note 4**) and adjust volume to 5.2 µL with DEPC-water in a sterile 0.5 ml reaction tube (*see* **Subheading 2.4.**).
2. Heat denature the reaction at 70° C for 5 min and chill on ice.
3. Prepare the following reagents in a reaction tube:
   - 2 µL 5X RT buffer (Gibco)
   - 0.8 µL 12.5 m*M* dNTP Mix
   - 1 µL MMLV Reverse Transcriptase (200 U/µL, Gibco)
   - 1 µL dithiothreitol [DTT] (Gibco)

   Transfer 4.8 µL of the total RT mix to the reaction tube, vortex and centrifuge briefly.
4. Incubate for 60 min at 37°C, and heat reaction for 10 min at 80°C to denature the reverse transcriptase and chill on ice. Briefly centrifuge and store at –20°C until needed.

3.4. Polymerase Chain Reaction

3.4.1. Incubation Mixture

1. The amplifications were performed using 10 µL of each cDNA sample in a 100 µL reaction mixture (final concentration) under a laminar flow. For efficient and reproducible amplification, prepare the components as follows:

cDNA	10 µL
1.25 m*M* dNTP mix (*see* **Subheading 2.4.**)	16 µL
primer HCV 101 (20 m*M*)	1 µL
primer HCV 102 (20 m*M*)	1 µL
10X PCR Buffer	10 µL
5 U/µL Taq-Polymerase	0.4 µL
sterile distilled H_2O	61.6 µL

   (0.2 m*M* of each primer, 50 m*M* KCl, 10 m*M* Tris-HCl, pH 8.3, 1.5 m*M* $MgCl_2$, 200 µ*M* dATP, 200 µ*M* dCTP, 200 µ*M* dGTP, 200 µ*M* dTTP, and 2.0 U of *Taq*-DNA polymerase).
2. Mix the reaction tube and spin down briefly in a microcentrifuge.

3.4.2. PCR Amplification Cycling Parameters

1. Incubate each sample mix at 37°C for 10 min and at 94°C for 10 min. Amplifiy using an automated thermocycler (Perkin-Elmer) for 30 cycles. Each cycle should

include a denaturation step at 94°C for 45 s, an annealing step at 60°C for 90 s, and a chain elongation step at 72°C for 90 s (see **Notes 4** and **5**). After the last cycle, include a final extension time of 6 min at 72°C.

3.5. Agarose Gel Electrophoresis

1. Mix 4.0 μL of the amplified PCR product with 4.0 μL distilled water and 2.0 μL of 5X loading buffer in a reaction tube.
2. Load the sample mixture on a 1.5% agarose/ethidium bromide gel in an electrophoresis chamber (GNA-100, Pharmacia) with 1X TAE electrophoresis buffer.
3. Verify the amplified 251 bp product size as shown on an agarose gel under an ultraviolet light illuminator at 302 nm relative to molecular weight marker standards. We use *Bgl*I and *Hinf*I digested pBR 328 DNA as molecular weight marker (Boehringer Mannheim).
4. Photograph with a Polaroid camera using Polaroid films (667, black/white, ISO 3000/36°).

4. Notes

1. Liver biopsy tissue and aliquoted serum samples should be immediately snap-frozen in liquid nitrogen and stored in an ultralow freezer at –80°C until further processing. Samples can be stored at –80°C up to 1 yr with no detectable loss of viral HCV RNA levels. Extreme caution should be undertaken avoiding freeze-thaw cycles and temperature changes to assure viral genomic RNA integrity. Inappropriate handling of samples may result to loss of viral HCV RNA leading to false-negativity.
2. Cryoglobulinemia occurs in as many as one-third of patients testing positive for HCV-RNA ***(5)***. Aliquoted frozen serum samples need to be prewarmed at 37°C in a water bath and resuspended prior to PCR analysis. Otherwise, precipitation of cryoglobulins directed at HCV epitopes present in the serum samples of these individuals with low HCV-RNA levels or large amount of cryoglobulins could lead to false-negative HCV-RNA PCR in 20% of cases (**Fig. 1A**) ***(5)***.
3. Place homogenized tissue and thawed serum samples immediately in the reaction tube containing extraction buffer. GTC is a strong RNase inhibitor and this would help prevent possible RNA degradation, particularly as liver tissue is rich in endogenous ribonucleases.
4. A control cDNA synthesis should be performed using antisense human β-actin primers (**Table 1**) for each RNA sample to be analyzed.
5. For every amplification performed, reagent controls containing all the necessary components for PCR but without the addition of template cDNA should be included which should serve as an internal standard control for contamination. All precautions for PCR contamination according to Kwok and Higuchi should be observed including specially designated pre-PCR and post-PCR rooms ***(6)***. Perform PCR preparation and store materials only under a laminar flow hood equipped with UV light. The PCR conditions for every primer pair were strictly optimized after carefully adjusting the $MgCl_2$, primer and *Taq* polymerase concentrations, pH of the PCR reaction buffer and primer annealing temperature

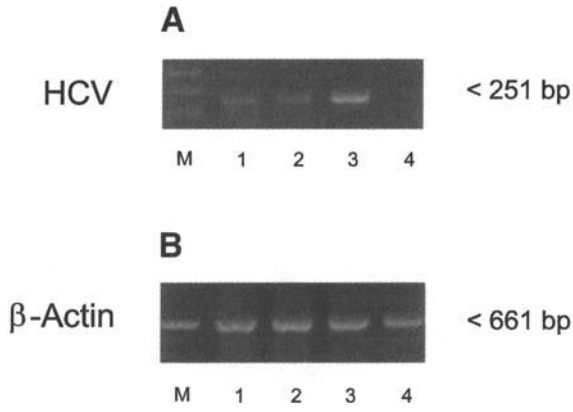

Fig. 1. (**A**) Amplified HCV-RNA PCR products (251 bp). *Bgl*I- and *Hinf*I-digested pBR 328 DNA as molecular weight marker (M, lane 1). Liver biopsy samples of patients TS (sample 1, lane 2) and JG (sample 2, lane 3) positive for anti-HCV ELISA 2 and RIBA 2 assay. Frozen sera obtained from patient TS (cryoglobulin-positive) with (sample 3, lane 4) and without (sample 4, lane 5) preheating at 37°C and resuspension prior to RNA extraction. (B) *Bgl*I- and *Hinf*I-digested pBR 328 DNA as molecular weight marker (lane 1). Amplified β-actin products (661 bp) as control for RNA extraction and cDNA synthesis efficiency for samples 1 to 4 (lanes 2–5).

Table 1
HCV and Human β-Actin Primer Sequences

Sense HCV 101 primer (nt 26–49) 5'-CAC TCC CCT GTG AGG AAC TAC TGT- 3'
Antisense HCV 102 primer (nt 244–276) 5'-TAC CAC AAG GCC TTT CGC GAC CCA ACA CTA CTC- 3'
Sense human β-actin primer *(7)* 5' -TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA- 3'
Antisense human β-actin primer 5' - CTA GAA GCA TTG CGG TGG ACG ATG GAG GG - 3'

producing distinct single bands of the amplified PCR products (**Fig. 1**). Efficiency of RNA and cDNA synthesis should be assessed by amplifying 10 μL cDNA of each RNA sample with human β-actin primers (**Table 1**).

References

1. Gurakar, A., Fagiuoli, S., Faruki, H., et al. (1995) Utility of hepatitis C virus RNA determinations in hepatic tissue as an end point for interferon treatment of chronic hepatitis C. *Hepatology* **22,** 1109–1112.

2. Kato, N., Hijikata, M., Ootsuyama, Y., et al. (1990) Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc. Natl. Acad. Sci. USA* **87,** 9524–9528.
3. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156–159.
4. Xie, W. Q. and Rothblum, L. I. (1991) Rapid, small-scale RNA isolation from tissue culture cells. *Biotechniques* **11,** 324–327.
5. Van Thiel, D. H., Fagiuoli, S., Caraceni, P., et al. (1995) Cryoglobulinemia: a cause for false negative polymerase chain reaction results in patients with hepatitis C virus positive chronic liver disease. *J. Hepatol.* **22,** 464–467.
6. Kwok, S. and Higuchi, R. (1989) Avoiding false positives with PCR. *Nature* **339,** 237, 238.
7. Ehlers, S. and Smith, K. A. (1991) Differentiation of T cell lymphokine gene expression: the in vitro acquisition of T cell memory. *J. Exp. Med.* **173,** 25–36.

15

Detection of *Legionella* Species in Bronchial Fluid by PCR

Gorm Lisby

1. Introduction

Patients suffering from Legionnaire's disease initially experience nonspecific symptoms such as fever, myalgia, and headache. The predominant clinical finding is pneumonia with symptoms ranging from a mild cough and slight fever to diffuse pulmonary infiltrates and multiorgan failure. The disease is caused by infection with *Legionella*, and since the outbreak of Legionnaire's disease in Philadelphia in 1976, resulting in 34 deaths among 221 cases ***(1)***, *Legionella pneumophila* has been established as an important cause of atypical pneumonia ***(2)***.

This short gram-negative bacterium infects alveolar macrophages, causing pneumonia by compromising the antigen presenting link of the pulmonary defence system. The family *Legionellaeceae*, genus *Legionella*, contains at least 32 species with 51 different serotypes. At least 16 species have been established as human pathogens with *L. pneumophila* responsible for the majority of the cases ***(3–6)***. *Legionellae* are normal inhabitants of fresh water, both in natural and man-made reservoirs ***(7)***, and the infection of humans occurs by inhalation of *Legionella*-infected water droplets ***(8,9)***.

The standard methods for detection of *Legionella* are growth on selective media ***(10)***, microscopic examination using indirect fluorescent antibody tests ***(11,12)*** and detection of *Legionella* antigens in urine ***(13)***. Culture is generally not considered to achieve 100% sensitivity and is time-consuming, thus delaying confirmation of the tentative clinical diagnosis. Immunofluorescence microscopy is hampered by the need for polyclonal antibodies in order to detect several serotypes, thus increasing the risk for false-positive reactions because of antigenic cross-reactivity ***(11)***. Detection of *Legionella* antigens in urine is

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

Table 1
***Legionella* Strains Analyzed in the Described *Legionella* PCR**

Detected	
L. pneumophila Togus 1, serogroup 2	ATCC no. 33154
L. pneumophila Knoxville 1, serogroup 1	ATCC no. 33153
L. pneumophila Bloomington 2, serogroup 3	ATCC no. 33155
L. pneumophila Los Angeles 1, serogroup 4	ATCC no. 33156
L. pneumophila Dallas 1, serogroup 5	ATCC no. 33216
L. pneumophila Chicago 2, serogroup 6	ATCC no. 33215
Legionella dumoffii NY-23, serogroup 1	ATCC no. 33279
Legionella gormanii LS-13, serogroup 1	ATCC no. 33297
Legionella longbeachae Tucker 1, serogroup 2	ATCC no. 33484
Legionella jordanis BL-540, serogroup 1	ATCC no. 33623
Legionella oakridgensis Oak Ridge 10, serogroup 1	ATCC no. 33761
Legionella feeleii WO-44C-C3, serogroup 1	ATCC no. 35072
Not detected	
Legionella micdadei TATLOCK, serogroup 1	ATCC no. 33218
Legionella bozemanii WIGA, serogroup 1	ATCC no. 33217
Legionella wadsworthii 81-716, serogroup 1	ATCC no. 33877

considered specific, but the clinical sensitivity is relatively low, as the test only detects *L. pneumophila* serogroup 1 and part of serogroup 3 ***(13)***.

The polymerase chain reaction technology (PCR) has been applied to detect *Legionella* by mainly two different approaches: the detection of *L. pneumophila* by amplification of a *L. pneumophila*-specific DNA fragment from the macrophage infectivity potentiator (mip) gene ***(14)***, or detection of several *Legionella* species by amplification of ribosomal RNA gene sequences ***(15)***.

The analysis described in this chapter specifically detects all tested serogroups of *L. pneumophila* as well as six of nine tested additional *Legionella* species (**Table 1**).

2. Materials

2.1. Sample Collection and DNA Extraction

1. Extraction buffer: 10 m*M* Tris-HCl, pH 7.4, 10 m*M* EDTA disodium salt, 150 m*M* NaCl, 0.4% SDS, 200 μg/μL Proteinase K.
2. SS-Phenol.
3. Chloroform.
4. 99% Ethanol.
5. 4 *M* Sodium acetate.

6. 70% Ethanol.
7. TE buffer: 10 m*M* Tris-HCl, pH 8.0, 0.1 m*M* EDTA disodium salt.
8. GT buffer: 4 *M* guanidinthiocyanate, 10 m*M* Tris-HCl, pH 6.0, 1 m*M* dithiothretiol.
9. Isopropanol.
10. Microcentrifuge.

2.2. PCR Amplification

1. Sterile water.
2. 10X PCR buffer: 500 m*M* KCl, 100 m*M* Tris-HCl, pH 8.3, 0.01% gelatin, 12.5 m*M* $MgCl_2$.
3. Nucleotide mix containing 1.25 m*M* each dATP, dCTP, dGTP, and dUTP.
4. Urasil *N*-glycosylase 1 U/μL, heat-labile (Boehringer Mannheim).
5. Primers at a concentration of 10 μ*M* each: LEP1: GTTAAGAGCTGA TTAACTG; LEP2: TCATATAACCAACAGCTAGTT. This primer set amplifies an approx 375 basepair fragment of the *Legionella* 16S ribosomal RNA gene.
6. Ampli*Taq* polymerase 5 U/μL.
7. Positive control DNA template (*see* **Note 1**).
8. Mineral oil (*see* **Note 2**).
9. Thermal cycler, Perkin Elmer 480 or Perkin Elmer 2400.

2.3. Agarose Gel Electrophoresis

1. Electrophoresis equipment (e.g., BioRad).
2. UV transilluminator including a Polaroid camera (e.g., PhotoDyne).
3. Agarose (Sigma).
4. Ethidium bromide (10 mg/mL).
5. 40X TAE buffer: Add 193.6 g Trisma base, 108.9 g sodium acatate, 15.2 g EDTA disodium salt, and sterile water to a total volume of 1 L. pH is adjusted to 7.2 with acetic acid.
6. 10X Gel loading buffer: 5 mL concentrated glycerol, 250 μL 40X TAE, 1 mL 2.5% bromphenol blue, sterile water to 10 mL.
7. DNA marker, e.g., *Phi*X174 (Gibco-BRL).

2.4. Southern Blot Hybridization

1. Alkaline solution: 20 g NaOH, 87.7 g NaCl, sterile water to 1 L.
2. Neutralizing solution: 87.7 g NaCl, 121.1 g Trisma base, sterile water to 1 L. pH is adjusted to 7.5 with concentrated HCl.
3. 10X SSC: 87.7 g NaCl, 44.1 g sodium citrate, sterile water to 1 L.
4. Nylon membranes, e.g., Nytran NY 13N (Schleicher and Schuell).
5. Bionick Labeling System (BRL).
6. Prehybridization solution: 500 mL concentrated formamide, 250 mL 20X SSC, 50 mL concentrated Denhardt's solution, 25 mL 1 *M* NaH_2PO_4, 50 mL 10% SDS, 50 mL concentrated Dextran sulfate, sterile water to 1 L.
7. Hybridizing solution: Prehybridizing solution with the addition of 0.5 mg/mL salmon sperm DNA and 1 μg biotin labeled probe (*see* **Note 3**).

8. Hybridization oven.
9. Washing buffers: 2X SSC, 0.1% SDS, and 0.1X SSC, 0.1% SDS.
10. PhotoGene kit (BRL).
11. X-ray films and cassettes.

3. Methods

3.1. Sample Collection and DNA Extraction

1. The sample (e.g., sputum) is collected in sterile 15-mL tubes.
2. Two equally efficient DNA extraction methods can be applied. **Steps 3–19** describe the classical phenol/chloroform extraction method, **steps 20–31** describe a method initially developed for RNA extraction, but the method works well with sputum.
3. 250 µL sputum plus 250 µL extraction buffer are mixed in an 1.5-mL microcentrifuge tube.
4. The sample is incubated for 4 h at 37°C followed by incubation for 2 h at 56°C.
5. 500 µL SS-phenol is added, and the sample is shaken carefully (e.g., by hand) for approx 1 min.
6. The sample is spun at 13,000*g* for 2 min.
7. The (top) waterphase is carefully pipeted to a new microcentrifuge tube. Avoid the interphase.
8. 500 µL chloroform is added, and the sample is shaken carefully (e.g., by hand) for approx 1 min.
9. **Steps 6** and **7** are repeated.
10. 0.1 vol 4 *M* sodium acetate plus 2.5 vol 99% ethanol are added.
11. If visible DNA precipitation, go to **step 12**, otherwise go to **step 15**.
12. Fish out the DNA precipitate with a needle or another suitable device.
13. Wash the precipitate in 70% ethanol.
14. Resuspend the dried precipitate in 25 µL TE buffer. Go to **step 19**.
15. Precipitate DNA for 15 min at –70°C or overnight at –20°C.
16. Spin the sample at 4°C for 30 min at 13,000*g*.
17. Wash the pellet in 70% ethanol.
18. Resuspend the dried precipitate in 25 µL TE buffer.
19. Optional: Determine the DNA concentration and purity by measuring the $(A_{260}–A_{300})/(A_{280}–A_{300})$ optical ratio with a UV-spectrophotometer. The protein absorbency at 300 nm is subtracted from the 260 nm as well as the 280 nm reading. The ratio should be approx 1.8 and 1 Absorbency unit at (A*260*-A*300*) equals 50 µg/µL DNA.
20. Mix 150 µL sputum and 600 µL GT buffer in a microcentrifuge tube.
21. Vortex for 10 s.
22. Add 750 µL isopropanol.
23. Vortex for 10 s.
24. Spin at 13,000*g* for 10 min at room temperature.
25. Remove the supernatant. The pellet may not be visible.
26. Add 1125 µL 70% ethanol.
27. Vortex for 10 s.
28. Spin at 13,000*g* for 10 min at room temperature.

29. Remove the supernatant.
30. Resuspend the pellet (may not be visible) in 25 µL TE buffer.
31. Optional: Determine the DNA concentration as described in **step 19**.

3.2. DNA Amplification (PCR)

1. Observe the general precautions taken to avoid aerosol contamination in the laboratory (*see* **Note 4**).
2. Label 0.5-mL PCR tubes (if Perkin Elmer model 480 is used) or 0.2-mL PCR tubes (if Perkin Elmer model 2400 is used) according to the clinical samples to be analyzed.
3. Prepare 97 µL mastermix for each sample plus one extra to ensure enough for all samples. The mastermix contains (per sample) 10 µL 10X PCR buffer, 4 µL each primer (0.4 µ*M* final concentration each of LEP1 and LEP2), 16 µL nucleotide mix (200 µ*M* final concentration each nucleotide), 0.5 µL (2.5 U total) Ampli*Taq* and 1 µL (1 U total) Urasil *N*-glycosylase (*see* **Note 5**). Add sterile water to 97 µL.
4. Add 97 µL mastermix to each sample. Place the samples on ice (*see* **Note 6**).
5. Add 3 µL extracted DNA to each corresponding PCR tube. It is very important that the total amount of DNA added to a sample is below 1 µg, as too much DNA can affect the sensitivity as well as the specificity of the PCR analysis. Add positive control DNA (*see* **Note 2**) to the positive control(s) and 3 µL sterile water to the negative controls interspersed between the clinical samples at an approx 1:5 ratio.
6. Transfer the tubes to the thermal cycler and run the appropriate program:
   PE model 480: Incubation at 37°C for 10 min followed by initial denaturation: 94°C for 2 min followed by 40 cycles of: 94°C, 60 s; 60°C, 90 s; 72°C, 60 s; and final extension: 72°C, 7 min–hold at 4°C.
   PE model 2400: Incubation at 37°C for 10 min followed by initial denaturation: 94°C for 2 min followed by 40 cycles of: 94°C, 20 s; 59°C, 45 s; 72°C, 60 s; and final extension: 72°C, 7 min–hold at 4°C.
7. Keep the samples at –20°C until electrophoresis analysis.

3.3. Agarose Gel Electrophoresis

1. Prepare a 1.5% agarose gel containing ethidium bromide (*see* **Note 7**).
2. Place the gel in the gel chamber and fill the chamber with 1X TAE buffer to a level 1–2 mm above the gel surface.
3. Mix PCR samples and DNA marker with 10X gel loading buffer (*see* **Note 8**).
4. Load the samples carefully into the wells.
5. Run the gel at a constant voltage (5 V/cm between the electrodes) for 30 min (*see* **Note 9**).
6. Place the agarose gel on a UV transilluminator and photograph the result with a polaroid camera using type 667 film. Experiment with aperture opening and exposure time, a 0.5–1 s exposure at f = 5.6 or 8 will produce excellent results.

3.4. Southern Blot Hybridization

1. The agarose gel is trimmed for excess agarose and the size is measured.
2. The gel is incubated for 5 min in the alkaline solution, for 5 min in the neutralizing solution and for 5 min in the transfer buffer (10X SSC).

3. The Southern blot is constructed with the following layers (from the bottom): 5 cm layer of thick filter paper (wider and longer than the gel); 3M filter paper (approx 1 cm wider and longer than the gel); nylon membrane (same area as the gel); the agarose gel; 3M filter paper (same area as the gel); 3–5 cm layer of paper tissue saturated with the transfer buffer (10X SSC) (same area or slightly smaller than the gel).
4. Transfer the DNA content of the gel overnight.
5. The nylon membrane is cleansed briefly in 5X SSC (to remove gel-pieces).
6. The membrane is baked at 80°C in vacuum for 2 h. Alternatively, the membrane (only if nylon) is UV-radiated for approx 5 min.
7. Salmon sperm DNA (10 mg/mL) is boiled for 10 min, cooled on ice for 10 min, then 0.5 mL is added to 9.5 mL prehybridization solution.
8. The nylon membrane is soaked for 2 min in 10 mL prehybridization solution made as described **step 7**.
9. The nylon membrane is placed on the inside of a 50-mL centrifugation tube. Overlapping is OK—avoid bubbles between the tube wall and the membrane.
10. The rest of the prehybridization solution is added to the tube.
11. The tube is placed on a wheel in a hybridization oven (at 42°C) in a horizontal position, and rotated slowly (5–10 rpm) for 2–4 h.
12. Hybridization solution (5–10 mL) is made by boiling premade hybridization solution (containing approx 1 μg biotinylated double-stranded specific DNA probe) for 10 min, followed by cooling on ice for 10 min.
13. The prehybridization solution is discarded (NB: chemical waste) and the hybridization solution with labeled probe is added.
14. The 50-mL tube is placed in a hybridization wheel at 42°C in a horizontal position and rotates slowly (5–10 rpm) overnight.
15. The hybridization solution is poured into a new 50-mL tube and kept for maximum 12 mo at –20°C. The solution can be reused many times (*see* **Note 10**).
16. The nylon membrane is washed in 40–50 mL 2X SSC, 0.1% SDS two times for 5 min at room temperature–followed by washing two times for 5 min in 0.15X SSC, 0.1% SDS at 65°C (stringent washing conditions).
17. The membrane is dried between two pieces of filterpaper at 80°C in vacuum for 2–5 min.
18. The biotin signal is visualized with the PhotoGene detection kit (BRL).

4. Notes

1. Positive control DNA is made by extraction of DNA from a single colony of *L. pneumophila* as described in **Subheading 3.1., steps 3–19** followed by DNA amplification as described in **Subheading 3.2.** Following electrophoresis, the resulting 375 basepair fragment is purified by GeneClean II (IBI) according to the manufacturer's instructions and diluted tenfold. Ten to 100 copies are used as positive control.
2. Mineral oil is not necessary if the thermal cycler (e.g., Perkin Elmer 2400 or 9600) is equipped with a heating element in the lid as well as in the block, thus preventing evaporation resulting from a temperature gradient.

3. One microgram double-stranded 375 basepair fragment amplified as described from DNA extracted as described from a single colony of *L. pneumophila* is labeled by nick-translation with biotin-7-dATP (Bionick Labeling System, BRL) as described by the manufacturer's instructions.
4. One of the major causes of problems during PCR is contamination. Besides the recommendations regarding laboratory design described in Chapter 1, some general guidelines should also be observed: The use of dedicated positive displacement pipeting devices in each laboratory, the use of gloves during all laboratory procedures, the use of filtertips in the preamplification areas and the use of containers with clorox or a related product for minimizing potential aerosol problems during disposal of pipet tips containing DNA. When adding sample or control DNA to the PCR tubes, never have more than one tube open at the same time and always pipet the positive control DNA as the last procedure to avoid carryover contamination. Furthermore, the use of aliquoted reagents and the use of a low copy number positive control (no more than 100 copies) are recommended to avoid aerosol contamination as well as to be able to detect emerging problems concerning sensitivity of the assay.
5. The carryover prevention system substitutes uracil for thymine in the PCR, and if the following PCR analyses are initiated with an incubation with a uracil degrading enzyme, such as uracil-*N*-glycosylase, contaminating, but not wild-type, DNA will be degraded ***(15)***. This system thus prevents false-positive samples resulting from contaminating DNA from previous analysis, but does not prevent carryover contamination within the actual setup because of contamination with thymine-containing DNA from a strongly positive patient sample or from the positive control. The system is to be regarded as an assurance against future contamination problems and cannot be used to solve an existing contamination problem.
6. To avoid nonstringent primer annealing at room temperature (and thus the possibility of unspecific DNA synthesis before reaching the thermal cycler), the hot-start or cold-start approach should be applied. The hot-start principle can be achieved by wax-bullets, anti-*Taq* antibodies, chemical hot-start by including the carryover prevention system or by substituting Ampli*Taq* polymerase by Ampli*Taq* Gold polymerase, which requires a 10 min incubation period at 94°C to regain its activity (and thus automatically includes a hot-start before the PCR). The cold-start approach can be achieved by mixing all components of the PCR analysis (including the sample DNA) at 0°C (on ice) and transferring the PCR tube directly from 0°C to a preheated thermal cycler.
7. If small PCR fragments (under 200 basepairs) are analyzed, use higher percentage agarose (e.g., 3 or 4% NuSieve agarose, FMC Bioproducts). After melting the agarose in 1X TAE buffer by boiling or microwave heating, cool the agarose to approx 50°C and add ethidium bromide to a concentration of 0.5 μg/mL. Pour the gel and insert the comb. Wait for the gel to solidify and remove the comb. Alternatively, the gel can be stained after electrophoresis with SYBR Green I (FMC Bioproducts) diluted 1:10,000 in 1X TAE buffer. Use enough buffer to cover the gel. This will result in at least five times higher sensitivity than ethidium bromide staining.

8. The mixing of 5–10 μL PCR sample and the sizemarker with 1/10 vol of 10X gel loading buffer can be performed in microtiter tubes (expensive), a microtiter plate (reusable after washing, which is less expensive) or by placing a drop of gel loading buffer for each sample on a strip of parafilm, and mixing 10 μL of each sample with the loading buffer just before loading the sample on the gel.
9. Run the gel for 0.5–2 h, depending upon the number and the size of the band(s) expected. In the case of the described *Legionella* PCR, a band of approx 375 basepairs will appear in positive samples.
10. If biotin or digoxigenin labelling is used, the hybridization probe can be reused many times for up to 12 mo. If ^{32}P is used as label, the half-time is only 14 d.

References

1. Ching, W. T. W. and Meyer, R. D. (1987) Legionella infections. *Infect. Dis. Clin. North Am.* **1,** 595–614.
2. Winn, W. C., Jr. (1988) Legionnaires disease: historical perspective. *Clin. Microbiol. Rev.* **1,** 60–81.
3. Benson, R. F., Thacker, W. L., Walters, R. P., Quinlivan, P. A., Mayberry, W. R., Brenner, D. J., and Wilkinson, H. W. (1989) *Legionella quinlivani* sp. nov. a new species of *Legionella* isolated from water. *Curr. Microbiol.* **18,** 195–197.
4. Benson, R. F., Thacker, W. L., Lanser, J. A., Sangster, N., Mayberry, W. R., and Brenner, D. J. (1991) *Legionella adelaidensis*, a new species isolated from cooling tower water. *J. Clin. Microbiol.* **29,** 1004–1006.
5. Bornstein, N., Marmet, D., Surgot, M., Nowicki, M., Meugnier, H., Fleurette, J., Ageron, E., Grimont, F., Grimont, P. A. D., Thacker, W. L., Benson, R. F., and Brenner, D. J. (1989) *Legionella gratiana* sp. nov. isolated from French spa water. *Res. Microbiol.* **140,** 541–552.
6. Wilkinson, H. W., Drasar, V., Thacker, W. L., Benson, R. F., Schindler, J., Potuznikova, B., Mayberry, W. R., and Brenner, D. J. (1988) *Legionella moravica* sp. nov. and *Legionella bruensis* sp. nov. isolated from cooling-tower water. *Ann. Inst. Pasteur/Microbiology (Paris)* **139,** 393–402.
7. Stout, J., Yu, V. L., Vickers, R. M., Zurevleff, J., Best, M., Brown, A., Yee, R. B., and Wadowsky, R. (1982) Ubiquitousness of *Legionella pneumophila* in the water supply of a hospital with endemic Legionnaire's disease. *N. Engl. J. Med.* **306,** 466–468.
8. Cianciotto, N. (1989) Genetics and molecular pathogenesis of *Legionella pneumophila*, an intracellular parasite of macrophages. *Mol. Biol. Med.* **6,** 409–424.
9. MacFarlaine, J. (1989) *Legionella.* The clinical picture. *Occup. Health* **6,** 157,158.
10. Feeley, J. C., Gibson, R. J., Gorman, G. W., Langford, N. C., Rasheed, J. K., Mackel, D. C., and Baine, W. B. (1979) Charcoal-yeast extract agar: primary isolation medium for *Legionella pneumophila. J. Clin. Microbiol.* **10,** 437.
11. Edelstein, P. H. and Edelstein, M. A. C. (1989) Evaluation of the Meriflour-Legionella immunofluorescent reagent for identifying and detecting 21 *Legionella* species. *J. Clin. Microbiol.* **27,** 2455–2458.
12. Ratshikhopha, M. E., Klugman, K. P., and Koornhof, H. J. (1990) An evaluation of two indirect fluorescent antibody tests for the diagnosis of Legionnaire's disease in South Africa. *S. Afr. Med. J.* **77,** 392–395.

13. Kashuba, A. D. and Ballow, C. H. (1996) *Legionella* urinary antigen testing: Potential impact on diagnosis and antibiotic therapy. *Diagn. Microbiol. Infect. Dis.* **24,** 129–139.
14. Mahbubani, M. H., Bej, A. K., Miller, R., Haff, L., DiCesare, J., and Atlas, R. M. (1990) Detection of *Legionella* with polymerase chain reaction and gene probe methods. *Mol. Cell. Prob.* **4,** 175–187.
15. Lisby, G. and Dessau, R. (1994) Construction of a DNA amplification assay for detection of *Legionella* species in clinical samples. *Eur. J. Clin. Microbiol. Infect. Dis.* **13,** 225–231.
16. Longo, M. C., Berninger, M. S., and Hartley, J. L. (1990) Use of urasil DNA glucosylase to control carry-over contamination in polymerase chain reactions. *Gene* **93,** 125–128.

16

Quantitative Measurement of mRNA Expression by Competitive RT-PCR

Joe O'Connell, Triona Goode, and Fergus Shanahan

1. RT-PCR

As a method of specific mRNA detection, the single most important advantage of RT-PCR is its sensitivity, because of the remarkable sensitivity of PCR. Isolation of polyA$^+$ mRNA is unnecessary, and minute amounts of total RNA suffice. If random primers are used in the reverse transcription reaction, a single cDNA preparation can be used for the detection of numerous different mRNAs in the same RNA sample. This sensitivity means that RT-PCR is a powerful technique, particularly when tissue availability is limiting, or when the mRNA to be detected is present in low abundance. We have routinely detected low abundance mRNAs for various cytokine receptors in RNA samples isolated from small pinch biopsies of human colonic mucosal epithelium. The technique has also been used for the sensitive detection of viral RNA, such as from HIV and HCV, in human serum, where the viral titer is frequently <1000 virus particles per milliliter of serum.

The technique is also rapid, facilitating a large throughput of samples. A quick RNA prep yields sufficient RNA of adequate purity for RT-PCR. RNA is purified by simple phenol extraction and ethanol precipitation from guanidine thiocyanate tissue lysates. By making up master mixes for both the reverse transcriptase and PCR reactions, 10–20 samples can easily be processed in an experiment. The entire process, from lysis of tissue to detection of mRNA-specific PCR products on an agarose gel, can be accomplished in a single working day.

The one major disadvantage of standard RT-PCR with respect to other mRNA detection techniques such as Northern blot is that it is only semiquantitative because of the kinetics of PCR product accumulation, whereby exponential amplification occurs only at early cycles below the level of detection by

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

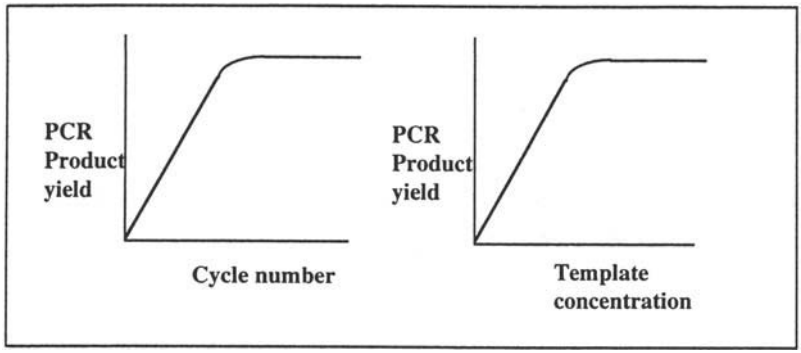

Fig. 1. The "plateau" in the kinetics of PCR amplification.

standard ethidium bromide-stained agarose gel electrophoresis (**Fig. 1**). As product accumulates in the later cycles, amplification efficiency tapers off because of exhaustion of nucleotides and primers, progressive denaturation of the polymerase and a shift in the equilibrium of template denaturation favoring association rather than denaturation of the template DNA strands as product concentration becomes high. Hence there is no linearity in the relationship of product yield to initial template. This results in the "plateau effect" of PCR whereby further product cannot be derived from increasing the initial template level. Hence, qualitative rather than quantitative data is obtained from standard RT-PCR.

2. Quantitative Competitive PCR (qcPCR)

A number of approaches have been employed in efforts to obtain quantitative data from PCR. These include incorporation of ^{32}P-labeled nucleotides during PCR to permit subplateau detection of amplification products after a small number of temperature cycles. Products are quantified either by liquid scintillation counting of bands excised from an agarose gel, or by comparative densitometry of bands following autoradiography of the gel. A related approach involves incorporation of a fluorescent-labeled nucleotide during PCR. Following PCR cycling to subplateau levels of product, the PCR product is detected using an automated DNA sequencer. Product is quantified by comparing the peak area to that of a known standard. Both of these techniques require the linear range of the PCR to be established for each sample. Many manipulations are required following PCR, and an absolute determination of target template quantity is not obtained.

One of the most widely employed quantitative approaches is that of competitive PCR (**Fig. 2**) ***(1)***. Essentially, a control PCR template is constructed such that it has identical primer sites to the target template, but has a differ-

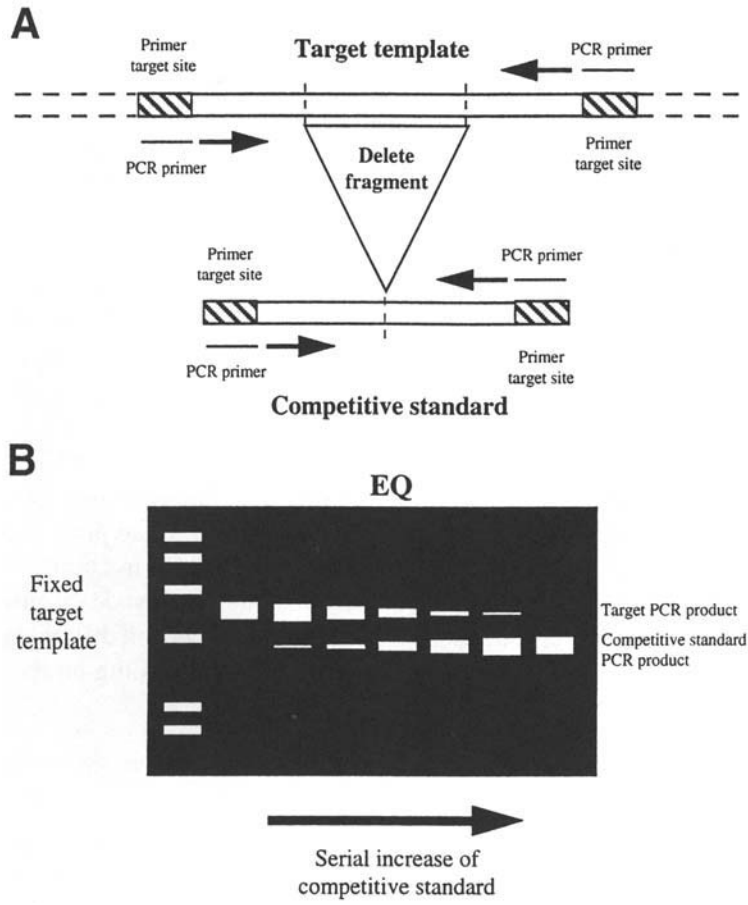

Fig. 2. Quantitative competitive (qc) PCR. **(A)** Construction of an internal standard for competitive PCR. **(B)** Use of internal standard in competitive PCR. Equivalence of PCR products (EQ) occurs when target and standard templates are present in equal initial concentration, permitting quantitation of the target template.

ence, usually simply in size, which allows amplification products from this control template to be distinguished from those of the target template. This standard template is used as an internal control in the amplification of the target template. As it shares the same primers as the target, it will compete directly with the target template during PCR amplification. If the starting amount of internal competitive standard is greater than that of the target, the PCR products from the competitive PCR will be predominantly derived from the standard. Similarly, if the target template is present initially at a higher concentration than the standard, the target template will out-compete during

PCR. If the internal competitive standard template is present in equal amount to the target template, equivalent PCR product is obtained from both. So, in practice, multiple PCR reactions (usually 5–7) are set containing a fixed source of target template, with serially increasing (2–10-fold, depending on the precision required and the range to be covered) known amounts of internal standard spiked into the reactions. Following PCR amplification, the relative amount of product from both templates in each reaction is ascertained from standard agarose gel electrophoresis. An equivalence point is usually obvious, where there is an equal yield of target and competitive standard PCR products. The number of copies of the specific target molecule in the template source (i.e., total tissue cDNA if RT-PCR is used) must be equivalent to the known number of competitive standard molecules spiked into this reaction, enabling quantification of target molecules.

With quantitative competitive RT-PCR, the linear phase of PCR does not need to be established for each sample, as direct competition between the target sequence and the internal standard will continue into the plateau phase of amplification. Also, unlike other quantitative approaches, in competitive RT-PCR, an RNA internal competitive standard may be employed that can control for the efficiency of reverse transcription of the target RNA in different samples and even for the recovery of RNA during isolation, depending on the point at which the standard is mixed with the sample.

3. Strategy for Construction of Internal Competitive Standard for qcRT-PCR

In our experience, the best method for generating a competitive standard is that in which a portion of the target template, usually <30%, is deleted to permit differentiation between amplification products of target and competitor on the basis of size. Unlike with other types of competitive standard, no processing of the resultant PCR products, by either restriction digestion or Southern hybridization, is required. No extraneous DNA sequence is introduced into the competitor, which is usually of sufficiently similar size and sequence composition to the target to result in identical efficiencies of amplification, and hence direct competition on an equal basis. Once the competitive internal standard has been constructed, no deviation is required from the standard RT-PCR protocol in order to achieve accurate quantitation of mRNA. No additional skills, equipment, reagents, or time are needed or further manipulations of the PCR products beyond detection of the equivalence point on a standard agarose gel.

4. Experimental Protocol

We have developed a relatively quick, straightforward, reliable protocol for generating competitive RNA standards for quantitative RT-PCR. The protocol

requires basic plasmid cloning techniques, including a number of steps that have been specifically found to result in efficient "trouble-free" cloning. Essentially, the target PCR product is cloned into a suitable plasmid vector, such as pBluescript, which has promoters for the T7 and the T3 RNA polymerases flanking the insertion site. The plasmid should include standard features, such as an antibiotic selection gene, as well as having the cloning site within the LacZ gene, thus permitting color selection of recombinants on X-Gal/IPTG. Using an appropriate pair of restriction enzymes, a suitably sized fragment (<30% of the target) is then excised from the cloned target sequence. Following Klenow-mediated fill-in of the incompatible sticky ends of the thus linearized recombinant plasmid, the plasmid is purified from the excised fragment and its blunt ends are religated to generate a deletion clone of the PCR target. Following determination of the orientation of the cloned insert relative to the T3 and T7 promoters on the plasmid, the appropriate polymerase is used to transcribe sense RNA copies in vitro from the deleted insert, which are then quantified and used as an internal competitive standard in the RT-PCR.

In the protocol that follows, standard procedures such as ligation, restriction digestion, electroporation, spun column chromatography, and so forth, are not described in detail. All such manipulations are performed either as recommended by the manufacturers of the reagents employed or else as described in **ref. *2***. The protocol is outlined diagrammatically in **Fig. 3**; **Fig. 4** shows the results obtained from monitoring an actual application of the protocol (development of an internal standard for measurement of mRNA for substance P receptor) and **Fig. 5** shows the result of an actual quantitation using this competitive standard.

1. Generate PCR product suitable for cloning. We use UlTma DNA polymerase (Perkin-Elmer, Norwalk, CT). This thermostable DNA polynerase has a 3'-exonuclease proofreading activity which results in a much lower misincorporation rate than *Taq* polymerase, thus ensuring amplification with maximal fidelity. This is important, as it ensures that the eventual clone will not contain sequence errors. This polymerase also generates amplified DNA with blunt ends (i.e., no 3' dA overhangs), which is an efficient substrate for blunt-ended cloning. Clean up PCR product by a single phenol extraction to remove the polymerase and other proteins such as BSA, followed by gel filtration through a Sephacryl S300 spun column (Pharmacia Biotech., Uppsala, Sweden). This eliminates unincorporated primers, dNTPs and changes the buffer to TE. It also removes traces of phenol, so that a chloroform extraction is obviated.
2. Generate cloning vector—we use pBluescript (Stratagene Cloning Systems, La Jolla, CA), a standard miniprep will suffice. Digest with *Eco*RV to linearize the plasmid giving blunt ends. It is vital that this digest goes to completion to avoid generating clones of the parent plasmid without inserted DNA. Usually a second shot of restriction enzyme for a further few hours ensures complete cutting. If not, further purification of the plasmid prior to digestion is required.

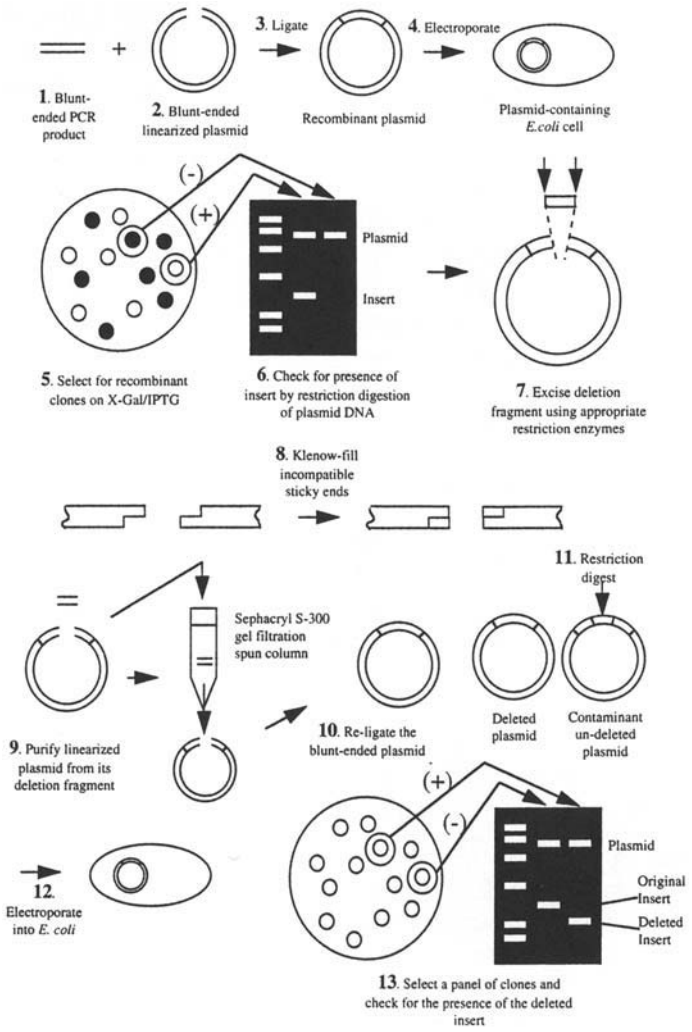

Fig. 3. Schematic diagrams outlining construction of an RNA competitive standard for quantitative competitive (qc) PCR.

3. Ligate the purified PCR product to the linearized plasmid. Because of the unphosphorylated nature of the PCR insert, which therefore cannot ligate to itself, a high insert:vector ratio may be used to favor insertion over religation of the parent plasmid. However, as it is difficult to predict the optimal ratio, we usually try three molar ratios of insert to vector: 50:1, 10:1, and 5:1. Also, 10 ng or less of the linearized plasmid is sufficient to obtain a good yield of clones. Although blunt-ended ligation is less efficient than sticky-ended ligation, the high

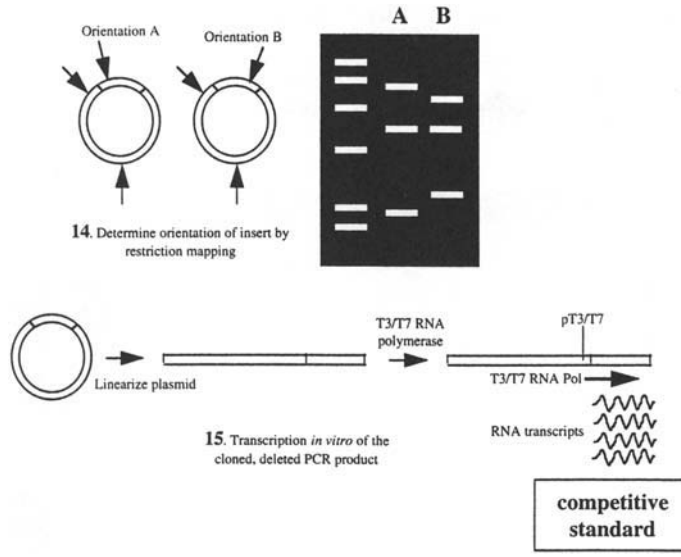

Fig. 3, part 2

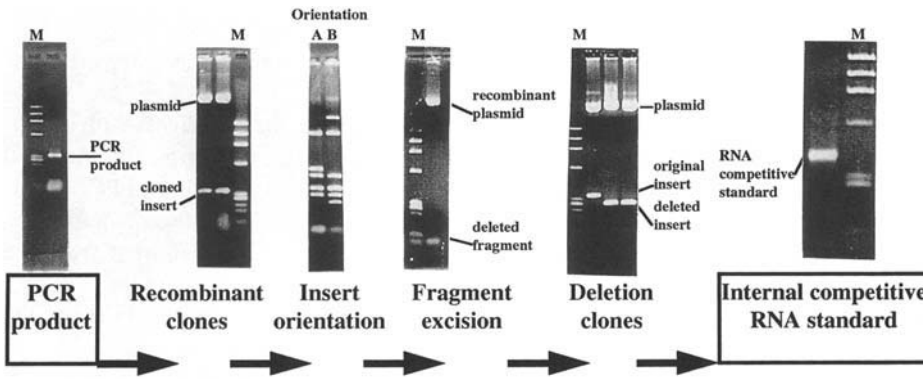

Fig. 4. Construction of a competitive standard for qcRT-PCR. The progress of competitive standard construction (in this case, for substance P receptor mRNA) is monitored by agarose gel analysis at various stages of the protocol.

insert:vector ratio forces the ligation toward insertion. Also, the volume of the ligation should be kept small (<10 μL) to maintain a high concentration of blunt ends of the DNA substrates, which also favors ligation.

4. Electroporate into *Escherichia coli* XL1 blue.
5. Select for clones on antibiotic plates containing X-Gal/IPTG.

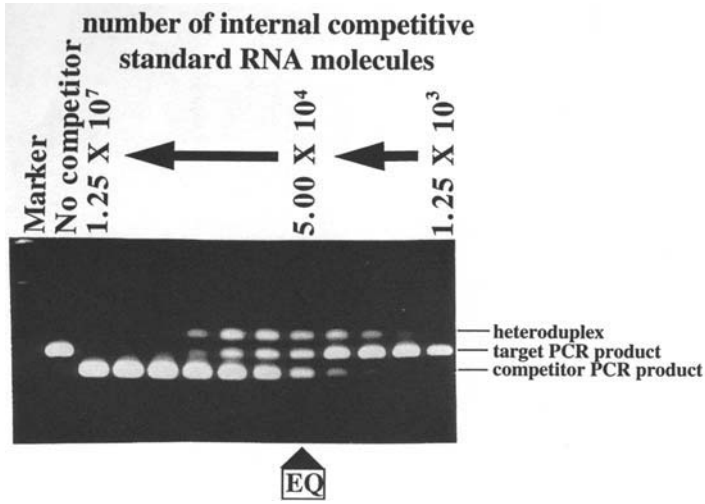

Fig. 5. qcRT-PCR. This gel shows quantitation of substance P receptor mRNA in a colonic mucosal pinch biopsy RNA sample. Prior to RT-PCR, competitive standard RNA molecules were added to fixed amounts of the test sample RNA in a 2.5-fold series of copy number. Equivalence between target and competitor PCR products occured at 5.0×10^4 copies of competitive standard.

6. Miniprep DNA from a panel of 10 positive (white) clones. Check for the presence of the cloned PCR insert by restriction analysis.
7. Take circular plasmid DNA from an insert-bearing clone and digest with appropriate restriction enzyme(s) to delete the required fragment from the inserted DNA. These enzymes must excise a fragment of <30% of the cloned PCR product, but do not cut the parent plasmid DNA. Also, the deletion fragment must be chosen to include within it a restriction site unique to itself (absent from the plasmid and remainder of the PCR insert) (*see* **step 11**).
8. Add Klenow DNA polymerase and deoxynucleotide triphosphates to fill in the incompatible sticky ends generated by restriction digestion.
9. Phenol extract and run the aqueous phase through a Sephacryl S300 spun column. This removes the small excised fragment from the remainder of the linearized recombinant plasmid DNA. This method results in greater purity, greater yield, and is quicker than agarose gel purification.
10. Religate the blunt ends of the purified, linearized recombinant plasmid to recircularize the DNA, minus the excised fragment.
11. Digest to linearize contaminant parent undeleted plasmids. Following excision of the deleted fragment, invariably some molecules of the parent, undeleted recombinant plasmid DNA persist. This usually results in a high background of undeleted clones, which cannot be distinguished from the required deleted clones by color selection on X-Gal/IPTG, as they are both recombinant (white) clones.

This problem is easily circumvented by subjecting the ligation from **step 10** to restriction with an enzyme which has a site unique to the sequence of the deleted fragment. Hence, only undeleted recombinant plasmid DNA will be linearized, leaving its deleted derivative intact in its religated circular form. As *E. coli* is transformed with negligible efficiency by linear DNA, the restricted DNA should predominantly yield clones of the deleted PCR product.

12. Re-electroporate the restricted DNA into *E. coli.*
13. Pick clones–check for deletion of fragment by restriction analysis.
14. Determine the orientation of the inserted PCR product relative to the plasmid sequence by restriction mapping, i.e., determine the position of restriction site(s) within the insert relative to restriction sites on the plasmid.
15. Run-off a sense RNA transcript in vitro using either T7 or T3 RNA polymerase, depending on the orientation of the inserted DNA relative to the T7 and T3 promoters that flank the inserted DNA. The plasmid is first linearized by restriction digestion at a site adjacent to the end of the insert distal to the chosen RNA polymerase promoter. This ensures that the RNA run-off transcripts will all terminate at the same site generating transcripts of uniform size, without containing plasmid sequences. The RNA transcript is purified by phenol extraction and Sephacryl S300 spun column chromatography following elimination of plasmid DNA using RNase-free DNase.
16. Quantify the purified RNA transcript spectrophotometrically. This permits the actual molarity and number of RNA copies in the transcript sample to be accurately determined (*see* **Note 2**), which is then diluted down according to the range of copy number to be spiked into the RT-PCR.
17. Spike the competitive standard into the aliquoted test RNA samples at a suitable range of concentrations prior to RT-PCR. The range needs to be established empirically, depending on the level of abundance of the mRNA in the tissue of interest. Initially, for this purpose, a 10-fold series of competitive standard may be useful (e.g., 10^2–10^7). Subsequently, a narrower range employing competitive standard in a series of fivefold or less (depending on the degree of precision sought and the number of individual competitive RT-PCR reactions considered practical to perform) will result in accurate quantitation (**Note 2**).
18. Following amplification using standard conditions, run the competitive RT-PCR products on an agarose gel. If an appropriate range of competitive standard is employed, the equivalence point is usually immediately obvious at which target and competitive standard product bands are of equal intensity. Although densitometry may be employed to confirm this, we consider this to be unnecessary as even a twofold difference in concentration of internal standard results in clearly obvious departure from equivalence and if the equivalence point is occasionally missed in the chosen series of competitive standard concentrations, or if greater precision is sought, a second set of competitive RT-PCRs can be performed using a narrower range of competitive standard focusing in around the equivalence point approximated from the first set of RT-PCRs (**Note 3**).

5. Notes

1. The following formula are useful for the calculation of the number of RNA copies present in a given amount of purified, in vitro transcribed RNA.

Spectrophotometrically, an $A_{260} = 1.0$ is equivalent to approx 40 μg/mL of RNA

The molecular weight of an RNA molecule, MW = number of nucleotides × 340

Number of moles = weight (g)/MW

Number of molecules = number of moles × 6.023×10^{23}

2. RNA quantitation. We usually express results of mRNA quantitation for a given cell or tissue as copy number of the mRNA per microgram of total RNA. For this purpose, total RNA must be carefully quantified. RNA quantitation kits are commercially available for the quantitation of small amounts of RNA. If spectrophotometric measurement of absorbance at 260 nm is used, it must be remembered that free nucleotides, which contaminate quick-preps of RNA, also absorb strongly at 260 nm. These must therefore be removed (e.g., by Sephadex G-50 gel filtration spun columns, Pharmacia Biotech) prior to spectrophotometric quantitation of RNA.
3. Heteroduplex formation. With some competitive PCRs, a heteroduplex arises during amplification, consisting of a hybrid between one strand of the target annealed to one strand of the smaller competitive standard (**Fig. 6**). The unannealed portion of the target strand corresponding to the sequence deleted from the competitive standard loops out to form a bulky secondary structure that results in slower mobility through agarose gel than either of the two linear products of the competitive PCR. Hence, a third band occurs in the gel above the target and competitor bands. That this band is due to heteroduplexing can be confirmed using the single-stranded DNA-specific S1 nuclease, which digests the unannealed portion of the hybrid, thus resolving this third band. Although measures such as the addition of formamide to the PCR may suppress the heteroduplex formation, as it consists of one strand from target and competitor products, it should not interfere with the ratio of target to competitor PCR products or bias the normal occurrence of equivalence between them, and can therefore essentially be ignored.

6. Conclusion

Although some labor is required to generate an internal competitive standard for quantitative RT-PCR, the protocol that we have presented has been optimized such that a standard can be constructed rapidly and reliably, and has allowed us to quantify many mRNAs in various areas of research. This protocol has enabled us to successfully quantify mRNA levels for substance P receptor in pinch biopsies of inflamed versus noninflamed human colonic mu-

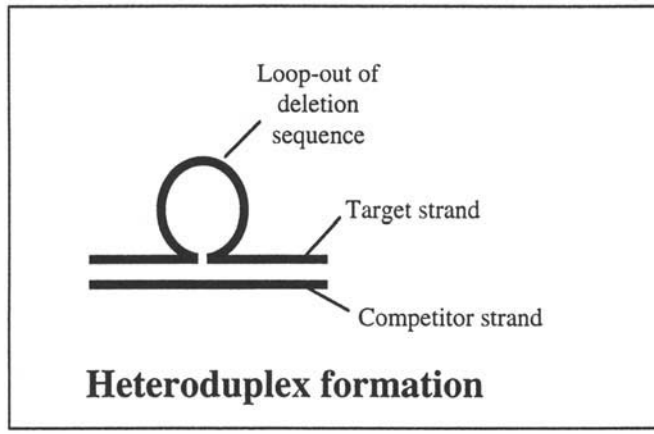

Fig. 6. Heteroduplex formation during competitve PCR between one strand of target and competitor.

cosa to investigate its role in inflammatory bowel disease. We have also used this technique to develop a quantitative RT-PCR assay for urokinase mRNA to investigate its role in the metastasis of esophageal cancer cells. Once the competitive standard has been developed, no skills, equipment, or reagents are necessary other than those required for standard RT-PCR, and absolute mRNA level can be easily, rapidly, and sensitively determined.

One of the by-products of our approach is a plasmid clone containing a fragment of the cDNA of interest. This is usually useful for other experiments also. We have used antisense RNA transcripts synthesized in vitro from the substance P receptor clone (undeleted clone!) as a probe for *in situ* hybridization. Labeled nucleotides can be incorporated to high specific activity during in vitro transcription, resulting in a long, specific, intensely labeled probe that we have found superior to short oligonucleotide probes for *in situ* hybridization. A sense RNA hybridization control probe can be run-off from the opposite strand of the clone using the opposite RNA polymerase promoter.

References

1. Zimmermann, K. and Mannhalter, J. W. (1996) Technical aspects of quantitative competitive PCR. *Biotechniques* **21,** 268–279.
2. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

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PCR Detection of *Toxoplasma gondii* in Human Fetal Tissues

Tamás Tóth, István Sziller, and Zoltán Papp

1. Introduction

Primary acquisition of *Toxoplasma gondii (T. gondii)* in healthy male and female adults is usually an asymptomatic infection (toxoplasma infection) or results in a harmless disease (toxoplasmosis) with nonspecific symptoms including headache, myalgia, fatigue, or a flu-like condition. In some cases, enlargement of lymph nodes in different body regions because of lymphatic spread of parasites together with slight fever might mimic infectious mononucleosis.

Since the parasite is immunogenic, production of specific immunoglobulins (IgM, IgA, and IgG) follows after the incubation period. These proteins may protect the affected individual from further progression of infection by slowing the rapid intracellular multiplication of the parasites but are unable to eradicate the pathogens. In this stage of infection, *T. gondii* may mostly persist in brain tissues, eyes, and muscles for many years and reactivate later in life. Immunocompromised patients with AIDS ***(1,2)*** and recipients of organ transplant ***(3)*** are those at highest risk for reactivation of life-threatening toxoplasmosis. In these patients encephalitis, myocarditis, pneumonitis, or a combination of these clinical manifestations may occur.

1.1. Toxoplasma Infection in Pregnancy

Acute toxoplasma infection may be acquired also in pregnancy by consumption of contaminated raw or undercooked meat, unwashed fruits and vegetables, or by contact of cat feces containing oocysts. The clinical picture and severity of infection is similar to that observed in nonpregnant individuals. In most cases, acquisition of primary infection during pregnancy is diagnosed by serologic evaluation of women at risk (seronegatives). Although reliable, screening of seronegative pregnant women remains a matter of debate ***(4,5)***.

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

As a consequence of parasitemia present in the acute phase of infection, large numbers of *T. gondii* may colonize the placenta. If maternal infection is not diagnosed and, left untreated, this organ may be the source of fetal infection throughout pregnancy. The risk of placental transmission depends on the gestational age when acute maternal infection with *T. gondii* has occurred: with the rate being low in the first trimester (about 10%) and increasing to about 80% by near term ***(6)***. Placental transmission of *T. gondii* may lead to congenital toxoplasmosis. Severity of congenital toxoplasmosis varies sharply from intrauterine fetal death through severe neurological sequel including hydrocephalus, bilateral ventriculomegaly, microcephaly, or blindness to an unapparent disease at birth with potential serious late consequences in the neonatal period or even 20 yr later ***(7,8)***. The severity of fetal damage associated with congenital toxoplasmosis decreases with advancing gestational age from when placental transmission occurred ***(9)***. Very early infection of the mother (within 2 wk after the first day of last menstrual period) poses little or no risk to the fetus ***(10)***. As many as 25% of primary maternal toxoplasma infections acquired during the first trimester of pregnancy may result in severe forms of congenital toxoplasmosis compared with a seeming lack of severe fetal damage when acute maternal disease occurred in the third trimester.

1.2. Prenatal Diagnosis of Toxoplasma Infection

Prenatal diagnosis of *T. gondii* infection should be thought in pregnancies in which: maternal infection was confirmed or strongly suspected, and routine ultrasound screening during pregnancy reveals signs resembling congenital toxoplasmosis (bilateral ventriculomegaly, hepatomegaly or ascites). Adoption of invasive prenatal diagnostic procedures with possible complications are justified by two facts: the extent of fetal damage can be reduced by early introduction of specific treatment, for which a rapid diagnostic is mandatory, and demonstration of lack of placental transmission of *T. gondii* is important to prevent unnecessary termination of pregnancy.

Prenatal diagnosis of toxoplasmosis is based on the demonstration of *T. gondii* in amniotic fluid or fetal tissues by inoculation of mice, tissue culture or in later years by the polymerase chain reaction (PCR) test ***(9)***. Specific IgM or IgA in fetal blood as demonstrated by an immunosorbent agglutination assay (ISAGA) together with a positive parasitological test is also considered as evidence of fetal infection. If therapeutic abortion was performed, *T. gondii* should be sought in the products of conception (placenta, amniotic fluid, fetal blood, fetal tissues) by PCR test. Besides specific diagnostic tests listed, nonspecific biologic parameters from fetal blood to evaluate the risk of fetal infection and determine the need for therapy, while awaiting the results of parasitologic tests have been long adopted ***(6)***. These included determination of total IgM con-

centration, γ-glutamyltransferase and lactate dehydrogenase activity, as well as leukocyte, eosinophil, and platelet counts from fetal blood. Prenatal diagnostic procedures include both invasive (amniocentesis, chorionic villus sampling or cordocentesis) and noninvasive methods (ultrasonography during pregnancy and after birth, as well as lumbar puncture of the neonate in some cases).

Detection of *T. gondii* by tissue culturing and inoculation of mouse are sensitive and specific but time-consuming methods. Furthermore, the sensitivity of these tests might depend on the number of parasites present in amniotic fluid or fetal tissues ***(10)***. Low numbers might result in lower sensitivity in both tests while very high number of *T. gondii* might have the possibility of an absence of reaction in mice ***(10)***.

PCR assays can substantially shorten the delay in obtaining a definitive diagnosis of toxoplasmosis. Using primers selected from the P30 ***(11)*** or B1 gene targets ***(12)*** or a segment of the 18S ribosomal DNA PCR ***(13)*** tests are sensitive, specific, and rapid methods for the demonstration of the parasite from liver, brain tissue, cerobrospinal fluid, myocardium, skeletal muscles, fetal blood, and amniotic fluid.

Data from a study including a large series of pregnant women with primary toxoplasma infection acquired during pregnancy suggest that the PCR test using primers of the B1 gene as target performed in amniotic fluid samples is a safe and sensitive method for the prenatal diagnosis congenital toxoplasmosis. This method can replace fetal blood sampling by cordocentesis which carries a higher risk for adverse pregnancy outcome. It is important to note that invasive prenatal diagnostic procedures should not be attempted until at least 4 wk after acute infection in the mother. Both amniocentesis and cordocentesis may be used from wk 18 of gestation until term ***(10)***. Clinical experiences for these procedures before wk 18 are lacking.

1.3. Detection of Toxoplasma gondii *DNA from Fetal Tissue*

Sometimes it is not possible to detect toxoplasma infection prenatally, because of miscarriage or induced abortion because of severe abnormality of the fetus. In these cases, the investigation of fetal tissues for the presence of toxoplasma DNA by PCR may provide the cause of the disease. Fetal liver and/or brain are the most suitable tissues for the diagnosis.

2. Materials

2.1. Tissue Collection and DNA Extraction

2.1.1. DNA from Amniotic Fluid

1. Ultrasound instrument, sterile biopsy needle.
2. Sterile 10-mL Falcon tubes.
3. ReadyAmp Genomic DNA Purification System (Promega, Madison, WI).

2.1.2. DNA from Fetal Tissue

1. Necropsy instruments (scissors, tweezers, forceps).
2. Sterile scalpel blades.
3. Tissue homogenizer.
4. Digestion buffer: 100 m*M* NaCl, 10 m*M* Tris-HCl, pH 8.0, 25 m*M* EDTA, 0.5% SDS.
5. Proteinase K, 20 mg/mL.
6. Phenol:chloroform:isoamylalcohol of 25:24:1 (Sigma, St. Louis, MO).
7. 3*M* Sodium acetate, pH 7.0.
8. Absolute EtOH.
9. TE buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0.

2.2. PCR Amplification

1. Sterile deionized water (Promega).
2. 10X PCR reaction buffer (Promega).
3. 10 m*M* stocks of deoxynucleotides dATP, dTTP, dCTP, dGTP (Pharmacia, Uppsala, Sweden).
4. Working stock of deoxynucleotides that contains 1.25 m*M* of each dNTP.
5. Primers diluted in sterile water to 10 μ*M* (*see* **Note 1**).
6. Sterile 0.2-mL PCR reaction tubes.
7. *Taq* polymerase (Promega, 5 U/μL).
8. Positive control *T. gondii* DNA (*see* **Note 2**).

2.3. Polyacrylamide Gel Electrophoresis

1. 40% Acrylamide/Bis solution 19:1 (Bio-Rad, Richmond, CA).
2. 5X TBE buffer: adding 54 g Tris base, 27.5 g boric acid, and 20 mL of 0.5 *M* EDTA (pH 8.0) to 1 L of ddH_2O ***(18)***.
3. 10% Ammonium persulfate.
4. TEMED.
5. Ethidium bromide (1 μg/mL) in 1X TBE buffer.
6. 6X gel loading dye (Promega).
7. 100 bp DNA ladder (Promega).

3. Methods

3.1. Tissue Collection and DNA Extraction

3.1.1. Amniotic Fluid

1. Ultrasound examination in order to determine the gestational age and optimal place for amniocentesis (*see* **Note 3**).
2. Clean the abdominal skin.
3. Aspirate the amniotic fluid.
4. Place the fluid into a separate, labeled, and sterile Falcon tube and use for DNA extraction or store at –20°C until use.
5. Pellet the amniotic cells by centrifugation at 7200*g* for 8 min.

6. Extract DNA from pelleted amniotic cells with ReadyAmp Genomic DNA Purification system (Promega) according to the manufacturer's instructions.
7. Use 4–6 μL of this ssDNA solution for PCR amplification (*see* **Note 4**).

3.1.2. Fetal Tissue

1. Decontaminate necropsy instruments and the necropsy table surface with 70% EtOH.
2. Collect small amount of tissues from fetal liver and/or brain (*see* **Note 5**).
3. Transfer the sample into a sterile 2 mL tube containing 500 μL of digestion buffer and homogenize.
4. Add proteinase K to a final concentration of 100 μg/mL.
5. Incubate at 55°C for 3 h (*see* **Note 5**).
6. Extract sample twice with 500 μL of phenol:chloroform:isoamylalcohol (25:24:1).
7. Add 30 μL of 3*M* sodium acetate and mix.
8. Add 500 μL of absolute EtOH and place on ice for 1 h.
9. Centrifuge for 2 min at 7200*g* and decant the supernatant.
10. Leave the pellet to dry the and dissolve in 200 μL of TE (*see* **Note 6**).

3.2. PCR Amplification

1. Prepare a master mix of PCR reagents containing (per 100 μL of PCR reaction) 10 μL of 10X PCR buffer, 100 pmol of each primer (*see* **Note 7**), and 16 μL of 1.25 m*M* dNTP working stock. Make enough for one extra PCR reaction to ensure there is enough for all samples.
2. Aliquot 6 μL of ReadyAmp prepared ssDNA from amniotic fluid or 1 μL of DNA derived from fetal tissue into labeled 0.2 mL PCR tubes. Add the volume of UV-irradiated sterile water required for each sample.
3. To the master mix, add 0.4 μL of *Taq* polymerase (2.0 U) for each reaction. Mix well and spin briefly.
4. Add the correct volume of master mix to each sample tube. Cap the PCR tubes and spin briefly in a microfuge.
5. Load the tubes into the thermal cycler block (*see* **Note 8**).
6. Amplify the samples according to the following cycling program: Initial denaturation: 95°C, 2 min; followed by 50 cycles of: 94°C, 30 s; 42°C, 30 s; 72°C, 45 s; extension: 72°C, 4 min; hold: 4°C.
7. Store PCR products at 4°C until ready for electrophoretic analysis.

3.3. Polyacrylamide Gel Electrophoresis

1. Set up glass plates with 1.0-mm spacers in the acrylamide gel casting stand (*see* **Note 9**).
2. Make up a 6% acrylamide gel solution in 1X TBE. 50 mL of this solution requires 7.5 mL 40% bis-acrylamide solution 19:1, 10 mL 5X TBE, 32.5 mL deionized distilled water, 250 μL 10% ammonium persulfate, and 30 μL TEMED (*see* **Note 10**).
3. Mix the solution and pipet it between the glass plates. When the plates are almost full, insert a 1.0-mm comb.

4. Allow for 1 h to polymerize the gel.
5. Prepare the samples by adding 2 μL of 6X gel loading dye per each 10 μL sample.
6. After standing for 1 h remove the comb from the gel and rinse the wells with distilled water.
7. Load the samples into the wells and run the gel on constant voltage at 10 V/cm for 4 h.
8. Remove the gel from the tank and place the opened plate with the gel in the staining tank. Stain the gel by shaking in 1 μg/mL ethidium bromide solution for 15 min (*see* **Note 11**).
9. Photograph the gel on a UV transilluminator with a Polaroid camera through an orange filter onto Polaroid type 667 film.

4. Notes

1. There are several primer sets selected from the P30 or B1 gene targets or a segment of the 18S ribosomal DNA. Some primer sequences used for detection of Toxoplasma DNA in human tissues can be seen here:

	Gene	Product size (bp)	Reference
5' - ACT GAT GTC GTT CTT GCG ATG TGG C - 3'			
5' - CGT CCA CCA GCT ATC TTC TGC TTC A - 3'	P30	282	17
5' - AAC GGG CGA GTA GCA CCT GAG GAG A - 3'			
5' - TGG GTC TAC GTC GAT GGC ATG ACA AC - 3'	B1	115	14
5' - TTG CAT AGG TTG CAG TCA CT - 3'			
5' - TCT TTA AAG CGT TCG TGG TC - 3'	B1	133	15
5' - GGC ATT CCT CGT TGA AGA TT - 3'			
5' - CCT TGG CCG ATA GGT CTA GG - 3'	rDNA	88	16
5' - TGC ATC CAA CGA GTT TAT AA - 3'			
5' - AGG CAT TCG GGT TAA AGA TT - 3'	rDNA	88	13

2. Toxoplasma positive control DNA (RH strain) could be derived from peritoneal fluid of intraperitoneally inoculated mice.
3. The amniocentesis procedure starts with an ultrasound examination. If the mother has had a previous ultrasound, this examination must be repeated immediately before the amniocentesis. The gestational age must be checked by comparison of

the menstrual age data and the ultrasound findings. The screen on which the fetal heartbeat will appear should be visible to the mother. The location and extent of the placenta is noted and the amount of amniotic fluid estimated. The placenta must be localized before the puncture site is chosen. The abdominal skin is cleaned and the puncture site isolated with a sterile cloth. Local anesthesia is not necessary, for the pain of amniocentesis is no more intense than that of an injection. The needle accepted as best for amniocentesis is 0.9 mm in external diameter and 9 cm in length, factory sterilized, and which has a stylet. The introduction of the needle must be such that the consecutive penetrations of skin, subcutis, fascia, abdominal musculature, abdominal peritoneum, wall of the uterus, and fetal membranes should be individually felt. The light point corresponding to the end of the stylet should be seen on the screen. Aspiration of fluid is accompanied by an intense streaming about the needle tip ***(19)***.

4. This type of DNA preparation method is very useful for PCR detection of *T. gondii* according to our observations.
5. A small amount of fetal tissue is the most appropriate. The best results can be obtained when about 10 mg of soft tissue is used. Overnight proteinase K digestion may improve the amount of isolated DNA.
6. It is very important to leave the samples at room temperature in order to evaporate the residual EtOH. Ethanol in DNA sample may inhibit *Taq* polymerase activity and lead to misdiagnosis.
7. We usually use primers described in **ref. *15*** that amplify a part of B1 gene of *T. gondii*. The B1 gene is about 35-fold repetitive and found to be the most sensitive target for detection of *T. gondii* by PCR.
8. The authors use a Perkin-Elmer model 2400 thermocycler.
9. Make sure the seals along the sides and bottom are tight.
10. This solution must be made up fresh for each gel.
11. More sensitive detection of PCR products can be obtained by silver staining of polyacrylamide gel ***(20)***.

References

1. Luft, B. J. and Hafner, R. (1990) Toxoplasmic encephalitis. *AIDS* **4,** 593–595.
2. Luft, B. J. and Remington, J. S. (1988) Toxoplasmic encephalitis. AIDS commentary. *J. Infect. Dis.* **157,** 1–6.
3. Heurkens, A. H. M., Koelma, I. A., de Planque M. M., Polderman, A. M., and van der Meer, J. W. M. (1989) Failure to diagnose fatal disseminated toxoplasmosis in a bone marrow transplant recipient: the possible significance of declining antibody titers. *J. Infect.* **18,** 283–288.
4. Daffos, F., Forestier, F., Capella-Pavlovsky, M., et al. (1988) Prenatal management of 746 pregnancies at risk for congenital toxoplasmosis. *N. Engl. J. Med.* **318,** 271–275.
5. McGabe, M. and Remington, J. S. (1988) Toxoplasmosis: the time has come. *N. Engl. J. Med.* **318,** 313–315.
6. Pratlong, F., Boulot, P., Villena, I., Issert, E., Tamby, I., Cazenave, J., and Dedet, J.-P. (1996) Antenatal diagnosis of congenital toxoplasmosis: evaluation of the

biological parameters in a cohort of 286 patients. *Br. J. Obstet. Gynaecol.* **103,** 552–557.
7. Hohlfeld, P., MacAleese, J., and Capella-Pavlovsky, M. (1991) Fetal toxoplasmosis: ultrasonographic signs. *Ultrasound Obstet. Gynecol.* **1,** 241–244.
8. Koppe, J. G., Loewer-Sieger, D. H., and de Roever-Bonnet, H. (1986) Results of 20-year follow-up of congenital toxoplasmosis. *Lancet* **ii,** 254, 255.
9. Remington, J. S. and Desmonts, G. (1990) Toxoplasmosis, in *Infectious Diseases of the Fetus and the Newborn Infant* (Remington, J. S. and Klein, J. O., eds.), Saunders, Philadelphia, pp. 89–195.
10. Hohlfeld, P., Daffos, F., Costa, J.-M., Thulliez, Ph., Forestier, F., and Vidaud, M. (1994) Prenatal diagnosis of congenital toxoplasmosis with a polymerase chain reaction test on amniotic fluid. *N. Engl. J. Med.* **331,** 695–699.
11. Savva, D., Morris, J. C., Johnson, J. D., and Hillman, R. E. (1990) Polymerase chain reaction for detection of *Toxoplasma gondii. J. Med. Microbiol.* **32,** 25–31.
12. Grover, C. M., Thulliez, P., Remington, J. S., and Boothroyd, J. C. (1990) Rapid prenatal diagnosis of congenital Toxoplasma infection by using polymerase chain reaction and amniotic fluid. *J. Clin. Microobiol.* **28,** 2297–2301.
13. Cazenave, J., Forestier, F., Bessieres, M. H., Broussin, B., and Begueret, J. (1992) Contribution of a new PCR assay to the prenatal diagnosis of congenital toxoplasmosis. *Prenat. Diagn.* **12,** 119–127.
14. Bretagne, S., Costa, J. M., Vidaud, M., Van Nhieu, J. T., and Fleury-Feith, J. (1993) Detection of *Toxoplasma gondii* by competetive DNA amplification of bronchoalveolar lavage samples. *J. Infect. Dis.* **168,** 1585–1588.
15. Van de Ven, E., Melchers, W., Galama, J., Camps, W., and Meuwissen, J. (1991) Identification of *Toxoplasma gondii* infections by B1 gene amplification. *J. Clin. Microbiol.* **29,** 2120–2124.
16. Dupon, M., Cazenave, J., Pellegrin, J. L., Ragnaud, J. M., Cheyrou, A., et al. (1995) Detection of *Toxoplasma gondii* by PCR and tissue culture in cerebrospinal fluid and blood of human immunodeficiency virus-seropositive patients. *J. Clin. Microbiol.* **33,** 2421–2426.
17. Dupouy-Camet, J., De Souza, S. L., Maslo, C., Paugam, A., Saimot, A. G., et al. (1993) Detection of *Toxoplasma gondii* in venous blood from AIDS patients by polymerase chain reaction. *J. Clin. Microbiol.* **31,** 1866–1869.
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
19. Papp, Z. (1990) Obsteric genetics, Akadémiai Kiadó, Budapest, Hungary.
20. Tóth, T., Németi, M., and Papp, Z. (1997) Detection of CAG repeats using silver staining in patients with Huntington disease in Hungary. *Am. J. Med. Genet.* (in press).

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Rapid Diagnosis of Pulmonary Tuberculosis Using Roche AMPLICOR™ *Mycobacterium tuberculosis* PCR Test

Richard F. D'Amato and Albert Miller

1. Introduction

The rapid diagnosis of infectious diseases, particularly those because of their communicability present a public health problem, is the objective of the clinical microbiologist and the practicing physician. The need to attain this goal is exemplified by the resurgence of tuberculosis and the increasing prevalence of drug-resistant isolates of *Mycobacterium tuberculosis*. The recent introduction of molecular technologies for the laboratory diagnosis of infectious diseases into the clinical microbiology laboratory now makes the goal of truly rapid diagnosis of infectious diseases attainable.

Molecular techniques, such as the polymerase chain reaction (PCR), circumvent the "pure culture dogma" that requires the isolation of a microorganism in pure culture prior to its definitive identification. PCR permits the direct detection and identification of infectious agents in clinical specimens, saving days to weeks in diagnostic time. Its application to infectious diseases caused by fastidious or slow-growing microorganisms, such as *M. tuberculosis*, has the potential to provide a truly rapid laboratory diagnosis with the attendant improvement in patient management and reduction in medical costs.

This chapter presents the impact of the Roche AMPLICOR™ *Mycobacterium tuberculosis* (AMPLICOR MTB) PCR test for the rapid diagnosis of pulmonary tuberculosis from a clinical, laboratory, and fiscal perspective.

The AMPLICOR MTB test is based on four major processes: specimen preparation, amplification of target nucleic acid by PCR in the presence of biotinylated primers; hybridization of the amplified products to oligonucle-

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

otide probes specific for *M. tuberculosis*-complex target DNA; and detection of the probe-bound amplification product by color formation.

The primers KY18 and KY75 are used to amplify a highly conserved region of the 16s rRNA gene ***(1,2)***. A primer is a short piece of synthetic DNA designed to complement a specific DNA sequence of the organism to be detected/identified. It serves as a starting point for the attachment of nucleotides complementary to the target DNA. In the case of *M. tuberculosis* a 584-base-pair region is amplified. The extracted specimen is added to a master mix reagent containing *Taq* polymerase, AmpErase®, biotinylated primers, and excess deoxynucleoside triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, and deoxyuridine (in place of thymidine) triphosphates and amplified in the GeneAmp® System 9600 (Perkin Elmer, Norwalk, CT). Each PCR cycle is comprised of three steps:

1. **Denaturation;** where double-stranded target DNA is denatured by heating at high temperatures;
2. **Annealing;** where biotinylated primers hybridize to the denatured target; and
3. **Extension;** where the excess dNTPs are added to the biotinylated primers by the enzyme *Taq* polymerase to produce a biotinylated copy of the target DNA known as an amplicon.

A 37-cycle profile is used with the AMPLICOR MTB test and within a matter of 2 h results in a theoretical amplification yield of over a billion-fold.

Selective amplification of target DNA, as opposed to amplicon carried over from previous amplification reactions, which can potentially lead to a false-positive result, is achieved by the use of AmpErase. AmpErase contains the enzyme uracil *N*-glycosylase (UNG) ***(3)***, which recognizes and catalyzes the destruction of deoxyuridine-containing amplicon DNA, but not deoxythymidine-containing target DNA. Deoxyuridine is not present in naturally occurring DNA, but is always present in amplicons as a result of the use of deoxyuridine triphosphate (in place of thymidine triphosphate) as one of the dNTPs in the master mix reagent. AmpErase catalyzes the cleavage of uracil-containing oligonucleotide at the deoxyuridine residues by opening the deoxyribose chain at the 1 position. When heated in the first thermal cycling step (at the alkaline pH of master mix), the amplicon's DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA nonamplifiable. The UNG enzyme is inactive at temperatures above 55°C, i.e., throughout the thermal cycling steps, thereby preventing the degradation of any "true" amplified products.

Specificity of the test for *M. tuberculosis* complex organisms is accomplished by hybridization of the amplified product to a DNA probe specific for organisms of the *M. tuberculosis* complex. Following amplification, denatur-

ation solution is added to all tubes, followed by a room temperature incubation to allow complete denaturation of the double stranded products. Hybridization buffer is then added to a microwell plate coated with a DNA probe specific for organisms of the *M. tuberculosis* complex. The denatured amplicons are then added to the microwell and hybridization takes place if the specimen contains *M. tuberculosis* DNA. Detection of hybridized duplexes is completed using an avidin–horseradish peroxidase conjugate-tetramethylbenzidine substrate system, which produces a colored reaction if *M. tuberculosis* is present.

2. Materials

2.1. Specimen Processing

The standard *N*-acetyl-L-cysteine-NaOH or NaOH decontamination and liquefaction method is used to process sputum specimens *(4)*.

2.2. PCR Amplification

The AMPLICOR *M. tuberculosis* test consists of the following kits:

1. AMPLICOR Sputum Specimen Preparation Kit.
2. AMPLICOR *Mycobacterium* Amplification Kit.
3. AMPLICOR *M. tuberculosis* Detection Kit.

Materials required but not provided with the AMPLICOR MTB test are as described in **Subheadings 2.2.1.–2.2.3.**

2.2.1. Reagent Preparation

1. Perkin-Elmer GeneAmp® PCR System 9600 MicroAmp™ consumables (tubes, caps, base, tray, and retainer).
2. Repeat pipeter and 1.25 mL individually wrapped Combitips.
3. Micropipets with plugged (aerosol barrier) or positive displacement tips (50 µL and 100 µL).
4. Plastic "zipper" bags.

2.2.2. Specimen Preparation

1. Microcentrifuge (maximum relative centrifugal force [RCF] 16,000*g*, minimum RCF 12,500*g*) Eppendorf 5415C, HERMLE Z230M, or equivalent.
2. Sarstedt 1.5 mL sterile screw-cap tubes (72.692.105).
3. Tube racks (Sarstedt 93.1428).
4. Sterile fine-tip transfer pipets, repeat pipeter with 12.5 mL individually-wrapped combitips, and vortex mixer.
5. Perkin-Elmer GeneAmp PCR System 9600 MicroAmp base and capping tool. Micropipets with plugged (aerosol barrier) or positive displacement tips (50 and 100 µL).
6. Dry heat blocks (60 ± 2°C).

2.2.3. Amplification and Detection

1. Perkin-Elmer GeneAmp PCR System 9600 thermal cycler.
2. Multichannel pipeter (25 and 100 µL) with plugged (aerosol barrier) tips (25 or 100 µL) and unplugged tips (100 µL).
3. Disposable reagent reservoirs.
4. Microwell plate lid.
5. Elisawell key for strip removal.
6. Incubator 37°C ± 2°C.
7. Distilled or deionized water.
8. Microwell plate washer (recommended but not required).
9. Microwell plate reader and printer.

3. Methods

3.1. Specimen Collection

Sputum specimens are collected from the patient, decontaminated, and concentrated according to a standard protocol formulated by the Centers for Disease Control *(4)*.

3.2. AMPLICOR Procedure

3.2.1. Reagent Preparation

If reagent preparation, specimen preparation, and amplification cannot be completed within 1 d, reagent preparation, amplification, and detection should be performed the following day.

1. Prepare Master Mix with AmpErase by adding 100 µL of AmpErase to one tube of master mix (the mixture is sufficient for 32 amplifications). Recap the master mix tube and mix well by inverting the tube 10–15 times. Discard the empty AmpErase tube. Record date of preparation on the tube.
2. Determine the appropriate number of PCR tubes needed for patient specimen and control testing. It is recommended that one (1) positive and three (3) negative controls be run in each amplification. Place tubes in the MicroAmp sample tray and lock in place with retainer.
3. Pipet 50 µL of master mix with AmpErase to each PCR tube using a repeat pipeter and 1.25 mL combitip or a micropipet with a plugged tip.
4. Place MicroAmp tray containing master mix in a "zipper" plastic bag. Include the appropriate number of strips of caps in the bag. Make sure the seal is secure and move to the Specimen Preparation Area (Area 2).

3.2.2. Specimen Preparation

1. Add 100 µL of decontaminated sputum or BAL to 500 µL of sputum wash solution in a 1.5 mL screw-cap tube. Vortex.
2. Centrifuge at 12,500*g* for 10 min.

3. Aspirate supernatant and add 100 µL of sputum lysis reagent to the cell pellet. Vortex to resuspend pellet.
4. Prepare positive and negative control stocks.
   a. Pipet 100 µL of negative control into a tube using a micropipet with a plugged tip. Add 400 pL of sputum lysis reagent. Vortex. This is the negative control stock.
   b. Pipet 100 µL of positive control into a tube using a micropipet with a plugged tip. Add 400 µL of sputum lysis reagent. Vortex. This is the positive control stock.
   c. Pipet 100 µL from each control stock and place each into a 1.5 mL screw-cap tube to be processed.
5. Incubate specimens and controls in 60 ± 2°C dry heat block (containing 0.5 cm sand) for 45 min.
6. Remove tubes from heat block and pulse-centrifuge the tubes for 5 s.
7. Add 100 µL of sputum neutralization reagent. Vortex.
8. Pipet 50 µL of prepared patient specimen(s) and prepared controls (one positive and three negative) to appropriate PCR tubes using a micropipet with plugged tip(s). Be careful to avoid transferring any material that may not have been resuspended. Record the positions of the controls and the patient specimens in the tray. Cap the tubes. Apply pressure for a tight seal using the GeneAmp PCR System 9600 cap installing tool.
9. Move prepared specimens in the sample tray to the Amplification and Detection Area.

3.2.3. Amplification

1. Place the sample tray into the thermal cycler sample block. Make certain that the notch in the sample tray is at the left of the block, and that the rim of the tray is seated in the channel around the block.
2. Make certain that the cover knob of the thermal cycler is turned completely counterclockwise. Slide the cover forward.
3. Turn the thermal cycler cover knob clockwise until tight. (The white mark on the cover knob should line up with the white mark on the cover.)
4. Program the GeneAmp PCR System 9600 thermal cycler for amplification of the Amplicor MTB test as follows (consult the GeneAmp PCR System 9600 User's Manual for additional information on programming and operation of the thermal cycler):
   Hold program: 2 min at 50°C.
   Cycle program (2 cycles): 20 s at 98°C, 20 s at 62°C, 45 s at 72°C.
   Cycle program (35 cycles): 20 s at 94°C, 20 s at 62°C, 45 s at 72°C.
   Hold program: 5 min at 72°C.
   Hold program: 72°C forever.

   In the cycle programs, the ramp times should be left at the default setting (0:00), which is the maximum rate, and the allowed setpoint error is at the default setting (2°C). Link the five programs together into a method program.

5. Start the method program. The program runs approx 1.5 h. Specimens may be removed at any time during the final hold program, but must be removed within 24 h.
6. Remove completed PCR amplification specimens (tray) from the thermal cycler. **Do not bring amplified DNA into Area 1 or Area 2. The amplified controls and specimens should be strictly confined to Area 3.** Remove caps carefully to avoid aerosolizing the contents of the PCR reaction tubes.
7. Immediately pipet 100 µL of denaturation solution to the first column (or row) of PCR reaction tubes using a multichannel pipeter with plugged tips and mix by pipeting up and down. For each column (or row), repeat this procedure using a fresh set of tips. Incubate for 10 min at room temperature to allow complete denaturation.
8. Store denatured, amplified specimens at room temperature only if the microwell plate detection will be performed within 1–2 h. If not, store the specimens at 2–8°C until the detection assay is performed. Amplicons may be stored for up to 1 wk at 2–8°C.

3.2.4. Detection

Warm all reagents to room temperature. Prepare working wash solution by adding 1 vol of 10X wash concentrate to 9 vol of distilled or deionized water. Mix well. For manual washing, prepare 40 mL of working wash solution for each 8-well microwell plate strip. For automated washing, prepare an amount according to the washer model being used.

1. Allow the microwell plate to warm to room temperature before removing from the foil pouch. Remove the appropriate number of 8-well microwell plate strips from the foil package and set into the microwell plate frame. Return unused strips to the pouch and reseal, making sure that the desiccant pillow remains in the pouch. (**Note:** Microwell strips must be handled carefully to avoid breakage. To remove strips from the frame, center the microwell plate on top of the Elisawell key and press down evenly on the corners of the frame. To lock strips in place, place the Elisawell key on top of the strips and press uniformly against the strips.)
2. Add 100 µL of *Mycobacterium* hybridization buffer to each well to be tested on the microwell plate.
3. Using plugged tips, pipet 25 µL of denatured amplification specimen to the appropriate well(s). Gently tap the plate approx 10–15 times until the color changes from blue to light yellow (this color change indicates sufficient mixing has occurred).
4. Cover the plate; incubate for 1.5 h at 37 ± 2°C.
5. Wash the plate five times manually or by using a Microwell Plate washer. Use the prepared working wash solution (10X concentrate diluted 1/10 with distilled/deionized water) for washing the plate.
   For manual washing:
   a. Empty contents of the plate and tap dry on paper towels.
   b. Pipet working wash solution to fill each well to the top (400–450 µL). Let soak for 30 s. Empty out contents and tap dry.
   c. Repeat **step 2** four additional times.

For automated washing, program the washer to:
   a. Aspirate contents of wells.
   b. Fill each well to top with working wash solution (approx 350–450 µL dependent on plate washer), soak for 30 s and aspirate dry.
   c. Repeat **step 2** four additional times.
6. Tap the plate dry.
7. Add 100 µL Avidin–HRP conjugate to each well. Cover the plate and incubate for 15 min at 37 ± 2°C).
8. Wash plate as described in **step 5**.
9. Prepare working substrate by mixing 2.0 mL of substrate A and 0.5 mL of substrate B for each multiple of two, 8-well microwell plate strips (16 tests). Prepare this reagent no more than 3 h before use and protect from exposure to direct light.
10. Pipet 100 µL of prepared working substrate reagent into each well being tested.
11. Allow color to develop for 10 min, at room temperature (20–25°C), in the dark.
12. Add 100 µL of stop reagent to each well.
13. Measure the optical density at A_{450} within 1 h of adding the stop reagent. Record the absorbance value for each patient specimen and control tested. Calculate the results. The presence of *M. tuberculosis* in the specimen is determined by relating the absorbance of the unknown specimen to that of the cutoff value. A value of 0.35 A_{450} has been selected as the cutoff for this assay. A clinical specimen with an A_{450} reading ≤0.35 is positive for the presence of *M. tuberculosis.* A clinical specimen with an A_{450} reading <0.35 is considered negative for *M. tuberculosis.*

3.3. Quality Control

It is recommended that at least 1 positive control and 3 negative controls be run each time the test is performed.

3.3.1. Negative Control

The assay result of each negative control should be <0.25 A_{450} U. If one or more of the negative control values are >0.25 A_{450} units, the entire run should be discarded and the entire assay, including amplification, should be repeated.

3.3.2. Positive Control

The response of thepositive control should be >3.0 A450 U. If the value of the positive control should fall below 2.0 A_{450} units, the entire run should be discarded and the entire assay, including amplification, should be repeated.

3.3.3. Sample Processing Control

To test the effectiveness of sample processing (recommended on a monthly basis), 10^4 *M. tuberculosis* cells should be processed as described in **Subheading 3.2.2.** and then treated as a normal clinical specimen. A positive signal above 3.0 A_{450} should be obtained on the microwell plate if the sample is properly processed.

3.4. Clinical Trials

1. Ultimately, the "value" of a diagnostic test is judged by its ability to improve patient care. This "value" can be in the form of improved accuracy over existing methodologies, improved turn-around time for test results, reduced costs, and simplification of test methodology, the latter two being of more indirect benefit to patient care.
2. To determine these parameters for the AMPLICOR MTB test we evaluated 985 sequential respiratory specimens from 372 patients suspected to have tuberculosis or who were being monitored for treatment with antituberculosis drugs ***(5)***. PCR results were compared with conventional smear and culture results and the final clinical diagnosis for each patient.

3.5. Cost Impact

We collaborated with Health Technology Associates to develop a mathematical model designed to assess the financial impact of the AMPLICOR MTB test at the Catholic Medical Center. Three scenarios were considered in which the AMPLICOR test could be used:

1. On all respiratory specimens;
2. Only on smear-positive specimens; and
3. On all "highly suspicious" cases, defined as either smear-positive or smear-negative but with high clinical suspicion of tuberculosis. The latter is the scenario we recommended for the Catholic Medical Center.

A net financial cost-benefit to the Catholic Medical Center was derived by comparing the costs of AMPLICOR MTB testing with the potential hospital cost savings that would be realized within the specific patient population in which the test would be used. The cost savings considered were estimated based on the impact of a rapid diagnostic result with AMPLICOR MTB in the clinical management of patients suspected to have tuberculosis infection. Savings in hospital costs were broken down by the savings associated with patients testing negative for AFB smear vs those testing positive.

4. Notes

1. **Table 1** compares AMPLICOR MTB results with resolved results, i.e., specimens that yielded *M. tuberculosis* on culture or were obtained from a patient with a definitive clinical diagnosis of tuberculosis. This is more meaningful than merely comparing PCR results with culture results because culture, as a test for the diagnosis of tuberculosis, is not 100% sensitive ***(1,6,7)***.

   The sensitivity, specificity, positive predictive value, and negative predictive value for the AMPLICOR MTB test were 66.7, 99.6, 91.7, and 97.7%, respectively. Interestingly, these results were comparable to those obtained from culture and to those reported by other investigators ***(7–10)***. However, AMPLICOR

Table 1
Comparison of Roche AMPLICOR MTB Results with Resolved Results[a]

Specimen category (no.)	Number of specimens: Resolved positive results PCR+	Resolved positive results PCR–	Resolved negative results PCR+	Resolved negative results PCR–	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
All specimens (985)	44	22	4[b]	915	66.7	99.6	91.7	97.7
Smear negative (949)	26	21	4[b]	898	55.3	99.6	86.7	97.7
Smear positive (36)	18	1	0	17[c]	94.7	100	100	94.7

[a]Culture positive for *M. tuberculosis* or diagnosis of tuberculosis.
[b]Repeat testing in two separate laboratories yielded negative PCR test results.
[c]All specimens were positive for mycobacteria other than *M. tuberculosis*.

results were available 6.5 h after specimen receipt in the laboratory, whereas a minimum of 2 wk were required before a definitive laboratory diagnosis was made with the conventional method. Additionally, AMPLICOR was positive in 14 culture-negative patients. In 10 of these patients, the diagnosis of tuberculosis was established by clinical findings (biopsy, response to antituberculosis medications). Five of the 10 patients also had positive smears. The availability of AMPLICOR provided specific evidence of tuberculosis in these patients when the culture was negative. Confirmation of this finding as experience increases and its application to clinical practice may obviate invasive biopsy procedures to establish the diagnosis of tuberculosis in such patients.

2. The 66.7% sensitivity of the AMPLICOR MTB test requires some discussion. In our study only 14 (25%) of the 55 culture-positive specimens were smear-positive. The sensitivity of detection tests, be they molecular, culture, or serologic, are influenced by the bioburden of the test sample. Usually, increases in the number of microorganisms present in a specimen result in greater test sensitivity. For example, with smear-positive specimens, the number of microorganisms in the patient sample is greater than for smear-negative specimens; the sensitivity of the AMPLICOR MTB test was an impressive 94.7%. Even when smears were negative, AMPLICOR was positive in 55.3% (26 of 47) of the specimens. Positive results in smear-negative specimens are very important to the clinician and infection control practitioner because prior to PCR, smears have been the only rapidly available clue for detecting unapparent cases or confirmation of clinically suspect cases of tuberculosis. We are now looking at clinical subsets of the smear-negative specimens to assess the predictive value of a positive PCR test when cavitary pulmonary disease or hematogenous spread is suggested by radiographic findings or when the patient is known or suspected to be immunocompromised.
3. The sensitivity of a diagnostic test is also influenced by how the test is used. The AMPLICOR test should be utilized in conjunction with, not in lieu of, culture. Therefore, a sensitivity of 66.7% does not mean that three out of every 10 specimens containing *M. tuberculosis* would be missed, because the AMPLICOR negative specimens would be cultured. The true significance of the 66.7% sensitivity is that seven of every 10 cases of tuberculosis would be diagnosed the day the test was performed. At the very least, the test should be performed for all smear-positive specimens and for all specimens from patients considered likely to have pulmonary tuberculosis. This approach not only will provide rapid, clinically significant results, but also has the ability to detect cases of tuberculosis missed by culture.
4. The rapidity and reliability of the AMPLICOR MTB test not only has a significant effect on the effectiveness of patient care but also impacts on the cost of providing that care. In all three scenarios, the AMPLICOR MTB was found to have a net positive cost-benefit at the Catholic Medical Center (*see* **Table 2**). The alternative with the greatest cost benefit was scenario 3 followed by scenario 2. The use of AMPLICOR on all respiratory specimens (scenario 1) was found to be the least cost-effective option. By implementing AMPLICOR MTB testing in all

Table 2
Impact of AMPLICOR MTB on Hospital Costs at the Catholic Medical Center

	Scenario 1	Scenario 2	Scenario 3
Hospital cost savings			
Smear-negative patients			
Decreased bronchoscopy	$25,125	$0	$25,125
Decreased lung biopsy	500	0	500
Subtotal	25,625	0	25,625
Smear-positive patients			
Decreased length of stay	144,900	144,900	144,900
Decreased use of isolation	58,498	58,498	58,498
Discontinuation of MTB drug therapy	12,627	12,627	12,627
Subtotal	216,025	216,025	216,025
Total cost savings	**241,652**	**216,025**	**241,650**
Cost of testing			
Cost of AMPLICOR	194,854	5,830	20,790
Amortization of thermalcycler	1,425	1,425	1,425
Total costs	196,279	7,255	22,215
Net cost-benefit	**45,371**	**219,432**	**219,435**

patients with a high clinical suspicion of tuberculosis infection or with a positive smear, not only may the cost savings associated with decreased use of isolation, early discontinuation of drug therapy, and earlier patient discharge be realized, but also certain costs associated with invasive diagnostic procedures may be avoided. The impact of these factors benefit not only the hospital, but the patient as well.

5. In summary, the definitive diagnosis of tuberculosis by PCR results in the following clinical and financial benefits:
   a. Immediate initiation of antituberculous therapy, with greater likelihood of control and cure of the disease;
   b. Effective use of isolation protocols, preventing the spread of disease to other patients and staff from patients whose smear-negative status may mislead the hospital staff from starting isolation;
   c. Termination of isolation when it is not necessary despite positive smears or cavitary disease caused by MOTT;
   d. Shorter period of isolation because of rapid effective therapy;
   e. Decreased hospital length of stay because of rapid effective therapy; and
   f. Obviation of invasive procedures to establish a diagnosis of tuberculosis (including bronchoscopies with or without bronchovaleolar lavage or transbronchial biopsy and thoracoscopic or open lung biopsy), with their attendant costs and risks.

References

1. Boddinghaus, B., Rogall, T., Flohr, T., Blocker, H., and Bottger, E. C. (1990) Detection and identification of mycobacteria by amplification of rRNA. *J. Clin. Microbiol.* **28,** 1751–1759.
2. Stahl, D. A. and Urbance, J. W. (1990) The division between fast- and slow-growing species corresponds to natural relationships among the mycobacteria. *J. Bact.* **172,** 116–124.
3. Longo, M. C., Berninger, M. S., and Hartley, J. L. (1990) Use of Uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* **93,** 125–128.
4. Kent, P. T. and Kubica, G. O. (1985) US DHHS. Public Health Mycobacteriology. A guide for the Level III laboratory. Centers for Disease Control, Atlanta, GA.
5. D'Amato, R. F., Wallman, A. A., Hochstein, L. H., Colaninno, P. M., Scardamaglia, M., Ardila, E., Ghouri, M., Kim, K., Patel, R. C., and Miller, A. (1995) Rapid diagnosis of pulmonary tuberculosis by using roche amplicor *Mycobacterium tuberculosis* PCR test. *J. Clin. Microbiol.* **33,** 2906–2908.
6. Abe, C., Hirano, K., Wada, M., Kazumi, Y., Takahashi, M., Fukasawa, Y., Yoshimura, T., Miyagi, C., and Goto, S. (1993) Detection of *Mycobacterium tuberculosis* in clinical specimens by polymerase chain reaction and Gen-Probe Amplified Tuberculosis Direct Test. *J. Clin. Microbiol.* **31,** 3270–3274.
7. Beavis, K. G., Lichty, M. B., Jungkind, D. L., and Giger, O. (1995) Evaluation of Amplicor PCR for direct detection of *Mycobacterium tuberculosis* from sputum specimens. *J. Clin. Microbiol.* **33,** 2582–2586.
8. Patel, R. C., Miller, A., Kim, K., Hochstein, L., D'Amato, R. F. (1995) Specificity of the PCR based Amplicor™ Mycobacterium tuberculosis test: Is there cross-reactivity with MOTT? Results from 108 culture-positive specimens for MOTT. *Am. J. Resp. Crit. Care Med.* **151,** A476.
9. Vuorinen, P., Miettinen, A., Vuento, R., and Hällström, O. (1995) Direct detection of *Mycobacterium tuberculosis* complex in respiratory specimens by Gen-Probe amplified Mycobacterium Tuberculosis Direct Test and Roche Amplicor Mycobacterium Tuberculosis Test. *J. Clin. Microbiol.* **33,** 1856–1859.
10. Wobeser, W. L., Krajden, M., Conly, J., Simpson, H., Yim, B., D'Costa, M., Fuksa, M., Hian-Cheong, C., Patterson, M., Phillips, A., Bannatyne, R., Haddad, A., Brunton, J., and Kraiden, S. (1996) Evaluation of Roche Amplicor PCR assay for *Mycobacterium tuberculosis. J. Clin. Microbiol.* **34,** 134–139.

19

The Use of Flow Cytometry and RT-PCR in the Detection of Circulating PSA-Positive Cells in Prostate Cancer

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1. Introduction

Prostate cancer is the most common malignancy diagnosed in men in the Western hemisphere. Once early tumors are detected, prognosis is largely unpredictable, and clinicians are currently unable to inform the patient whether his tumour is likely to progress. New criteria to define the aggressive and metastatic potential of prostate cancer are needed, particularly in view of the recent controversies and evidence from North American studies questioning the benefits of radical surgery over observation in early-stage prostatic adenocarcinoma ***(1–3)***. Furthermore, even in patients where the disease appears to be confined to the prostate, cancers can be understaged in over 50% of cases, with resulting positive surgical margins, extracapsular extension, and potential treatment failure ***(4)***.

The formation of metastasis is a significant, rate determining event in the progression of cancer. It is a complex, nonrandom phenomenon involving a cascade of multisequential events, including tumor cell detachment from the primary lesion into the blood and lymphatic channels, survival of malignant cells in a hostile environment, extravasation at a chosen site, and the final formation of a secondary deposit ***(5)***. Previous studies in animals have shown that metastasis does not rely on the random survival of cells released from the primary tumor, but from the selective growth of specialized subpopulations of highly metastatic cells endowed with properties that will allow them to successfully complete each step of the metastatic cascade ***(6,7)***.

The detection of circulating tumor cells in cancer patients is not, by any means, a new phenomenon. Indeed, several previous studies, some dating back

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

over half a century, have elegantly demonstrated the presence of circulating malignant cells in the peripheral blood of patients with advanced disease ***(6,8–10)***, without the help of current sophisticated and molecular biology techniques. In recent years, however, new technology, including flow cytometry (FC) and the reverse transcription polymerase chain reaction (RT-PCR), has allowed the design of several new methodologies to detect circulating tumor cells with high levels of sensitivity.

The discovery of prostate-specific antigen (PSA) by Wang and colleagues ***(11)*** has provided clinicians and researchers with a valuable marker. PSA is a serine protease, expressed almost exclusively in the cytoplasm of epithelial cells of the prostate, that prevents semen coagulation. It is present in small amounts in the serum, and its levels increase in the presence of carcinoma, infection, large volume glands, and other nonmalignant pathologies. It is important to emphasize that PSA is tissue- and not tumor-specific, a major drawback and limitation for all techniques using PSA as a marker. A PSA-positive cell, therefore, does not necessarily mean a prostate tumor cell, but a cell expressing PSA, which is likely to be of prostatic origin, in particular if the cell is found to express the gene constitutively, i.e., mRNA for PSA.

Based on these principles, analytical FC and RT-PCR have been used in an attempt to detect and isolate circulating tumor cells from patients with prostate cancer. Studies have shown that although quantification of circulating PSA-positive cells by FC was a better predictor of skeletal metastases than isotope bone scanning, the majority of these cells were not of prostatic origin, raising important questions regarding the role of nonprostatic circulating PSA-positive cells in patients with prostate cancer ***(12,13)***. RT-PCR methods, on the other hand, are considerably more sensitive in identifying cells of prostatic origin by detecting mRNA for PSA. The methodology, however, is unable to provide firm evidence that these cells represent circulating micrometastases. Despite this limitation, Moreno et al. ***(14)*** suggested that RT-PCR was able to detect hematogenous micrometastases in patients with advanced prostate cancer. Their study failed to identify any of these cells in patients with clinically nonmetastatic disease. Katz et al. ***(15)***, also using RT-PCR, were able to detect circulating prostate cells in patients with apparently localized disease undergoing radical prostatectomy, and found a strong correlation among a positive PCR reaction, capsular tumor penetration, and positive surgical margins, suggesting the potential of this technique to be used for "molecular staging" of prostate cancer. Two further studies by Israeli et al. ***(16,17)*** used nested RT-PCR to compare the sensitivity of PSA with a more recently identified marker, prostate-specific membrane antigen (PSMA), in the detection of circulating prostatic cells. The studies showed that PSMA was significantly more sensitive than PSA, although some of their patients, with pathologically organ-confined

disease and an undetectable serum PSA following radical prostatectomy, had a positive RT-PCR assay for PSMA, the significance of which has not been established. In turn, Cama et al. ***(18)*** repeated the initial experiments made by Katz and colleagues ***(15)*** using the same patients' samples, comparing PSA with PSMA, and found, in contrast with Israeli et al. ***(17)***, that PSA was more sensitive than PSMA in predicting local tumor penetration, adding further controversy to the possible value of these sensitive assays in staging prostate cancer.

It is interesting to note that the authors of all these studies assume that circulating PSA-positive cells are endowed with metastatic propensity, despite the fact that the results only demonstrate the presence of cells of prostatic origin. Furthermore, the specificity of PSA mRNA in identifying cells of prostatic origin has been questioned in a study that demonstrated PSA mRNA in nonprostatic cell-lines, including ovarian, lung, myeloid leukemia, and normal blood ***(19)***. More recently, Brandt et al. ***(20)*** were able to use analytical FC with cell sorting, RT-PCR, and combined buoyant density gradient and immunomagnetic separation to discriminate prostatic from nonprostatic circulating PSA-positive cells. The techniques will allow further investigation of these putative circulating micrometastatic cells found in prostate cancer. In this chapter, the authors describe, step-by-step, the use of analytical FC and RT-PCR in the detection of PSA-positive cells in the blood stream of patients with prostate cancer.

2. Materials

2.1. Tissue Collection

1. EDTA vacutainers.
2. Sterile phosphate-buffered saline (PBS).
3. Histopaque 1077 (Sigma, Poole, UK).
4. Sterile scalpels and blades, NFC microfilament filter cloth (25 μm, Cadish Precision Mesh, London, UK).
5. 1% Trypan blue in PBS.
6. Ice, plastic bags.

2.2. Flow Cytometric Analysis

1. Falcon 6 mL tubes (Becton Dickinson, Cowley, UK; **Note 1**).
2. Anti-PSA monoclonal antibody (MoAb), Anti-LCA MoAb, IgG isotype control MoAb, FITC-conjugated Fab_2 (Dakopatts, High Wickham, UK; **Note 2**).
3. PBS 1% bovine serum albumin (BSA).
4. PBS 1% paraformaldehyde.

2.3. RNA Extraction

1. Sterile diethylpyrocarbonate-treated distilled deionized water (DEPC-treated water).
2. RNA Stat-60 isolation reagent (Tel-Test B, Friendswood, TX; **Note 3**).
3. Isopropanol.

4. 75% Ethanol in DEPC-treated water.
5. Chloroform.
6. 1 m*M* EDTA in DEPC-treated water.
7. Ice.

2.4. cDNA Synthesis

1. Sterile DEPC-treated water.
2. Monkey moloney virus reverse transcriptase (M-MLV), 5X buffer, 250 m*M* Tris-HCl (pH 8.3), 375 m*M* KCl, 15 m*M* $MgCl_2$, 100 m*M* DDT (Life Technologies, Gibco, Paisley, UK; **Note 4**).
3. Random primer pd(N_6) (Pharmacia, Uppsala, Sweden).
4. Nucleotides, 100 m*M* stock solutions of dATP, dCTP, dTTP, dGTP, each used at 0.2 m*M* final concentration (Pharmacia).
5. Mineral oil.
6. Positive control RNA, extracted from prostate tissue (**Note 5**).
7. Ice.

2.5. PCR Amplification

1. Sterile DEPC-treated water.
2. *Taq* polymerase, 10X incubation buffer, $MgCl_2$ (Kit, Boehringer Mannheim, Mannheim, Germany; **Note 4**).
3. Nucleotides, 100 m*M* stock solutions of dATP, dCTP, dTTP, dGTP, each used at 0.2 m*M* final concentration (Pharmacia).
4. Oligonucleotide primers (**Note 6**).
5. Mineral oil.
6. cDNA preparation (5 μL/test).

2.6. Digoxigenin (DIG) Labeling of Probe

1. Sterile DEPC-treated water.
2. DIG DNA labeling and detection kit (Boehringer, **Note 7**).
3. Oligonucleotide probe (1 μg).
4. LiCl (4 mol/L).
5. EDTA (0.2 mol/L).
6. 70% Ethanol in sterile DEPC-treated water.
7. TE buffer: 10 mmol Tris-HCl, 1 mmol EDTA (pH 8.0).

2.7. Agarose Gel Electrophoresis

1. NuSeive agarose (Sea Kern GTG, Flowgen Instruments).
2. Ethidium bromide (5 mg/mL) in sterile water.
3. 50X TAE buffer: 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 *M* EDTA (pH 8.0) dissolved in a final volume of 1 L of distilled deionized water. Dilute to 1X working concentration in distilled deionized water.
4. Gel loading dye: 0.1% bromophenol blue, 50% glycerol in distilled deionized water.
5. Any commercially available DNA marker preparation.

6. DIG-labeled markers (Boehringer).
7. Chloroform.
8. Polaroid camera and film.

2.8. Southern Blot

1. 0.4 *M* Sodium hydroxide.
2. Positively charged nylon membrane (Boehringer).
3. 10X TSB: 1.5 *M* sodium chloride, 150 m*M* Tris-HCl, pH 7.5.
4. 10X TBS: 1.5 *M* sodium chloride, 1 *M* Tris-HCl, pH 7.5.
5. Substrate buffer: 100 m*M* sodium chloride, 100 m*M* Tris-HCl pH 9.5.
6. Tween-20.
7. Sonicated salmon sperm DNA.
8. DIG-labeled probe, DIG detection kit (part of the labeling kit, Boehringer).
9. Hyperfilm MP (Amersham, Aylesbury, UK).

2.9. Oligonucleotide Primers and Probe

1. Antisense PCR primer (exon 3): 5'-ACTCCTCTGGTTCAATGCTG-3'
2. Sense PCR primer (exon 2): 5'-TCATCCTGTCTCGGATTGTC-3': Product size: 426 base pairs.
3. Exon 3 probe: 5'-CCGACCCAGCAAGATCACGC-3'

3. Methods

3.1. Tissue Preparation

3.1.1. Preparation of Tumor Cell Suspension for Titration of the PCR

1. Collect the tumor tissue by needle core biopsy, put into cryovials on ice, use fresh or store at –80°C.
2. Place the tissue on a plastic Petri dish sitting on ice. Cover the tissue with a small volume of PBS and mince with the scalpel blades.
3. Filter the tissue through 25 μm NFC mesh into a new Petri dish, to obtain a single cell suspension.
4. Harvest cells into test tubes, wash the Petri dish with PBS and harvest the supernatant.
5. Centrifuge the cells at 600*g* for 6 min at 4°C.
6. Discard the supernatant, resuspend the pellet in 0.5 mL PBS.
7. Count the cells using trypan blue.

3.1.2. Preparation of Tumor Cells for RNA Extraction

1. Mince the core biopsy, with scalpel blades, in a Petri dish on ice.
2. Collect the tissue into a 1.5-mL microcentrifuge tube.
3. Add 1 mL of RNA Stat-60 solution to the minced tissue.

3.1.3. Preparation MNC for Flow Cytometry and RNA Extraction

1. Collect 20 mL of blood by venepuncture into EDTA vacutainers.
2. Decant blood into a universal and dilute with an equal volume of PBS, mix well.

3. Overlay two parts diluted blood onto 1 part Histopaque (14 mL blood:7 mL Histopaque).
4. Centrifuge at 400*g* for 25 min at room temperature.
5. Harvest the MNC from the interface between the plasma and centrifugation media.
6. Wash twice in cold PBS at 600*g* for 6 min at 4°C.
7. Resuspend cells in cold PBS and count using 1% trypan blue.
8. Aliquot cells at 1×10^6, 5×10^6, and 1×10^7 cells.

3.2. Flow Cytometric Analysis

1. Aliquot MNC at 1×10^6 cells/tube, pellet by centrifugation at 600*g* for 6 min at 4C.
2. Blot tubes to remove excess PBS, resuspend the remaining pellet.
3. Add 10 mL of MoAb, incubate at 4°C for 20–30 min.
4. Wash twice in 1 mL PBS 1% BSA at 600*g* for 6 min at 4°C.
5. Blot the tubes to remove excess PBS 1% BSA, resuspend the remaining pellet.
6. Add 100 μL of FITC-Fab_2 (1/100 dilution in PBS 1% BSA) per tube.
7. Incubate at 4°C for 20–30 min.
8. Wash twice in 1 mL of PBS 1% BSA at 600*g* for 6 min at 4°C.
9. Blot the tubes and resuspend the cells in 300 μL of PBS 1% BSA. Store in the dark at 4°C until acquired on the flow cytometer.
10. If the samples are to be stored for longer than 6 h, fix with PBS 1% paraformaldehyde, and store at 4°C in the dark.

3.3. RNA Extraction

1. Add 1 mL of RNA Stat-60, per 5–10 × 10^6 pelleted MNC or for up to 100 mg of minced tumor tissue, in a 1.5-mL microcentrifuge tube. Lyse by passing several times through a pipet (**Note 8**).
2. Leave at room temperature for 5 min.
3. Add 200 μL of chloroform, cover, and shake vigorously for 15 s.
4. Leave at room temperature for a further 3 min.
5. Centrifuge at 12,000*g* (max.) for 15 min at 4°C.
6. Carefully remove the top, colorless, aqueous phase (approx 60% of the total volume) and place in a microcentrifuge tube.
7. Add 0.5 mL isopropanol to the aqueous phase. Incubate at room temperature for 5–10 min.
8. Centrifuge at 12,000*g* for 10 min at 4°C.
9. Discard the aqueous phase, the RNA precipitate forms a white/opaque pellet.
10. To wash the RNA pellet add 1 mL of 75% ethanol and vortex.
11. Centrifuge at 7500*g* for 5 min at 4°C.
12. Carefully remove the supernatant and allow the pellet to air-dry (**Note 9**).
13. Dissolve the RNA in 20 μL of 1 m*M* EDTA. Dissolving may require vortexing or an incubation at 55–60°C for 10–15 min.
14. Determine the RNA content and extract purity by measuring the optical density ratio at A_{260}/A_{280} with a UV-vis spectrophotometer.
15. A ratio of ≥1.8 is considered to be a pure RNA sample free of contaminating protein and/or DNA.

3.4. cDNA Synthesis

1. Master mix (50 μL/reaction): 10 μL 5X buffer, 1 m*M* DDT, 0.2 m*M* of each dNTP, 10 μg of random primer $pd(N)_6$, 400 U M-MLV, DEPC-treated water to a final volume of 30 μL. Prepare one extra aliquot of master mix to ensure that there is sufficient for all the samples (**Note 10**).
2. Prepare the RNA at 1–5 μg in 20 μL DEPC-treated water (**Note 11**).
3. Denature the RNA at 65°C for 10 min. Place on ice.
4. Add 30 μL of the master mix to each microcentrifuge tube, and to this add the 20 μL prepared RNA, vortex. One prostate tissue RNA, positive control, and at least one DEPC-treated water, negative control, should be included.
5. Place 100 μL of mineral oil into each microcentrifuge tube, cover.
6. Incubate at 37°C for 1 h in a water bath.
7. Samples may be used immediately or stored at –20°C until use.

3.5. PCR Amplification

1. Master mix (50 μL/reaction): 5 μL 10X buffer, 2.5 m*M* $MgCl_2$, 0.2 m*M* of each dNTP, 100 pmol of each oligonucleotide primer, 1 U of *Taq* polymerase, DEPC-treated water to a final volume of 45 μL. Prepare one extra aliquot of master mix to ensure that there is sufficient for all samples.
2. Aliquot 45 μL of master mix into each 0.5-mL microcentrifuge tube.
3. Add 100 μL of mineral oil.
4. Place 5 μL of cDNA into the cap of the microcentrifuge tube. Cover immediately. At least two DEPC-treated water negative controls should be included, the first and last samples (**Note 12**).
5. Pulse at 12,000*g* to mix the reagents.
6. Load the samples onto the thermal cycler block (**Note 13**).
7. Samples are amplified according to the following program: denaturation: 2 min at 95°C; 35 cycles: 30 s at 95°C; 30 s at 55°C; and 1.5 min at 72°C, increasing by 6 s per cycle; extension: 15 min at 72°C; and hold: 4°C.
8. Store the PCR products at –20°C until analyzed.

3.6. DIG-Labeling of Probe

1. Denature DNA by heating at 100°C for 10 min and chilling rapidly by placing on ice.
2. Prepare the reaction mix in a microcentrifuge tube: 2 mL hexanucleotide mixture, 2 μL dNTP, 1 μL Klenow enzyme.
3. Add 1 mg of oligonucleotide probe to the reaction mix and make up to 20 μL with water, mix well.
4. Incubate for 3 h at 37°C in a water bath (**Note 14**).
5. Add 2 μL of the EDTA solution to stop the reaction.
6. Add 2.5 μL of LiCl and 75 μL of cold ethanol (stored at –20°C) to precipitate the labeled DNA, mix well.
7. Leave for 30 min at –70°C or for 2 h at –20°C.
8. Centrifuge at 12,000*g* for 10 min.
9. Wash the pellet with 50 μL of cold 70% ethanol.

10. Dry under vacuum, and dissolve in 50 μL TE buffer.
11. Store at –20°C.

3.7. Agarose Gel Electrophoresis

1. Add 100 μL of chloroform and 13 μL of gel loading dye to the PCR product.
2. Centrifuge at 7000*g* for 1 min. The PCR product will be in the top fraction.
3. Prepare 1.5% agarose gel containing 1X TAE buffer. Dissolve the agarose.
4. Once cool pour the agarose carefully into a prepared gel box with a comb inserted. Remove any bubbles (**Note 15**).
5. When the gel has solidified remove the comb and cover the well with 1X TAE buffer.
6. Carefully load each well with the molecular weight markers or the PCR product (**Note 16**).
7. Perform the electrophoresis at 100 V for 3 h. Check to ensure that the product has not run off the gel.
8. Stain the gel with 0.5 μg/mL ethidium bromide in 1X TAE for 30 min.
9. Examine the gel under UV, and photograph the gel with a Polaroid camera.

3.8. Southern Blot

1 Wash gel twice for 10 min in 0.4 *M* sodium hydroxide.
2 Blot overnight onto positively charged nylon membrane.
3. Wash membrane twice for 10 min in 2X TSB.
4. Air-dry and bake for 45 min at 95°C.
5. Prepare prehybridization solution: 4X TSB-0.1% Tween-20, 1% blocking reagent (from kit), 100 μg/mL salmon sperm DNA.
6. Prehybridize the blot for at least 2 h at 55°C.
7. Add the DIG-labeled probe at a final concentration of 2 ng/mL.
8. Perform the hybridization for 16 h at 55°C.
9. Wash the blot in decreasing TSB concentrations: 4X TSB, 2X TSB, 0.5X TSB, 0.1X TSB for 15 min at room temperature.
10. Wash in blocking solution: 1X TBS-0.1% Tween-20, 1% blocking reagent (from kit) for 30 min at room temperature
11. Add 3.75 U alkaline phosphatase-conjugated sheep anti-DIG-labeled IgG Fab fragment (from kit).
12. Incubate for 30 min at room temperature.
13. Wash twice, to remove unbound antibody, in 1X TBS for 15 min at room temperature.
14. Equilibrate for 5 min in substrate buffer.
15. Soak the membrane in substrate buffer containing 0.1 mg/mL Lumigen PPD (from Kit).
16. Place the membrane between two acetate sheets and expose to preflashed film for 10–30 min at 37°C.

4. Notes

1. Any flow cytometer may be used for the acquiring and analysis of the stained cells. Cells should be stained and processed in the tubes recommended for use with the flow cytometer. At least 10,000 cells should be analyzed.

2. The recommended amount of antibody required per test is usually given on the product data sheet provided. However, it is recommended that all the antibodies are titrated prior to use. If directly conjugated antibodies are used, only the first step of the staining protocol needs to be followed, after washing and resuspension in PBS 1% BSA the cells may be analyzed.
3. A variety of protocols for the extraction of RNA and kits are available. The best technique may depend on the tissue being studied. The reagent outlined gave good, pure yields of RNA with both the MNC and tumor tissue.
4. Many different cDNA synthesis and PCR amplification kits are available commercially, any kit may be used. Components may be purchased from different suppliers but it is important to use enzymes with buffers supplied by the same manufacturer as these may not be interchangeable.
5. The sensitivity of the PCR should be determined by spiking MNC preparations with single cell preparations of prostate tissue or cell lines (e.g., LnCaP) and then extracting the RNA as outlined. Suggested ranges are 1 tumor cell in 10 MNC, $1{:}10^2$, $1{:}10^3$, $1{:}10^4$, $1{:}10^5$, and $1{:}10^6$.
6. A variety of primer sequences specific to PSA may be used for the amplification of cDNA. These may be devised using the commercially available computer packages, where the melting temperature (T_m), suitability and specificity of the sequences are generated automatically. Otherwise the gene sequences may be accessed via data bases, such as Daresbury (UK), using the Internet. Registration for such data bases is required and is free.
7. The DIG DNA labeling kit is a nonradioactive labeling and detection system that is both faster and safer than the radioactive system. DIG-labeled DNA is stable for at least 1 yr if stored in TE buffer or hybridization solution at –20°C.
8. RNase contamination must be avoided, change gloves frequently, use RNase-free sterile plastic ware and RNase-free tips throughout the preparation and handling of the RNA.
9. The pellet may also be washed in a vacuum (5–10 min). It is important not to dry the pellet completely as the solubility of the RNA will be reduced. RNA guard (Boehringer) may also be added to the RNA preparation to protect the sample.
10. In order to avoid crosscontamination different sets of pipeters, positive displacement pipets and aerosol-resistant tips should be designated for preparation of the cDNA and PCR reagents. RNA and DNA samples should be handled in separate areas, with designated pipeters. In order to reduce crosscontamination, gloves should be changed frequently, tips should be changed between each sample, microcentrifuge tubes containing the cDNA should be opened prior to addition to the reaction mix, and the reaction mix tubes should be covered immediately after the addition of cDNA.
11. The amount of RNA required should be titrated for each study to ensure that sufficient RNA is added for the generation of detectable PCR products.
12. Experiments with more than 10 samples should have additional negative controls distributed between the samples under test, e.g., every 5 samples. These samples are a reference for crosscontamination.
13. In this study we used a Perkin-Elmer thermal cycler block. To optimize the system, in order to obtain a single band of the correct size, several parameters may

need to be titrated. The annealing temperatures should be titrated first, starting with the lowest T_m of the primers, next the amount of primer and lastly $MgCl_2$. The optimal number of cycles may also be determined by plotting log signal vs number of cycles, this should give a sigmoidal curve, use the midpoint as the starting number.

14. Labeling may be carried out for 1 h minimum up to 20 h. Extended labeling times increase the yield of labeled DNA.
15. Biorad mini gels require 50 mL of 1.5% agarose and the miniwide gels require 125 mL of 1.5% agarose. The gel should be cast on a level casting stand and will solidify within 30 min.
16. It is suggested that the molecular weight markers are loaded in the first well, followed by the controls, then PCR products. For miniwide gels it is recommended that molecular weight markers are also loaded in the last well for easier comparison.

References

1. Fleming, C., Wasson, J. H., Albertsen, P. C., Barry, M. J., and Wennberg, J. E. (1993) A decision analysis of alternative treatment strategies for clinically localised prostate cancer. Prostate Patient Outcomes Research Team. *JAMA* **269,** 2650–2658.
2. Lu-Yao, G. L., Mclerran, D., Wasson, J., and Wennberg, J. E. (1993) An assessment of Radical Prostatectomy. Time trends, geographic variation and outcomes. The Prostate Patient Outcomes Research Team. *JAMA* **269,** 2633–2636.
3. Chodak, G. W., Thisted, R. A., Gerber, G. S., Johansson, J. E., Adolfsson, J., Jones, G. W., Chisholm, G. D., Moskovitz, B., Livne, P. M., and Warner, J. (1994) Outcome following conservative management of patients with clinically localized prostate cancer. *N. Engl. J. Med.* **330,** 242–248.
4. Epstein, J. I., Carmichael, M. J., Pizov, G., and Walsh, P. C. (1993) Influence of capsular penetration on progression following radical prostatectomy: a study of 196 cases with long-term follow-up. *J. Urol.* **150,** 135–141.
5. Poste, G. and Fidler, I. J. (1980) The pathogenesis of cancer metastasis. *Nature* **283,** 139–146.
6. Fidler, I. J. (1970) Metastasis: quantitative analysis of distribution and fate of tumour emboli labelled with ^{125}I-5-iodo-2'-deoxyuridine. *J. Natl. Cancer Inst.* **45,** 773–782.
7. Fidler, I. J. and Kripke, M. L. (1977) Metastasis results from pre-existing variant cells within a malignant tumour. *Science* **197,** 893–895.
8. Warren, S. and Gates, O. (1936) Fate of intravenously injected tumour cells. *Am. J. Cancer* **27,** 485–492.
9. Engell, H. C. (1955) Cancer cells in the circulating blood. Clinical study on occurrence of cancer cells in peripheral blood and in venous blood draining tumour area at operation. *Acta Chir. Scand., Suppl.* **201,** 1–70.
10. Schwartz, R., Walk, A., Toomes, H., and Schirrmacher, V. (1985) Assay for the determination of human carcinoma cells in circulating blood. *J. Cancer Res. Clin. Oncol.* **109,** 122–129.

11. Wang, M. C., Valenzuela, L. A., Murphy, G. P., and Chu, T. M. (1979) Purification of a human prostate specific antigen. *Invest. Urol.* **17,** 159–163.
12. Hamdy, F. C., Lawry, J., Anderson, J. B., Parsons, M. A., Rees, R. C., and Williams, J. L. (1992) Circulating prostate specific antigen-positive cells correlate with metastatic prostate cancer. *Br. J. Urol.* **69,** 392–196.
13. Fadlon, E. J., Rees, R. C., McIntyre, C., Sharrard, R. M., Lawry, J., and Hamdy, F. C. (1996) Detection of circulating prostate-specific antigen-positive cells in patients with prostate cancer by flow cytometry and reverse transcription polymerase chain reaction. *Br. J. Cancer* **74,** 400–405.
14. Moreno, J. G., Croce, C. M., Fischer, R., Monne, M., Vihko, P., Mulholland, S. G., and Gomella, L. G. (1992) Detection of hematogenous micrometastasis in patients with prostate cancer. *Cancer Res.* **52,** 6110–6112.
15. Katz, A. E., Olsson, C. A., Raffo, A. J., Cama, C., Perlman, H., Seaman, E., O'Toole, K. M., McMahon, D., Benson, M. C., and Buttyan, R. (1994) Molecular staging of prostate cancer with the use of an enhanced reverse transcriptase-PCR assay. *Urology* **43,** 765–774.
16. Israeli, R. S., Miller, W. H., Su, S. L., Powell, T., Fair, W. R., Samadi, D. S., Huryk, R. F., Deblasio, A., Edwards, E. T., Wise, G. J., and Heston, W. D. W. (1994) Sensitive nested reverse transcription polymerase chain reaction detection of circulating prostatic tumour cells: comparison of prostate-specific membrane antigen and prostate-specific antigen-based assays. *Cancer Res.* **54,** 6306–6310.
17. Israeli, R. S., Miller, W. H., Su, S. L., Samadi, D. S., Powell, C. T., Heston, W. D. W., Wise, G. J., and Fair, W. R. (1995) Sensitive detection of prostatic haematogenous tumor cell dissemination using prostate specific antigen and prostate specific membrane-derived primers in the polymerase chain reaction. *J. Urol.* **153,** 573–577.
18. Cama, C., Olsson, C. A., Raffo, A. J., Perlman, H., Buttyan, R., O'Toole, K., McMahon, D., Benson, M. C., and Katz, A. E. (1995) Molecular staging of prostate cancer. II. A comparison of the application of an enhanced reverse transcriptase polymerase chain reaction assay for prostate specific antigen versus prostate specific membrane antigen. *J. Urol.* **153,** 1373–1378.
19. Smith, M. R., Biggar, S., and Hussain, M. (1995) Prostate-specific antigen messenger RNA is expressed in non-prostate cells: implications for detection of micrometastases. *Cancer Res.* **55,** 2640–2644.
20. Brandt, B., Junker, R., Griwatz, C., Heidl, S., Brinkmann, O., Semjonow, A., Assman, G., and Zänker, K. (1996) Isolation of prostate-derived single cells and cell clusters from human peripheral blood. *Cancer Res.* **56,** 4556–4561.

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Detection of *Chlamydia trachomatis* and *Trichomonas vaginalis* in the Vaginal Introitus, Posterior Vagina, and Endocervix by Polymerase Chain Reaction

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1. Introduction

Chlamydia trachomatis, an obligate intracellular bacterium, and *Trichomonas vaginalis*, a protozoan, are two of the most prevalent sexually transmitted pathogenic microorganisms worldwide. *C. trachomatis* is a major cause of pelvic inflammatory disease, occluded fallopian tubes resulting in infertility, and ectopic pregnancy ***(1)*** whereas *T. vaginalis* frequently induces vaginitis, cervicitis, and urethritis ***(2)***. Both organisms are also associated with adverse pregnancy outcomes ***(3)***.

Infections of the female genital tract by *C. trachomatis* and *T. vaginalis* are often asymptomatic. Therefore, it is difficult to make a specific diagnosis based on symptoms or clinical findings. In addition, detection of either infection by various methods is of low sensitivity or specificity or requires time consuming cultures. The most prevalent method to diagnose a *T. vaginalis* infection is to place a drop of posterior vaginal or cervical secretion on a slide and to search under the microscope for motile trichomonads. This technique has a markedly low sensitivity in most clinical situations ***(4)***. The alternative, to inoculate culture medium with the sample and observe for *T. vaginalis* growth, requires a 7 d incubation period before one can conclude that the organism is not present ***(5)***.

An endocervical sample is the most frequent site tested for *C. trachomatis*. Since the organism is an obligate intracellular microbe the samples must be inoculated onto monolayers of in vitro grown cell lines. The presence in the inoculum of inhibitors of mammalian cell growth can therefore lead to false negative results ***(6)***. For low concentrations of *Chlamydia*, multiple passages may be

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

necessary before the organism is identified. Methods are also available for the direct detection of *C. trachomatis* from cervical samples. Antigen detection methods utilize a monoclonal antibody to a chlamydial surface component while DNA detection utilizes a probe that hybridizes with chlamydial DNA. Both methods are less sensitive than culture and can yield false positive results resulting from crossreactivities with other microorganisms ***(6)***.

The polymerase chain reaction (PCR) has been utilized to detect *C. trachomatis* ***(7)*** and *T. vaginalis* ***(8)*** in female lower genital tract samples ***(9,10)***. A *C. trachomatis* PCR assay (Amplicor, Roche Molecular Systems, Branchburg, NJ) has been approved by the United States Food and Drug Administration and is commercially available ***(7)***. PCR assays for both organisms offer increased sensitivity over previous assays coupled with rapid detection of the organism in 1 d. An alternative DNA amplification assay, the ligase chain reaction, has also been introduced for *C. trachomatis* detection ***(11)***.

Screening women for *C. trachomatis* and *T. vaginalis* typically requires the insertion of a speculum into the vagina for collection of samples. This may be unavailable or unacceptable to some women for a variety of reasons. Identification of an alternate means of sample collection, or the ability of the woman to collect her own sample in privacy for detection of both *T. vaginalis* and *C. trachomatis* infections, would increase the numbers of women who could be tested for these pathogens.

The entrance to the vagina (the introitus) has recently been identified as an alternative site for detection of both *C. trachomatis* and *T. vaginalis* infections ***(12,13)***. When utilized in conjunction with PCR, introital sampling was shown to be as sensitive as internally collected samples for the identification of both organisms. Other investigators have similarly shown that first morning urine samples is another external sample that can be analyzed for these microorganisms with high specificity ***(14)***. However, because of the need for more extensive processing of urine samples as compared to introital samples, and the decrease in sensitivity of urine sampling if processing is delayed, introital sampling may be preferable.

In the Department of Obstetrics and Gynecology at the New York Hospital-Cornell Medical Center, introital, vaginal, and endocervical swabs are collected. For introital testing, a sterile Dacron swab is placed just within the vaginal opening, twirled and withdrawn. A vaginal sample is obtained by inserting a Dacron swab 4–6 cm into the posterior vaginal fornix and rotating the swab to obtain cellular material. An endocervical specimen is obtained after inserting a sterile speculum to view the cervix. After wiping away any mucopus, the Dacron swab is inserted 2–3 cm into the endocervix and rotated. Each swab is placed directly into an individual Amplicor specimen tubes for detection of both *T. vaginalis* and *C. trachomatis* (*see* **Subheading 2.1.1., item**

3). A single sample can thus be tested for both organisms. For *T. vaginalis* we utilize oligonucleotide primer pairs coding for a region of the ferredoxin gene *(8)*. Specificity is verified by subjecting the PCR product to endonuclease treatment and sizing of the final fragments on polyacrylamide gels. For *C. trachomatis*, the primer pairs utilized code for a segment of the chlamydial cryptic plasmid. Specificity is verified by hybridization of the PCR product with an internal probe; detection of the hybridized pair is by ELISA.

2. Materials

2.1. Chlamydia trachomatis

2.1.1. Sample Processing and DNA Extraction

1. Biological cabinet: Area #2 (*see* **Note 5**).
2. Powder-free gloves.
3. Amplicor specimen tubes (Roche Diagnostic Systems, Branchburg, NJ): Tris-HCl solution containing <1% solubilizer.
4. Amplicor control diluent (Roche Diagnostic Systems): 6 m*M* $MgCl_2$, >10% detergent, and 0.05% sodium azide in a Tris-HCl solution.
5. Amplicor specimen diluent (Roche Diagnostic Systems): 6 m*M* $MgCl_2$, >10% detergent, and 0.05% sodium azide in a Tris-HCl solution.

2.1.2. PCR Amplification

1. Dead air box with UV light: Area #1 (*see* **Note 5**).
2. Biological cabinet: Area #2 (*see* **Note 5**).
3. 0.2 mL MicroAmp tubes.
4. MicroAmp tray.
5. Plastic ziplock bags.
6. AmpErase (Roche Diagnostic Systems): <1% EDTA, dithiotheitol, NaCl, 0.01% uracil *N*-glycosylase, 5% glycerol, and 0.5% detergent in Tris-HCl solution.
7. Master mix solution (Roche Diagnostic Systems): 20% glycerol, <0.001% deoxynucleoside triphosphates dATP, dGTP, dCTP, and dUTP, biotinylated primers, <0.01% Ampli*Taq* (*Taq* polymerase), <1% EDTA, KCl, and 0.05% sodium azide in a Tris-HCl solution.
8. Working master mix: Prepare the working master mix solution by adding 100 µL of AmpErase to the 1.7 mL vial of master mix reagent. Mix by inverting 10–15 times.
9. Positive control: noninfectious *C. trachomatis* template DNA.
10. Negative control: nonspecific DNA.

2.1.3. Detection of PCR Product by ELISA

1. Denaturation solution: 1.6% NaOH and thymol blue diluted in an EDTA solution.
2. Hybridization solution: <0.2% solubilizer and <25% chaotrope in a sodium phosphate solution.
3. A microwell plate coated with *C. trachomatis* DNA probe.

4. Wash buffer concentrate: a sodium phosphate and sodium salt solution containing EDTA, 2% detergent, and 0.5% Proclin 300 (as a preservative).
5. Working wash buffer solution: Dilute 10X concentrated wash buffer to 1X with sterile deionized water.
6. Avidin-HRP conjugate: Avidin-horseradish peroxidase conjugate in a Tris-HCl solution containing 0.1% Proclin 150 emulsifier, bovine γ-globulin, and 0.1% phenol.
7. Substrate solution A: 0.01% H_2O_2 and 0.1% Proclin 100 in a citrate solution.
8. Substrate solution B: 0.1% 3, 3', 5, 5'-tetramethylbenzadine in 40% dimethylformamide.
9. Working substrate solution: prepared by combining four parts substrate A (Chromogen) to one part substrate B (Substrate).
10. Stop reagent: 4.9% sulfuric acid.

2.2. Trichomonas vaginalis

2.2.1. Sample Preparation

1. Amplicor specimen tubes (Roche Diagnostic Systems).
2. Amplicor specimen diluent (Roche Diagnostic Systems).
3. Sterile 1.5 mL and 0.5 mL microcentrifuge tubes.
4. Sterile phosphate-buffered saline (PBS).
5. Sterile $5^3/_4$ in. glass Pasteur pipets.
6. Mineral oil.
7. Nonionic detergent buffer: 50 m*M* KCl, 10 m*M* Tris-HCl, pH 8.3, 2.5 m*M* $MgCl_2$, 1% Brij 35, diluted in sterile deionized water.
8. Proteinase K: Prepare a 5 mg/mL solution in sterile deionized water.

2.2.2. PCR Amplification

1. Sterile deionized water.
2. 10X PCR buffer: 100 m*M* Tris-HCl, 15 m*M* $MgCl_2$, 500 m*M* KCl, and 0.01% gelatin (Perkin Elmer-Roche Molecular Systems)
3. 10 m*M* stocks of deoxynucleoside triphosphates dATP, dTTP, dCTP, and dGTP (Perkin Elmer).
4. Working stock that contains 1.25 m*M* of each dNTP. Prepare by adding 62.5 μL each 10 m*M* dNTP stock to 250 μL sterile deionized water.
5. Oligonucleotide primers diluted in sterile deionized water to 10 μ*M*.
6. Sterile 0.5 μL microcentrifuge tubes.
7. *Taq* DNA Polymerase 5 U/μL.
8. Positive control DNA template diluted 1:10 in sterile deionized water.

2.2.3. Restriction Enzyme Analysis

1. 10X restriction enzyme buffer: 500 m*M* NaCl, 100 m*M* Tris-HCl, 100 m*M* $MgCl_2$, and 100 m*M* DTT (New England Biolabs, Beverly, MA).
2. *Hin*fI restriction endonuclease 10 U/μL (New England Biolabs).

2.2.4. Acrylamide Gel Electrophoresis

1. Acrylamide/Bis 19:1 (Bio-Rad, Hercules, CA).
2. Acrylamide/Bis diluted in sterile deionized water to 40% (store at 4°C for 3 mo).
3. Ethidium bromide 10 mg/mL in sterile deionized water.
4. 1X and 5X TBE buffer: Prepare a 10X TBE solution by adding 242 g Tris base, 123.5 g boric acid, and 14.9 g EDTA to 2 L of sterile deionized water (pH 8.0).
5. 1X TE buffer: Prepare a 1X TE solution by adding 5 mL 1 *M* Tris-HCl (pH 8.0), 1 mL 0.5*M* EDTA (pH 8.0), and 494 mL sterile deionized water.
6. Gel loading dye: 0.25% bromophenol blue, 0.25% xylene cylanole FF, 25% glycerol, diluted in sterile deionized water.
7. 25% Ammonium Persulfate: 0.25 g ammonium persulfate diluted in 1 mL sterile deionized water (store at room temperature for no more than 1 mo).
8. TEMED (*N,N,N′,N′*-tetramethylethylenediamine).
9. DNA markers. Numerous commercially available DNA marker preparations that contain fragments ranging from 100–1000 bp may be used. We currently use ϕX174 RF DNA *Hae*III digest (New England BioLabs) which contains 11 fragments ranging from 72 to 1353 bp.

3. Methods

3.1. Chlamydia trachomatis

3.1.1. Sample Processing and DNA Extraction

3.1.1.1. Introitus and Endocervical Swabs

1. Pipet 1 mL specimen diluent into each Amplicor specimen tube (Area #2, **Note 5**).
2. Recap the tube and vortex each sample for 10–15 s.
3. Incubate the samples at room temperature for 10 min.

3.1.1.2. Control Preparation

1. Prepare the positive and negative control by adding 750 µL control diluent to each vial (Area #2, **Note 5**).
2. Recap the vial and vortex the diluted control for at least 10 s.
3. Incubate for 10 min at room temperature.

3.1.2. PCR Amplification

1. Determine the number of samples and controls to be tested. Every PCR assay should include at least one positive and three negative controls.
2. Fill out a tray map for control and sample identification.
3. Assemble the appropriate number of MicroAmp tubes in a rack and secure with the retainer (Area #2, **Note 5**).
4. Pipet 50 µL of working master mix into each MicroAmp tube.
5. Place MicroAmp tray into a plastic ziplock bag and move to Area #2.
6. Aliquot 50 µL of diluted controls into the appropriate MicroAmp tubes.
7. Pipet 50 µL of processed sample into the appropriate MicroAmp tube.

8. Cap the tubes tightly with the capping tool and carry the tray to Area #3.
9. Remove the MicroAmp tray from the base and place the tray into the thermal cycler block.
10. Amplify the samples according to the following cycling program. 1 cycle of: 95°C 5 min; 60°C 1 min; 29 cycles of: 95°C 30 s; 60°C 1 min; hold program: 72°C 5 min; hold program: 72°C indefinitely.

3.1.3. Detection of PCR Product by ELISA

1. Remove the sample tray and place it in the holding base.
2. Carefully remove the caps and immediately add 100 µL denaturation solution (blue) to each tube using a multichannel pipet.
3. Incubate at room temp for 10 min.
4. Remove the appropriate number of microwell strips from the foil pouch and insert the strips into the frame.
5. Add 100 µL hybridization solution into each empty microtiter plate well using a multichannel pipet.
6. Transfer 25 µL of denatured amplicon from the tubes to the wells using a multichannel pipet (*see* **Note 11**).
7. Tap the plate until the color changes from blue to yellow.
8. Cover the plate and incubate for 1 h at 37°C.
9. Using the 1X working wash buffer wash the plate five times.
10. Tap the plate dry and add 100 µL of Avidin-HRP conjugate to each well using a multichannel pipet.
11. Cover the plate and incubate at 37°C for 15 min.
12. Prepare the working substrate solution.
13. Remove the plate from the incubator and repeat the wash step as in **step 9**.
14. Pipet 100 µL of the working substrate into each well using a multichannel pipet.
15. Incubate for 10 min at room temperature in the dark.
16. Add 100 µL of stop reagent to each well using a multichannel pipet.
17. Place the plate in a photometer and read the absorbance at 450 nm within 1 h.
18. Analyze the results (*see* **Note 15–18**).

3.2. Trichomonas vaginalis

3.2.1. Sample Collection and DNA Extraction

1. Vaginal and introital samples are received in Amplicor specimen collection tubes.
2. In a clean, sterile biological cabinet pipet 1 mL of Amplicor specimen diluent into each tube.
3. Recap the tube and vortex each sample for 10–15 s.
4. Incubate the samples at room temperature for 10 min.

3.2.2. Preparation of Positive Control

1. *Trichomonas vaginalis* may be propagated easily and may be obtained from a clinical microbiology laboratory.

2. Add two drops of mineral oil and 10 μL of proteinase K to a 0.5 mL sterile microcentrifuge tube. Cap this tube and set it aside.
3. Use a sterile glass Pasteur pipet to transfer the *T. vaginalis* into a 1.5 mL microcentrifuge tube and centrifuge the sample at 6500*g* for 5 min.
4. Remove all the culture media and wash the pellet three times with sterile 1X PBS (5 min at 6500*g*).
5. Remove all the PBS from the *T. vaginalis* pellet.
6. Resuspend the pellet in 120 μL of nonionic detergent buffer and transfer it to the microcentrifuge tube that contains the proteinase K.
7. Vortex and centrifuge sample briefly.
8. Place the sample in the thermal cycler for 56°C for 60 min, followed by 95°C for 10 min. Hold at 4°C.
9. Dilute the positive control 1:10 and freeze at –80°C in 5 μL volumes.

3.2.3. PCR Amplification

1. Aliquot 1.5 mL sterile autoclaved filtered deionized water into a 1.5 mL microcentrifuge tube.
2. Each patient sample, and the positive and negative controls are tested in duplicate. Assemble and label the appropriate number of 0.5 mL microcentrifuge tubes.
3. Add one drop of sterile mineral oil to every tube.
4. Add 10 μL sterile deionized water to all sample tubes, 24 μL sterile deionized water to the positive control tube, and 25 μL sterile deionized water to the negative control tube.
5. Prepare a master mix of reagents containing, per PCR reaction, 7.9 μL sterile deionized water, 5.0 μL 10X buffer, 8.0 μL of 1.25 m*M* dNTP working solution, and 2.0 μL of each oligonucleotide primer. Calculate the number of PCR reactions needed and make enough reaction mixture for two additional samples to ensure there is enough master mix for all the samples. Store the master mix on ice.
6. Add 15 μL of sample to the appropriate tubes.
7. Add 1 μL of positive control to the appropriate tube.
8. Cap the tubes, vortex each sample, and centrifuge the samples briefly.
9. Denature each DNA sample in a thermal cycler at 94°C for 7 min; immediately place the samples on ice.
10. 0.1 μL DNA *Taq* polymerase per PCR reaction is needed. Calculate the number of samples plus two and multiply this number by 0.1 add this amount of *Taq* to the master mix tube.
11. Add 25 μL master mix to each sample tube, starting with the negative control and working backwards to the positive control.
12. Cap the samples, vortex and centrifuge the samples briefly.
13. Load the samples into the thermal cycler block.
14. Amplify the samples according to the following cycling program: 40 cycles of 94°C for 1 min; 47°C for 1 min; 67°C for 1 min, followed by an extension cycle: 67°C for 7 min. Hold at 4°C until adding enzyme (up to 12 h).

3.2.4. Restriction Enzyme Analysis

1. Prepare a master mix of enzyme reagents containing, per PCR reaction, 2.0 µL *Hin*fI, and 5.5 µL 10X restriction enzyme buffer. Calculate the number of PCR reactions needed and make enough reaction mixture for two additional samples to ensure there is enough for all samples. Store master mix on ice.
2. Remove the samples from the thermal cycler and place them on ice.
3. Add 7.5 µL enzyme mix to one of the duplicate tubes for each sample, positive and negative controls.
4. Incubate all the samples (± enzyme) in a 37°C water bath for 4 h to overnight.

3.2.5. Acrylamide Gel

1. Prepare a 6% acrylamide gel (*see* **Note 27**).
2. Label a 0.5 mL microcentrifuge tube standard and add 18 µL 1X TE.
3. Add 4 µL loading buffer to the standard tube and 15 µL loading buffer to all the sample tubes.
4. Add 0.3 µL *Hae*III digest to the standard tube.
5. Remove the well comb from the gel and wash the wells two times with 1X TBE.
6. Fill the wells with 1X TBE and using a pipetman remove 40 µL of TBE.
7. Add 20 µL of sample to the appropriate well.
8. Remove the gel unit from the base and attach it to the buffer chamber.
9. Fill the buffer chamber with 1X TBE.
10. Run the gel at a voltage of 300 V, 100 mA for approx 60 min.
11. Place 1 L of tap water in a basin and add 30 µL ethidium bromide (*see* **Note 30**).
12. Place the gel in the ethidium bromide solution for 15 min.
13. Photograph the gel on a UV transilluminator with a Polaroid camera through a red filter onto Polaroid type 57 film.
14. Examine the control lanes to confirm that the positive and negative control samples worked. If the controls worked properly, inspect the sample lanes for DNA fragments of the correct size.

4. Notes

4.1. Chlamydia trachomatis

1. Before all procedures the biological cabinet should be decontaminated by cleaning all surfaces with a 10% bleach solution and a 70% ethanol solution.
2. Store the specimens separately from the reagents.
3. A dry temperature stable 37°C incubator is preferred over a wet bath.
4. A set of pipets should be dedicated exclusively for specimen preparation and should not be used in the PCR reagent preparation.
5. The procedure is divided into three components and each should be performed in a different laboratory area. Dead air box with UV light, Area #1. Biological cabinet, Area #2. Amplification and detection area, Area #3.
6. Always work in a one way direction (Area #1 to Area #2 to Area #3)
7. Area #1 should be far from Area #3, if possible try to have these areas in separate rooms.

8. Always wear powder-free gloves.
9. Allow all the reagents to reach room temperature.
10. Prepare the substrate immediately before it is needed and store away from light.
11. When transferring the denatured amplicon from the tubes to the wells, using a multichannel pipet, making sure to change the tips between rows.
12. We use a GeneAmp PCR system 9600 (Perkin Elmer, Roche Molecular Systems).
12. After each PCR run review the thermal cycler record on Run Quality Control Log.
13. Amplicor will not detect rare plasmid-free variants of *C. trachomatis*.
14. The presence of PCR inhibitors may cause false-negative results. The presence of spermicides in excess of 1% or surgical lubricants in excess of 10% may have an inhibitory effect on this procedure. The presence of blood or mucus in cervical samples has not been demonstrated to have any direct biochemical effect on this PCR procedure. Powder from gloves may produce false-negative results.
15. All clinical samples with an absorbance <0.2 are negative, an absorbance >0.5 are positive, and an absorbance between 0.2 and 0.5 are to be repeated.
16. Repeat the questionable samples in duplicate, within 4 d of adding the specimen diluent. The sample is positive if 2 out of 3 assays (original plus duplicates) result in an absorbance >0.25. If 2 out of 3 assays (original plus duplicates) result in an absorbance <0.25 the sample is negative.
17. The values for the negative control should fall within 25% of the mean value for negative controls. If one or more negative control value(s) is >25% of the mean value, the entire procedure, including amplification should be repeated.
18. The positive control values should have an absorbance >2.0. If the value falls below 2.0 the entire run, including amplification should be repeated.
19. A sample processing control should be run once a month to test the Amplicor procedure. A 10^3–10^4 concentration of HeLa cells infected with *C. trachomatis* should be added to a fresh sample tube with standard transport medium and processed as a normal clinical specimen. A positive signal above an absorbance of 0.25 should be obtained if the sample is processed properly.

4.2. Trichomonas vaginalis

20. To remove any potential DNA contamination all racks are soaked for at least 1 h in 10% bleach.
21. Filter pipet tips are used in all PCR reagent preparations and amplification procedures.
22. Change pipet tips between the addition of each sample and between the adding of the PCR and enzyme master mixes to each tube.
23. Be extremely careful in washing and working with the positive control. Contamination with aerosolized DNA can be a horrific problem and can be very difficult to remedy.
24. Add the mineral oil to the PCR reaction tubes and prepare the PCR master mix in a clean area or sterile hood to limit contamination problems.
25. When adding the restriction enzyme make sure that you go beneath the oil layer and use the tip to mix the enzyme with the PCR reaction.
26. Clean all parts of the gel aparatus with water and 70% ethanol and dry very well.

27. For acrylamide gels add in the following order 15.8 mL sterile deionized water, 4.2 mL 40% bis-acrylamide, 5 mL 5X TBE, 100 μL 25% ammonium persulfate, and 20 μL TEMED to a 50 mL tube. Mix the solution very well with a 10 mL pipet. Quickly add the acrylamide solution to the gel casting unit and insert a well comb. Allow the gel to solidify (approx 10 min).
28. When inserting the comb ensure that there are no bubbles on the bottom of the comb. This will help to guarantee that the DNA in different wells migrates equally.
29. To ensure uniform staining of the gel the ethidium bromide and water solution should be mixed very well.
30. If more than one gel will be used run a standard and a positive control on every gel.
31. Each negative control should produce no DNA bands, if a band is detected the entire run should be repeated.
32. The *T. vaginalis* specific oligonucleotide primers are: TVA5: 5' GAT CAT GTT CTA TCT TTT CA 3' and TVA6: 5' GAT CAC CAC CTT AGT TTA CA 3'.
33. The positive control should produce a 102 base pair band that is cleaved by *Hin*fI to 56 and 46 base pair bands. If the positive control sample does not produce the appropriate bands, the entire run should be repeated.
34. This PCR has been tested using female vaginal samples. Performance with other specimens has not been evaluated and may result in false-negative or positive-results.
35. Presence of PCR inhibitors may cause false negative results. The presence of spermicides in excess of 1% or surgical lubricants in excess of 10% may have an inhibitory effect on this procedure. Blood does have an inhibitory effect on this PCR. Samples with blood contamination should not be tested.
36. Powder from gloves may produce false negative results, powder-free gloves should only be used in this procedure.

References

1. Cates, W., Jr. and Wasserheit, J. N. (1991) Genital chlamydial infections: epidemiology and reproductive sequelae. *Am. J. Obstet. Gynecol.* **164,** 1771–1781.
2. Hammill, H. A. (1989) Trichomonas vaginalis. *Obstet. Gynecol. Clin. North Am.* **16,** 531–540.
3. McGregor, J. A., French, J. I., Lawellin, D., and Todd, J. K. (1988) Preterm birth and infection: pathogenic possibilities. *Am. J. Reprod. Immunol. Microbiol.* **16,** 123–132.
4. McMillian, A. (1991) Laboratory diagnostic methods and cryopreservation of trichomonads, in *Trichomonads Parasitic in Humans* (Honigsberg, H. M., ed.), Springer-Verlag, New York, pp. 297–310.
5. Draper, D., Parker, R., Patterson, E., Jones, W., Beutz, M., French, J., Borchardt, K., and McGregor, J. (1993) Detection of *Trichomonas vaginalis* in pregnant women with the InPouch TV culture system. *J. Clin. Microbiol.* **30,** 465–472.
6. Quinn, T. C. (1994) Recent advances in diagnosis of sexually transmitted diseases. *Sex. Trans. Dis.* **21,** S19–S27.
7. Bass, C. A., Jungkind, D. L., Silverman, N. S., and Bondi, J. M. (1993) Clinical evaluation of a new polymerase chain reaction assay for detection of *Chlamydia trachomatis* in endocervical specimens. *J. Clin. Microbiol.* **31,** 2648–2653.

8. Riley, D. E., Roberts, M. C., Takayama, T., and Krieger, J. N. (1992) Development of a polymerase chain reaction-based diagnosis of *Trichomonas vaginalis. J. Clin. Microbiol.* **30,** 465–472.
9. Witkin, S. S., Jeremias, J., Toth, M., and Ledger, W. J. (1993) Detection of *Chlamydia trachomatis* by the polymerase chain reaction in the cervices of women with acute salpingitis. *Am. J. Obstet. Gynecol.* **168,** 1438–1442.
10. Jeremias, J., Draper, D., Ziegert, M., Jones, W., Inglis, S., McGregor, J. A., and Witkin, S. S. (1994) Detection of *Trichomonas vaginalis* using the polymerase chain reaction in pregnant and non-pregnant women. *Infect. Dis. Obstet. Gynecol.* **2,** 16–19.
11. Schachter, J., Stamm, W. E., and Quinn, T. C. (1994) Ligase chain reaction to detect *Chlamydia trachomatis* infection of the cervix. *J. Clin. Microbiol.* **32,** 2540–2543.
12. Witkin, S. S., Inglis, S. R., and Polaneczky, M. (1996) Detection of *Chlamydia trachomatis* and *Trichomonas vaginalis* by polymerase chain reaction in introital specimens from pregnant women. *Am. J. Obstet. Gynecol.* **175,** 165–167.
13. Weisenfeld, H. C., Heine, R. P., DiBiasi, F., Rideout, A., Macio, I., and Sweet, R. L. (1996) The vaginal introitus: a novel site for *Chlamydia trachomatis* testing. *Am. J. Obstet. Gynecol.* **174,** 1542–1546.
14. Quinn, T. C., Welsh, L., Lentz, A., Crotchfelt, K., Zenilman, J., Newhall, J., and Gaydos, C. (1996) Diagnosis by AMPLICOR PCR of *Chlamydia trachomatis* infection in urine samples from women and men attending sexually transmitted disease clinics. *J. Clin. Microbiol.* **34,** 1401–1406.

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Detection and Isolation of Differentially Expressed Genes by PCR

John M. Abraham

1. Introduction

The approx 3×10^9 basepairs that comprise a human genome are believed to contain at least 100,000 different genes ***(1)***. Many of these genes and the proteins that they encode have been isolated and identified, but the vast majority of these genes have yet to be characterized. A typical mammalian cell is believed to express approx 15,000–25,000 different proteins. A significant percentage of these proteins are present in virtually all cell types and they are generally designated as "housekeeping genes." These include many of the various proteins involved in energy production, cellular biosynthesis, or the regulation of cell growth and division. The remainder of the proteins that the genome is capable of encoding are those that are differentially expressed. The expression of these proteins is often restricted to a few cell types or even just one particular cell type. For example, genes that are expressed only in a muscle or a kidney cell, or those that are found in a T or B lymphocyte, but not in both types of cells are differentially expressed.

The phenotype of a cell and the functions that it is capable of performing is the collective result of the actions of all of the expressed proteins in that cell. In some cases, the presence of a particular protein is not required for the proper function of that cell and its deletion or "knockout" does not result in any detectable change in phenotype. On the other hand, for some genes, the expression of that gene is absolutely essential for the viability and function of the cell and the loss of even one of these essential expressed proteins can lead to the absence of cellular function or even cellular death. By comparing the expression patterns of genes in one cell type versus another, it is possible to identify genes that are important to the function and activity of that particular cell. Many

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

different research groups have been pursuing the identification of genes that are differentially expressed during transformation in an attempt to gain insight in the mechanisms involved in the establishment of the malignant state ***(2–11)***.

The classical approach that was developed to isolate differentially expressed genes relied on duplicate nitrocellulose (or nylon) lifts of plated phage cDNA libraries ***(12,13)***. Each lift was probed with ^{32}P labeled cDNA produced from mRNA isolated from the two different cell or organ types that were being compared. After high stringency washing, a positive hybridization signal on one lift or the other would lead to secondary and tertiary rounds of screening and the eventual isolation of the cDNA clone that represented the mRNA that was differentially expressed in comparing the two cell populations. At this point, the size of the cDNA insert was determined and DNA sequencing indicated whether the mRNA had been previously reported.

The advent of PCR technology has allowed several improvements in terms of the time and effort required to isolate differentially expressed genes. A recent PCR-based technique developed by Liang and Pardee ***(14–16)*** utilizes random primer hybridization to visualize mRNA transcripts with different expression patterns in different cellular sources of mRNA. This conversion of the mRNA patterns into corresponding cDNA bands is relatively fast, but only a portion of the mRNA transcript, usually on the order of a few hundred basepairs or less, is actually represented and detected on the acrylamide gel. This DNA fragment is sequenced and if the cDNA is of interest, it is generally necessary to rescreen a cDNA library by conventional means in order to obtain a full-length or even a sufficiently large partial open reading frame clone.

We have developed an approach that utilizes two duplicate agarose gels, with each gel lane being loaded with the PCR-amplified inserts of many cDNA clones. Phage plaques are first picked, eluted individually in the wells of a 96-well microtiter plate, groups of 12 clones are pooled together, and the cDNA inserts are PCR amplified. Equal amounts of this DNA is run on duplicate agarose gels, the gels are Southern blotted, and each nylon filter is hybridized with ^{32}P labeled cDNA produced from different mRNA populations that are being compared. The resulting positive hybridization band is easy to detect and the size of the cDNA is immediately known. After a lane, which originally contained twelve pooled clones, has been identified to contain a differentially expressed cDNA, one returns to the original 96-well plate and individually PCR amplifies each of the twelve clones. These cDNA inserts are run separately in their own lanes and the above described procedure is repeated. It is now possible to identify the clone representing the differentially expressed mRNA. The size of the insert is immediately known from the Southern blot and the PCR amplified insert can be directly sequenced and compared with GenBank to determine whether it is a known gene or not.

We have used this technique to screen and identify differentially expressed genes in comparing normal tissue to tumor tissue isolated from the same patient, nonirra-

diated vs irradiated cell lines, and resting versus mitogen stimulated lymphocyte cell lines. This experimental approach is very reproducible and allows the screening of large numbers of cDNA clones in a relatively short period of time.

2. Materials

2.1. Preparation of Duplicate Southern Blots

1. A cDNA library from the appropriate tissue or organ is required. This can be purchased from a variety of companies or the investigator may prefer to produce a cDNA library of their own if the required library is not available commercially (*see* **Note 1**).
2. Standard LB plates, LB broth, LB top agar (0.7%), and 37°C stationary and shaking incubators are required to grow and propagate the cDNA phage libraries ***(17)***.
3. Agarose for gel electrophoresis, such as Ultrapure Agarose (Gibco-BRL, Gaithersburg, MD).
4. Phage dilution buffer: Add 6 g NaCl, 2 g $MgCl_2$, 20 mL of 1 *M* Tris-HCl (pH 7.4), and 0.1 g of gelatin (Sigma, St. Louis, MO) to 1 L of water.
5. 10X TBE buffer: Add 108 g Tris base, 55 g boric acid, and 20 mL of 0.5 *M* EDTA (pH 8.0) to 1 L of H_2O.
6. Gel loading dye: 0.25% bromphenol blue (w/v), 50% glycerol.
7. 10X PCR buffer: 200 m*M* Tris-HCl (pH 8.4), and 500 m*M* KCl (Gibco-BRL).
8. 50 m*M* $MgCl_2$ (Gibco-BRL).
9. 10 m*M* stocks of dGTP, dATP, dTTP, dCTP.
10. *Taq* polymerase (5 U/μL) (Gibco-BRL).
11. Hybond-N (Amersham, Arlington Heights, IL) or another nylon or nitrocellulose membrane which can be purchased from a variety of suppliers.

2.2. Preparation of cDNA Probe and Hybridization

1. Trizol (Gibco-BRL).
2. Poly(A+)Quik push column (Stratagene, La Jolla, CA) or other similar system available from a variety of suppliers.
3. SuperScript RNase H Reverse Transcriptase kit (Gibco-BRL).
4. Multiprime DNA labeling System (Amersham) or other similar system avaiable from a variety of suppliers.
5. X-Omat Film (Kodak, Rochester, NY).

3. Methods

3.1. Preparation of Duplicate Southern Blots

1. Libraries containing cDNA clones of interest may be purchased or made following the instructions provided by a number of different companies. We have found the cDNA library systems from Stratagene to be very reliable (*see* **Note 1**).
2. The phage are plated using the manufacturer's suggested bacterial strain and following standard methods ***(17)***. A dilution should be used that will result in a plating of low density (3–4 plaques per cm^2) (*see* **Note 2**).

3. Plaques are picked at random and each one is placed in 200 µL of phage dilution buffer ***(17)*** (*see* **Note 3**).
4. Elute the phage for at least 3 h at room temperature or overnight at 4°C.
5. For the primary screen, pool the randomly selected phage with twelve different cDNA clones in each pool.
6. Use 50 µL of this pooled eluted phage in a 100 µL PCR reaction.
7. A 100 µL PCR reaction contains: 10 µL of 10X PCR buffer, 0.2 m*M* each dNTP, 1.5 m*M* $MgCl_2$, 0.5 µ*M* each primer, 0.5 µL *Taq* polymerase, 50 µL of the pooled eluted cDNA phage, and H_2O to 100 µL.
8. Perform 25 cycles of PCR consisting of 1 min at 94°C, 1 min at 45°C, and 3 min at 72°C (*see* **Note 4**).
9. Add 15 µL of loading buffer to 50 µL of PCR product.
10. Load 30 µL in one lane of a 1% agarose gel containing and 30 µL in a corresponding lane of an identical gel, leaving 5 µL to discard. One may upscale the number of pooled PCR amplified cDNA inserts in the procedure in order to load all of the wells of a gel apparatus.
11. Electrophorese the samples until the bromphenol blue marker is near the bottom of the gel.
12. Denature the gel for 45 min in 0.5 *M* NaOH, 1.5 *M* NaCl.
13. Neutralize the gel for 45 min in 1 *M* Tris (pH 7.0), 1.5 *M* NaCl.
14. Soak the gel for 45 min in 20X SSC.
15. Transfer the blot by capillary action ***(17)*** to Hybond-N (Amersham) and crosslink the DNA with a UV Stratalinker (Stratagene) (*see* **Note 5**).

3.2. Preparation of Single-Stranded cDNA Probe Hybridization

1. Prepare total RNA from tissue or cell lines using Trizol reagent and the instructions provided by the manufacturer (Gibco-BRL) ***(18)*** (*see* **Note 6**).
2. Isolate mRNA by passing the total RNA through a Poly(A)+ Quik push column (Stratagene).
3. Synthesize cDNA from the mRNA using the SuperScript RNase H Reverse Transcriptase kit (Gibco-BRL).
4. Label the cDNA with ^{32}P using the random prime labeling kit from Amersham ***(19)***.
5. Each of the two labeled cDNAs that are being compared is boiled and hybridized overnight at 42°C to one of the duplicate blots (Maniatis).
6. The blots are washed at high stringency conditions with the last two washes at 68°C in 0.1X SSC and 0.5% SDS.
7. Dry the blots and expose overnight with X-Omat film (Kodak).
8. Compare the two autoradiographs. If any of the lanes suggest differentially expressed genes, it is necessary to repeat the above procedure, but this time generating PCR amplified cDNA inserts from the proper individual clones indicated by the lanes of the duplicate gels. These individual cDNA inserts are each loaded into their own lanes of duplicate agarose gels. After hybridization, if bands are detected showing differential expression, one may now analyze the single pure phage clone indicated by the blot.
9. Sequence the cDNA clone of interest using the dideoxy termination sequencing method (USB, Cleveland, OH) and an aliquot of the PCR product used in the Southern blot described above (*see* **Note 7**).

4. Notes

1. Many different companies now offer cDNA library synthesis. In general, these kits are relatively straight forward and easy to use. For the appproximate cost of purchasing one pre-made cDNA library, it is possible to purchase a kit that will allow the investigator to produce five high quality libraries of their own.
2. It is important to plate the plaques such that they are clearly separated from one another. It is possible for phage to diffuse through the agarose and contaminate neighboring plaques that are close.
3. The PCR amplification of cDNA inserts found in phage plaques is extremely reproducible and easy. It is interesting that the plaques can be directly selected from the lysis plate, eluted in phage dilution buffer and then used successfully in the PCR amplification of the inserts without any type of cleanup procedure required.
4. As mentioned previously, the PCR amplification of phage eluted from plaques is usually successful. There is no need to begin with a longer denaturation step or to end with a longer synthesis step.
5. Although the use of a UV crosslinker is the quickest method, one may also bake the blots in an 80ºC vacuum oven for 2 h.
6. There are many warnings in various RNA extraction protocols about the danger of RNase contamination from hands and skin and the use of gloves to counter this. Often overlooked is the presence of RNase that results from the careless preparation of solutions and buffers used in plasmid isolation procedures. The custom of some laboratories is to prepare stock solutions of RNase of high concentrations by measuring several hundred milligrams of the very light and fluffy RNase powder on an open-air balance and then tapping the contents of the weigh boat into the solution bottle or flask. This can readily result in an aerosol of RNase powder that is distributed by air throughout the laboratory. Unlike most other proteins, a protein like RNase A that is so resistant to degradation that it can withstand boiling in water, can survive on the laboratory bench for a lengthy period of time.
7. One may subclone the PCR product into any number of good commercially available vectors and then determine the DNA sequence. USB Biochemical has developed an excellent DNA sequencing kit that allow the determination of the DNA sequence directly from the PCR product without any subcloning step.

References

1. Lewin, B. (1994) *Genes V.* Oxford University Press, Oxford, UK.
2. Gebert, J. F., Moghal, N., Frangioni, J. V., Sugarbaker, D. J., and Neel, B.G. (1991) High frequency of retinoic acid beta abnormalities in human lung cancer. *Oncogene* **6,** 1859–1868.
3. Oyama, F., Hirohashe, S., Shimosato, Y., Titani, K., and Sekiguchi, K. (1991) Qualitative and quantitative changes of human tenascin expression in transformed lung fibroblast and lung tumor tissue: comparison with fibronectin. *Cancer Res.* **51,** 4876–4881.
4. Rosengard, A. M., Krutzsch, H. C., Shearn, A., Biggs, J. R., Barker, E., Marguilies, I. M. K., King, C. R., Liotta, L. A., and Steeg, P. S. (1989) Reduced nm23/Awd protein in tumor metastatis and aberrant drosophila development. *Nature* **342,** 177–180.

5. Lumelsky, N. L. and Forget, B. G. (1991) Negative regulation of globin gene expression during megakaryocytic differentiation of a human erythroleukemic cell line. *Mol. Cell. Biol.* **11,** 3528–3536.
6. Chaganti, R. S., Nishiyama, M., Ozturk, M., Frohlich, M., and Mafune, M. (1990) Expression of human alpha-actinin in human hepatocellular carcinoma. *Cancer Res.* **50,** 6291–6294.
7. Reed, J. C., Ekstrand, A. J., James, C. D., Cavenee, W. K., Seliger, B., and Pettersson, H. (1991) Genes for epidermal growth factor receptor, transforming growth factor alpha, and epidermal growth factor and their expression in human gliomas in vivo. *Cancer Res.* **51,** 2164–2172.
8. Shitivelman, E. and Bishop, J. M. (1991) Expression of CD44 is repressed in neuroblastoma cells. *Mol. Cell. Biol.* **11,** 5446–5453.
9. Eilers, M., Schirm, S., and Bishop, J. M. (1991) The MYC protein activates transcription of the alpha-prothymosin gene. *EMBO J.* **10,** 133–141.
10. Luo, X. N., Arcasoy, M. O., Brickner, H. E., Mistry, S., and Schechter, A. D. (1991) Regulated expression of p18, a major phosphoprotein of leukemic cells. *J. Biol. Chem.* **266,** 21,004–21,010.
11. Finch, J., Andrews, K., Krieg, P., Furstenberger, G., Slaga, T., and Oostsuyama, K. (1991) Identification of a cloned sequence activated during multi-stage carcinogenesis in mouse skin. *Carcinogenesis* **12,** 1519–1522.
12. Maniatis, T., Kee, S. G., Efstratiadis, A., and Kafatos, F. C. (1976) Amplification and characterization of a beta-globin gene synthesized in vitro. *Cell* **8,** 1630–1641.
13. Maniatis, T., Hardison, R. C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, D. K., and Estratiadis, A. (1978) The isolation of structural genes from libraries of eucaryotic DNA. *Cell* **15,** 687–698.
14. Liang. P. L., Averboukh, K., Keyomarsi, T., Sager, R., and Pardee, A. B. (1992) Differential display and cloning of mRNAs from human breast cancer versus mammary epithelial cells. *Cancer Res.* **52,** 6966–6968.
15. Liang, P., Averboukh, L., and Pardee, A. B. (1993) Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimisations. *Nucleic Acids Res.* **21,** 3269–3275.
16. Liang, P. and Pardee, A. B. (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257,** 967–971.
17. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd Edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
18. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156–159.
19. Feinberg, A. P. and Vogelstein, B. (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132,** 6–13.

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Detection of Transgene Integrants and Homologous Recombinants in Mice by Polymerase Chain Reaction

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1. Introduction

The use of genetically altered mice in research has increased exponentially since the production of the first transgenic mouse 15 yr ago. Within the past decade, the technique of targeted mutagenesis in mice has seen a similar rapid expansion in use, becoming a strategy widespread throughout a number of laboratories studying a variety of experimental systems. In addition to the large number of new transgenic and knockout models being generated, many investigators are now performing combinatorial experiments in which various transgenic and/or knockout mice are intercrossed to produce animals with complex genotypes. These rapid experimental advances have necessitated the development of tools that permit efficient and precise identification of genetically altered alleles in mice. Many investigators use Southern blotting to identify transgenic founder mice or mice bearing homologous disruptions; however, this strategy often becomes laborious when large breeding programs are involved. PCR is a technique that lends itself very well to rapid identification of large numbers of mutant mice bearing complex genotypes. These large-scale analyses are often required when performing experiments with transgenic and knockout animals.

A variety of methods can be used to prepare mouse tissue DNA suitable for PCR. Many of these strategies rely upon proteinase K digestion followed by organic extraction and ethanol precipitation, a procedure yielding DNA suitable for Southern blotting, as well as PCR *(1)*. With more widespread use of PCR, more rapid methods for DNA recovery have been developed. For example, boiling of tissue in lysis buffer is a technique that has been adapted

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

for DNA recovery from tail tissue as well as ear tissue taken at the time of ear punching for identification *(2–4)*. In addition, a variety of commercial column or affinity resin-based kits are available for extracting DNA. A new method for extracting DNA from saliva samples has also been developed, representing the most noninvasive method reported to date for generating PCR-ready mouse DNA *(5)*. Most of these methods produce DNA quantities sufficient for PCR reactions, and the choice of method is governed by issues such as convenience, cost, and age of animals at sampling.

Strategies for transgene or knockout allele detection are also quite varied and depend on the type of detection required. For example, murine transgenes are often detected using primer sets incapable of detecting endogenous gene sequences, or which yield a transgene-specific product that can be distinguished from the product of the endogenous gene by its characteristic size. This technique is readily applied to transgenes consisting of cDNA copies of endogenous genes, as primers specific for sequences in different exons may easily distinguish the cDNA transgene from genomic DNA. In another strategy, transgene constructs are tagged with heterologous oligonucleotide sequences to serve as primer binding sites for PCR. Finally, transgene expression constructs that place the gene of interest downstream from heterologous promoters are often detected using primer pairs that bind within the promoter and insert DNA. In general, knockout alleles are detected using similar strategies. If the allele has been disrupted because of insertion of a neomycin resistance gene in the coding sequence (Neo), primer pairs may be chosen that bind within the Neo gene and within regions of the targeted locus outside of the region of homology carried in the targeting construct. In this strategy, generation of a PCR product is only possible if the targeted disruption has occurred. Mice heterozygous or homozygous for the disrupted allele can then be distinguished by including a primer, which when used in conjunction with the locus-specific primer, will amplify a product of a specific size from only the nontargeted allele *(6)*. In general, PCR primers may be chosen that yield products that range from 200 to 800 bp. Although longer products are attainable, appropriate targets must often be identified empirically, and should be chosen based on their ability to be amplified reliably. Control reactions should always include nontransgenic mouse DNA as well as transgene-positive DNA, which may be obtained from known transgenic samples, or from nontransgenic DNA spiked with small amounts (<1 ng) of the transgene construct. Assays differentiating homozygous and heterozygous knockouts from wild-type animals should include both wild-type DNA and the targeted allele as controls. In general, the targeted allele may be obtained by recovery of DNA from the embryonic stem cell clones originally used for

blastocyst injection. What follows is a commonly used procedure for recovery and analysis of mouse DNAs bearing transgenes or homologous recombination constructs. This procedure is relatively rapid, reliable, and yields DNA suitable for screening of DNAs by PCR and for examining integration sites or transgene copy numbers by Southern blotting. The PCR protocol described is designed to detect transgenes containing the human growth hormon gene (hGH) in constructs commonly used to promote expression of cDNAs in thymocytes of transgenic mice ***(7–9)***.

2. Materials

2.1. DNA Preparation

1. Sharp stainless steel scissors.
2. Proteinase K Buffer: 50 m*M* Tris, pH 8.0, 100 m*M* EDTA, 100 m*M* NaCl, 1% SDS.
3. 10 mg/mL proteinase K (made up in dH_2O and stored at –20°C)
4. 10 mg/mL RNase A (stock solution stored at –20°C).
5. Phenol/$CHCl_3$/isoamyl alcohol (25/24/1).
6. $CHCl_3$/isoamyl alcohol (24/1).
7. Isopropanol.
8. 70% Ethanol, 95% ethanol.
9. TE: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA.

2.2. PCR Amplification

1. Sterile dH_2O.
2. 10X PCR buffer.
3. 1.25 m*M* dNTPs (dATP, dGTP, dCTP, dGTP).
4. 50 m*M* $MgCl_2$.
5. Oligonucleotide primers diluted in sterile dH_2O to 100 μ*M*. Four amplimers corresponding to sequences contained in the human growth hormone gene were designed that generate two PCR products of 396 and 275 bp following amplification of genomic DNA:
   hGH-B: 5' AGGACAAGGCTGGTGGGCACTG 3'
   hGH-C: 5' GTTTGGATGCCTTCCTCTAGGT 3'
   hGH-D: 5' TCCTCTAGCCTTTCTCTACACC 3'
   hGH-E: 5' CAACAGGGAGGAAACACAACAG 3'
6. Sterile PCR tubes.
7. *Taq* polymerase (5 U/μL).

2.3. Agarose Gel Electrophoresis

1. Agarose.
2. 1X TBE.
3. 6X DNA gel loading dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol (v/v) in dH_2O.

3. Methods

3.1. Sample Collection and DNA Extraction

3.1.1. Day 1

1. Place approx 1 cm of tail (or other) tissue directly into an Eppendorf tube.
2. Add 700 µL of Proteinase K buffer.
3. Mince the tissue using small, sharp, stainless steel scissors (*see* **Note 1**).
4. Add 35 µL of 10 mg/mL proteinase K to the tissue slurry and incubate at 55°C overnight.

3.1.2. Day 2

1. Add 10 µL of 10 mg/mL RNase A to the samples and incubate at 37°C for 1 h.
2. Extract with an equal volume of phenol/$CHCl_3$/isoamyl alcohol (approx 745 µL). Vortex 10–20 s to mix the organic and aqueous phases well. Spin the tubes in a microcentrifuge for 15 min.
3. Using a large orifice pipet tip, remove the upper, aqueous layer to a new tube without taking any of the white interphase. Add an equal volume of $CHCl_3$/isoamyl alcohol (24/1), mix well and centrifuge for 10 min. Remove the upper aqueous layer to a new tube. Continue extracting until no white material remains at the interphase.
4. Add 0.6 vol (450 µL) of isopropanol and invert to mix. The high molecular weight DNA should form a stringy precipitate in the solution. The tubes may be centrifuged immediately, or retained at room temperature for 1–2 h.
5. Spin the tubes at full speed in the microfuge for 10–15 min. Pipet off the isopropanol manually (do not aspirate), wash the pellet once with 70% ethanol and once with 95% ethanol. Remove residual alcohol by drying the samples using a Speed-Vac.
6. Resuspend the dried DNA pellet in 150 µL TE, and dissolve at 65°C for 2–3 h, or overnight at 55°C. The fully dissolved DNA pellet is now suitable for enzymatic digestion (as for Southern blotting) or PCR analysis (*see* **Subheading 3.2.**).

3.2. PCR Amplification

1. Each PCR amplification is performed in a 25 µL total volume.
2. Prepare a master mix containing PCR amplification reagents suitable for amplifying each sample, including positive and negative (no DNA or nontransgenic DNA) controls. The master mix consists of (per sample): 2.5 µL 10X PCR buffer, 1.5 µL 50 m*M* $MgCl_2$, 1.5 µL 1.25 m*M* dNTP stock solution, 0.25 µL of each 100 m*M* primer stock solution (hGH B, C, D, E), and 18.50 µL sterile dH_2O.
3. Aliquot 1.0 µL DNA sample per PCR tube (*see* **Note 3**). Samples should be included which contain 1.0 µL sterile dH_2O or nontransgenic DNA as a negative control, and 1 ng transgene vector DNA (or known transgene positive tail DNA) as a positive control (*see* **Note 4**).
4. Add *Taq* polymerase to complete the PCR master mix and mix thoroughly (*Taq* polymerase per sample: 0.25 µL = 1.25 U).

5. Add 24 μL of the PCR master mix containing *Taq* polymerase to each sample tube containing 1 μL of DNA and mix thoroughly.
6. Add a single drop of sterile mineral oil to cover each sample (if necessary), cap and place in the thermal cycler block (*see* **Note 5**).
7. Amplify using a thermal cycler programmed as follows: 2 min preincubation at 94°C followed by 32 cycles consisting of denaturation at 94°C for 2 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min. Samples may be held at 4°C until analysis.

3.3. Analysis of PCR Products

1. Remove 20 μL of PCR sample from under the oil overlay and add to 4 μL of 6X gel loading buffer.
2. Load the PCR samples and a sample containing DNA migration markers into wells of a preformed gel containing 1% agarose in 1X TBE running buffer and 100 ng/mL ethidium bromide.
3. Electrophorese the samples using 1X TBE running buffer at constant voltage until the bromophenol blue dye front has migrated to within 2–3 cm of the end of the gel.
4. Remove the gel from the electrophoresis tank, expose using a shortwave UV transilluminator and photograph.

4. Notes

1. To minimize crosscontamination of samples prior to enzymatic digestion it is recommended that the scissors used to mince the tissue be decontaminated between samples by immersion in a 70% ethanol wash.
2. Aerosol-resistant pipet tips are recommended for use in all sample preparation and PCR procedures.
3. Amounts of DNA recovered using this protocol vary, but samples normally contain between 25 and 50 μg of DNA per 150 μL TE.
4. A positive control template should be analyzed in parallel with the test samples in each analysis to monitor satisfactory execution of the PCR assay. Positive controls may consist of the vectors themselves (transgenes) diluted in wild-type genomic DNA, or of DNA recovered from embryonic stem cells bearing the homologous disruption (ES cells). To recover genomic DNA from ES cell clones for use, cell pellets containing 10^5–10^6 cells may be subjected to proteinase K and RNase digestion and nucleic acid purified as outlined above.
5. The authors use a Thermolyne Amplitron II equipped with a heated lid that allows amplifications to be performed without the addition of mineral oil to the samples or block. However, many thermocylers require that a small volume of oil be added to sample wells in the block, and that samples be overlayed with a small volume of sterile mineral oil to minimize volume losses due to evaporation.

References

1. Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994) *Manipulating the Mouse Embryo*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

2. Chen, S. and Evans, G. A. (1990) A simple screening method for transgenic mice using the polymerase chain reaction. *BioTechniques* **8,** 32,33.
3. Hanley, T. and Merlie, J. P. (1991) Transgene detection in unpurified mouse tail DNA by polymerase chain reaction. *BioTechniques* **10,** 56,57.
4. Drews, R., Drohan, W. N., and Lubon, H. (1994) Transgene detection in mouse tail digests. *BioTechniques* **17,** 866,867.
5. Irwin, M. H., Moffatt, R. J., and Pinkert, C. A. (1996) Identification of transgenic mice by PCR analysis of saliva. *Nature Biotechnol.* **14,** 1146–1148.
6. Busler, D. E. and Li, S. W. (1996) Rapid screening of transgenic Type II and Type XI collagen knock-out mice with three-Primer PCR. *BioTechniques* **21,** 1002–1004.
7. Chaffin, K. E., Beals, C. R., Wilkie, T. M., Forbush, K. A., Simon, M. I., and Perlmutter, R. M. (1990) Dissection of signaling pathways by in vivo expression of pertussis toxin ADP-ribosyl-transferase. *EMBO J.* **9,** 3821–3829.
8. Cooke, M. P., Abraham, K. M., Forbush, K. A., and Perlmutter, R. M. (1991) Regulation of T cell receptor signaling by a src family protein-tyrosine kinase (p59fyn). *Cell* **65,** 281–291.
9. Wildin, R. S., Wang, H. U., Forbush, K. A., and Perlmutter, R. M. (1995) Functional dissection of the murine lck distal promoter. *J. Immunol.* **155,** 1286–1295.

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Direct Analysis for Familial Adenomatous Polyposis Mutations

Steven M. Powell

1. Introduction

Over the past decade, the genes that underlie the development of many human diseases have been identified and the diseases causing mutations within these genes have been unveiled. Many genetic alterations responsible for a variety of human disorders have been characterized. These alterations range from simple Mendelian inherited syndromes to more complex traits such as cancers that involve multiple genetic and environmental factors. Identification and characterization of disease-causing mutations has practical as well as biological implications. As our understanding of these alterations advances, the potential for developing molecular genetic markers with clinical applications increases. This improved understanding also opens new avenues for advances in diagnostic testing, prognostication, and design of preventative strategies or therapeutic interventions. Indeed, direct genetic testing for an inherited colorectal cancer predisposition syndromes, Familial Adenomatous Polyposis (FAP) is currently available to the medical community with appropriate genetic counseling ***(1)***.

This chapter describes the application of the in vitro synthesis (IVS) protein assay, which is a sensitive and rapid method for detecting truncating gene mutations ***(1,2)***. The importance of mutational analyses that can be applied routinely in clinical practice is highlighted by the IVS protein assay's current use to FAP presymptomatically. This assay may also potentially aid in the diagnosis and management of many other diseases that involve truncating genetic mutations (*see* **Table 1**).

We may soon be entering into an era where mutational analysis and detection become the limiting steps in our diagnosis and care of patients. For instance, we may know the gene(s) involved in a disease, but not have the

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

Table 1
Applications of the IVS Protein Assay

Current	
Familial adenomatous polyposis syndromes	APC
Emerging	
Hereditary nonpolyposis colon	DNA repair genes (MMR)[a]
Neurofibromatosis type 1	NF1
Hereditary breast/ovarian cancer	BCRA1
Duchenne muscular	Dystrophin
Potential	
Neurofibromatosis type 2	NF2
Von Hippel-Lindau	VHL
Retinoblastoma	Rb
Becker muscular dystrophy	Dystrophin
β-Thalassemia	β-Globin
Hemophilia B	Factor IX
Cystic fibrosis	CFTR
Osteogenesis Imperfecta	COLIA1/COLIA2
Werner's syndrome	WRN

[a]MMR = mismatch repair genes that include: hMSH2, hM:LH1, hPMS1, hPMS2.

ability to conveniently test those individuals who may have or are at risk for having the disease for causative alterations in the responsible gene(s). Currently, identifying the appropriate clinical setting for genetic testing is of paramount importance. The group of patients and relatives for whom genetic testing will be beneficial is presently being defined as we better understand genotype to phenotype relationships and the penetrance of pathologic traits.

Many conventional techniques of mutational analysis, such as direct nucleotide sequencing, ribonuclease protection assays, or other chemical cleavage of nucleotide mismatch methods (i.e., hydroxylamine and osmium tetroxide) that can identify genetic mutations sensitively are labor intensive and usually reserved for the research setting (reviewed in **ref. *3***). Other methods of detecting gene mutations, such as single-strand conformation polymorphism (SSCP) analysis ***(4)***, denaturing gradient gel electrophoresis (DGGE) analysis ***(5)***, or heteroduplex analysis, may require only a few steps but are limited in their sensitivity of mutation detection. The narrow range of a gene's size that can be analyzed by these methods at any one time also restricts their use for mutation identification, specifically limited are those assays that involve altered heteroduplex migration on gel electrophoresis analysis. Moreover, some methods such as allele-specific amplification (ASA), allele-specific hybridization (ASH), ligation amplification reactions (LAR) ***(6)***, or restriction site amplifi-

cations are relatively simple to perform, but they are designed to detect only a specific nucleotide change. This specificity limits their usefulness for screening genes that tend to have multiple types of mutations occurring at different locations in the gene.

Additionally, genes can be altered in the noncoding region with important functional effects. For example, changes that occur in the promoter or enhancer regions of a gene or alterations that change methylation patterns might result in abnormal gene expression. Moreover, gross allelic or chromosomal deletions, amplifications, or rearrangements are known to occur at gene loci that result in the loss, disruption, or increased expression of its product. High-resolution cytogenetic analyses such as those involving fluorescent *in situ* hybridization (FISH) and Southern blot-based restriction fragment length polymorphism (RFLP) analysis can facilitate the detection of these alterations ***(7)***; however, only a few genes presently allow routine identification of such alterations. Thus, none of these conventional methods of mutation detection are readily applicable for routine screening of large genes with a wide distribution and spectrum of mutations. Therefore, one can see how efficient mutational analysis has become a pressing issue.

The *APC* gene was isolated in 1991 ***(8,9)*** and so named *Adenomatous Polyposis Coli* when it was found to be altered in the germline of FAP patients and cosegregated with this disease ***(10,11)***. FAP is a clinically well-described highly penetrant autosomal dominant trait that has been reported for over a century (reviewed in **ref. *12***). Affected individuals develop hundreds to thousands of colorectal adenomatous polyps, some of which inevitably progress to colorectal carcinomas unless they are removed surgically.

FAP patients harbored multiple types of nucleotide changes widely distributed throughout *APC*'s relatively large coding region with some trend toward concentrating in its mid-portion (the 5' end of the last large exon 15). Conventional genetic screening methods were applied in early research-based studies of the coding region of the *APC* gene. They could detect mutations in the range of 30–60% of patients with FAP depending on the technique used ***(13–15)***. The variegated nucleotide changes and wide distribution of *APC* gene mutations presented a formidable obstacle in the development of a rapid mutational assay for this gene by conventional approaches.

It was observed that the overwhelming majority of these *APC* gene mutations would result in a truncated gene product when expressed because of small insertions or deletions producing frameshifts and subsequent premature stop codons, nonsense point mutations, or splice site alterations (reviewed in **ref. *16***). Thus, it was surmised that the examination of an individual's *APC* protein would identify the majority of *APC* mutations. A novel assay was developed to examine *APC*'s gene based on IVS of its protein from a PCR-amplified prod-

uct ***(2,3)***. In this assay, an individual's gene or mRNA transcript, amplified by polymerase chain reaction (PCR) or reverse transcription and polymerase chain reaction (RT-PCR) serves as a nucleotide surrogate template of the *APC* gene for rapid in vitro transcription and translation. The protein synthesized in vitro is then analyzed electrophoretically for its size. This method of mutational detection was shown to sensitively identify the germline truncating *APC* mutations in 82% of 62 unique FAP kindreds.

The IVS protein assay originated in an effort to efficiently identify truncating *APC* mutations. This test was first validated in the analysis of sporadic colorectal tumors containing known truncating *APC* gene mutations. The accuracy of identifying *APC* mutations in this manner was illustrated by the clearly visible mutant protein bands in these samples. The sensitivity of this assay was demonstrated in the detection of *APC* mutations in tiny dysplastic colonic polyps and aberrant crypt lesions ***(17)***.

The strength of the IVS protein test lies in its ability to rapidly identify truncating gene mutations irrespective of their origin or nature at the nucleotide level. Truncation of a gene's product is a drastic alteration that is expected generally to have critical effects on the protein's normal function in a cell. Therefore, the ability to identify only these truncating kinds of alterations, while avoiding numerous inconsequential polymorphisms or rare variant changes, is a significant advantage offered by the IVS protein assay in mutational screening.

A variety of mutations at the genetic level such as nonsense point mutations, frameshifts, or alterations producing splice abnormalities that result in a truncated gene product, can be detected sensitively all at once by this method. Additionally, the IVS protein assay can be used to analyze relatively long gene segments. This is especially advantageous for large genes having a widespread distribution of mutations. The ability to generate cDNA from mRNA transcripts by RT-PCR reactions for use as a template in this assay facilitates rapid screening of multiple exons and long regions of coding sequence at one time as well as of the splicing pattern of a particular gene.

Limitations of the IVS protein assay include its inability to identify nontruncating genetic mutations such as missense point mutations. Gross allelic loss, insertion, or rearrangement, which may prohibit the amplification of a genetic locus, also would not be detected by the PCR-based IVS protein assay. Furthermore, alterations in noncoding regions such as those that may occur in the promoter or intron regions and affect gene product expression would not be detected by the IVS protein assay.

Finally, the IVS protein assay would not detect epigenetic alterations, such as methylation changes and imprinting abnormalities, that might affect gene expression. Therefore, additional more broad analyses, such as the allele-spe-

cific expression (ASK) assay ***(2)***, Southern blot analysis, or Western blot analysis, are needed when these types of mutations are sought. Interestingly, a novel strategy, termed monoallelic mutation analysis (MAMA), which is based on somatic cell hybridization technology, was recently reported to identify germline mutations sensitively and specifically ***(18)***.

This assay was readily applied to FAP patients' blood samples in the original quest to identify *APC* mutations efficiently and sensitively for clinical use. This assay lends itself to routine use by utilization of supplies and equipment that are commonly available in most molecular biology laboratories. Moreover, RNA and especially DNA can be extracted by standard means from routinely available clinical samples such as blood and stored stably for analysis at convenient times.

At-risk family members are commonly the greatest beneficiaries of using the IVS protein assay to make a molecular diagnosis of FAP patients (*see* **Fig. 1**). Once a causative *APC* mutation is identified with this assay, one can employ the test presymptomatically to determine with virtually 100% accuracy whether or not a family member has inherited the specific genetic abnormality and the resultant risk of neoplasia associated with this disease. Presymptomatic direct genetic testing greatly aids in the clinical management of FAP kindred members and allows more directed screening for cancer development. Genetic counseling is a prerequisite for this type of testing to convey information appropriately to these patients ***(19)***.

Since its emergence in 1993, the IVS protein assay has also been used to identify truncating genetic mutations in other genes, most notably the DNA mismatch repair genes, the Duchennes muscular dystrophy gene, *BRCA1,* and *NF1.* HNPCC is a cancer predisposition syndrome inherited as an autosomal dominant trait with fairly high penetrance which is associated with colorectal and other cancer development (reviewed in **ref. *20***). This disease was recently demonstrated to result from alterations in DNA mismatch repair genes ***(21–25)***. The IVS protein assay was used initially to screen the candidate genes in HNPCC patients for deleterious mutations and revealed germline truncating alterations of varied genetic origins in four different genes, namely *hMSH2, hMLHl, hPMSI, and hPMS2,* which reflect the heterogeneity of this disease.

The clinical utility of the IVS protein test in identifying alterations in DNA repair genes is just beginning to be established ***(26,27)***. The spectrum of mutations in the DNA repair genes in HNPCC patients suggests that more than half of those identified are truncating in nature and would be amenable to detection by the IVS protein assay. A clinically useful genetic test to identify an HNPCC kindred's causative mutation would have important implications for presymptomatic screening of at-risk family members similar to those described for FAP. An additional subgroup of patients that might benefit from the use of

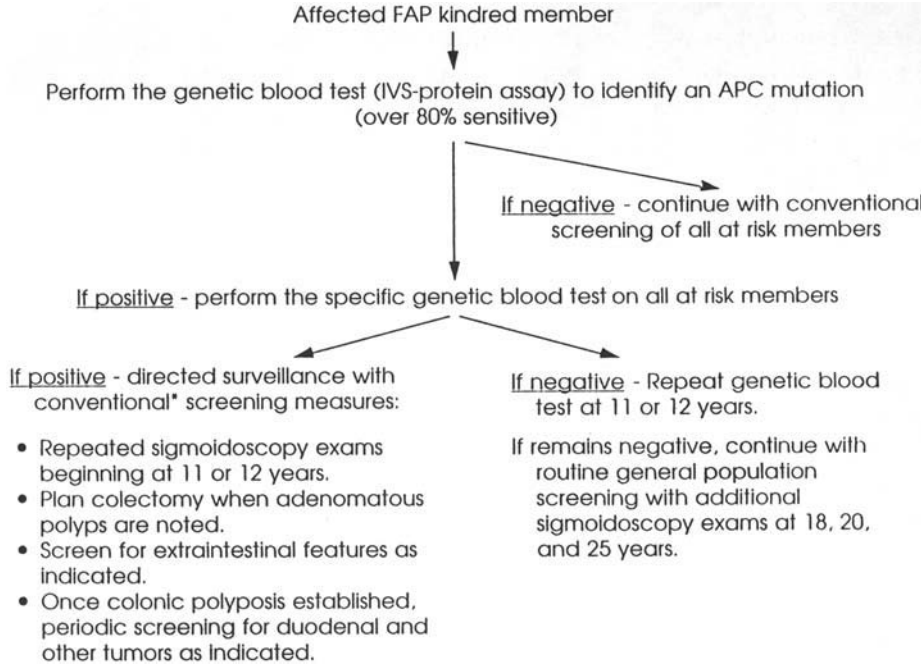

Fig. 1. Algorithm for the management of FAP kindreds. These management guidelines of FAP kindreds incorporate presymptomatic direct genetic testing for *APC* mutations. The conventional measures of screening for members of FAP kindreds at risk may vary in frequency (e.g., sigmoidoscopic exams usually performed annually until approx 40 yr of age or until significant adenomatous polyposis is noted). Endoscopic surveillance exams once colectomy has been performed is dependent on the surgical procedure performed, severity of polyposis, and amount of remaining colon mucosa left at risk (e.g., sigmoidoscopic exams every 6 mo if the rectum is intact vs annual exams after ileoanal anastomosis procedures. Extraintestina screening examinations advocated by some physicians inclucle fundoscopic exams and radiologic exams of the skull, mandible, and teeth. Once colonic adenomatous polyposis is established, surveillance for duodenal polyposis is considered every 1–3 yr, although cost to benefit ratios are not well established. Surveillance for other extraintestinal tumors, such as brain, thyroid, and soft tissues (e.g., desmoids), must then be considered, especially in kindreds already manifesting these features (e.g., Gardner's or Turcot's syndrome). (Adapted from **ref. *44*** with permission).

the IVS protein assay to identify DNA repair gene mutations are those individuals who display microsatellite instability in their colon tumor and are diagnosed with colorectal cancer at <35 yr of age. A study found that 5 of 12 such subjects, who were examined for DNA mismatch repair gene abnormalities, harbored germline truncating alterations in *hMSH2* or *hMLH1* ***(28)***.

Other genes that potentially lend themselves to clinically applicable mutational screening by the IVS protein assay include: the neurofibromatosis 2 gene *(NF2)* ***(29,30)***, the von Hippel- Lindau gene ***(31)***, Duchenne and Becker muscular dystrophy gene ***(32)***, *BCM1* ***(33)*** collagen genes (e.g., *COLlA1* or *COLlA2*, which cause osteogenesis imperfecta when altered ***[34]***), the retinoblastoma gene ***(35)***, the beta-thalassemia gene ***(36)***, and the hemophilia B gene ***(37)***(*see* **Table 1**). All of these genes have a significant proportion (many greater than 50%) of truncating intragenic mutations in the patients examined so far. These mutations appear to be detectable by the IVS protein test. Over 50% of the various cystic fibrosis mutations that have been characterized, other than the common phenylalanine deletion at codon 508, appear to be detectable by the IVS protein assay as well ***(38–40)***.

The Neurofibromatosis l gene *(NF1),* Duchenne muscular dystrophy gene, and *BCRA1* have been screened for mutations using the methodology of the IVS protein test with successful identification of truncating mutations ***(41–43)***. Many of these genes are quite large with widespread genetic changes that could not be screened easily for mutations by conventional approaches, as they are too laborious or cumbersome for routine clinical use.

Of course, before one would decide to perform a genetic test, such as the IVS protein assay, to identify a causative mutation clinically, a benefit would have to be gained in doing so (e.g., enabling more directed screening measures or allowing earlier preventive or therapeutic interventions to be given). Studies are also needed to determine which individuals would be the best to screen and who would gain the most from these direct mutational tests. Sensitivity and cost-to-benefit ratio analyses are needed to help address these issues.

2. Materials

2.1. Blood Processing

1. EDTA anticoagulated (lavender top) blood tubes filled with whole blood (Becton Dickinson [Bedford, MA], Vacutainer Brand).
2. Histopaque-1077 (Sigma, St. Louis, MO).
3. 50-mL and 15-mL polypropylene conical tubes and 1.5-mL tubes (Marsh, Rochester, NY).
4. Hanks balanced saline solution (Gibco-BRL, Gaithersburg, MD).
5. Table-top centrifuge and plastic transfer pipets and 1.5-mL Eppendorf tubes.

2.2. DNA Extraction

1. Chelex-100 (5% stock solution made with sterile water) (Bio-Rad, Hercules, CA).
2. Sterile water (Gibco-BRL, HPLC-purified).
3. Microfuge and Eppendorf tubes.

2.3. RNA Extraction

1. Promega RNagents kit (Promega, Madison, WI).
2. 70% Ethanol solution.
3. Microfuge, Eppendorf tubes, and snap-cap tubes.

2.4. First-Strand cDNA Synthesis

1. Superscript II reverse transcriptase (200 U/μL) (Gibco-BRL).
2. Random hexamers (1 mg/mL stock) (Pharmacia Biotech, Piscataway, NJ).
3. 5X first-strand buffer (Gibco-BRL).
4. dNTPs (100 m*M* stock) (Pharmacia Biotech).
5. RNasin (40 U/μL) (Promega).
6. Bind-Aid (0.5 U/μL) (USB, Arlington Heights, IL).
7. DTT (0.1 *M* stock) (Gibco-BRL).
8. Template (Total RNA).
9. Heating block (VWR, Bridgeport, NJ).

2.5. PCR Amplifications

1. Ampli*Taq* DNA polymerase (Perkin Elmer, Foster City, CA).
2. Bind-Aid amplification kit (USB).
3. Ampliwax gem beads (Perkin Elmer).
4. Oligonucleotides (10 μ*M* stocks) (*see* **Table 2**).
5. Sterile water.
6. Thermocycler.
7. PCR tubes (Marsh).
8. Template (genomic DNA or cDNA).

2.6. Coupled In Vitro Transcription/Translation

1. TnT-coupled T7 transcription/translation system (Promega).
2. L-[^{35}S]-Methionine Tran^{35}S-label, >1000 Ci/mmol) (ICN, Los Angeles, CA).
3. RNasin (40 U/μL) (Promega).
4. Template (PCR product).
5. Heating block (VWR).

2.7. Gel Electrophoresis and Fluorography

1. Protein sample buffer: 10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 62.5 m*M* Tris-HCl, pH 6.8, and 0.002% bromophenol blue.
2. Gel electrophoresis rig, accessories, and power supply.
3. SDS-polyacrylamide gel, 10–20% gradient.
4. Stacking gel (5% stock solution, 500 m*M* Tris-HCl, pH 6.8).
5. Eletrophoresis buffer: 25 m*M* Tris-HCl, 0.192 *M* glycine, 0.1% SDS.
6. Ammonium persulfate (10% stock solution) and TEMED (Bio-Rad, Richmond, CA).
7. 10% SDS solution (Gibco-BRL).
8. Fixative solution: 30% methanol, 10% acetic acid.

Table 2
APC Gene PCR Amplification Oligonucleotide Primers

Primer[a]	Sequence (5' to 3')
Segment 1:	
Stage I (Outside-F)	CAA GGG TAG CCA AGG ATG GC
Stage I (Outside-A)	TTG CTA GAC CAA TTC CGC G
Stage II (internal-F)	GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGC TGC AGC TTC ATA TGA TC
Stage II (internal-R)	CTG ACC TAT TAT CAT CAT GTC G
Segment 2:	
F	GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGA TGC ATG TGG AAC TTT GTG G
R	GAG GAT CCA TTA GAT GAA GGT GTG GAC G
Segment 3:	
F	GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGT TTC TCC ATA CAG GTC ACG G
R	GGA GGA TCC TGT AGG AAT GGT ATC TCG
Segment 4:	
F	GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGA AAA CCA AGA GAA AGA GGC AG
R	TTC ACT AGG GCT TTT GGA GGC
Segment 5:	
F	GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGG TTT ATC TAG ACA AGC TTC G
R	GGA GTG GAT CCC AAA ATA AGA CC

[a]F = forward primers, R = reverse primers of a pair for amplification. All forward primers except stage I outside primer have the T7 transcription and translation nucleotide sequences at its 5' end.

9. ENHANCE (Dupont, NEN, Boston, MA).
10. Gel dryer, autoradiography film (Kodak [Rochester, NY] X-Omat), developer, and processor.
11. ^{14}C-labeled protein molecular weight standards (Gibco-BRL).

3. Methods

3.1. Blood Processing

1. Pipet 30 µL of EDTA anticoagulated whole blood into 1 mL of sterile water for Chelex DNA extraction (*see* **Subheading 3.3.**) (*see* **Note 1**).
2. Pour the rest of the EDTA anticoagulated whole blood (approx 10–20 mL) into a 50-mL conical tube.
3. Carefully pipet 12–15 mL of Histopaque-1077 into the bottom of the same 50-mL conical tube (*see* **Note 2**).
4. Centrifuge the 50-mL conical tube at 400*g* for 30 min at room temperature.

5. Transfer the opaque mononuclear layer (approx 5 mL) with plastic transfer pipet into a 15-mL conical tube and fill tube with Hank's balanced saline solution (HBSS) and invert mix several times (*see* **Note 3**).
6. Centrifuge the 15-mL conical tube at 400*g* for 5 min at room temperature.
7. Aspirate supernatant off and repeat wash of pellet with HBSS one more time.
8. Resuspend cell pellet in 1–2 mL of guanidinium isothiocyanate solution from Promega RNAgents kit (*see* **Note 4**).

3.2. DNA Extraction

1. Let mix of whole blood and water from **Subheading 3.1., step 1** sit at room temperature for 30 min with occasional mixing.
2. Microfuge at 12,000*g* for 3 min and pipet off supernatant.
3. Pipet 180 μL of a 5% Chelex solution in and tap mix.
4. Incubate at 56°C for 30 min, then briefly vortex.
5. Seal lids and boil for 8 min.
6. Briefly vortex, then centrifuge at 12,000*g* for 3 min (*see* **Note 5**).

3.3. RNA Extraction

1. Process 0.5 mL of guanidinium solution from **Subheading 3.1., step 8** above with Promega's RNagents reagents according to manufactures directions (acid quanidinium isothiocyanate-phenol-chloroforrn extraction method).
2. Wash precipitated RNA pellet with 70% ethanol.
3. Resuspend RNA precipitate in 100 μL of sterile water.

3.4. Amplification of APC Gene Segment 1 (RT-PCR)

3.4.1. First-Strand cDNA Synthesis

1. Total RNA of 5–10 μL from **Subheading 3.3., step 3** (approx 5 μg) is mixed with 1 μg of random hexamer, 1 μL of Bind-Aid, and 300 μL of superscript II reverse transcriptase and appropriate buffer containing dNTPs, DTT, and RNasin according to manufacturer's instructions in a 20 μL reaction (*see* **Note 6**).
2. Incubate reaction for 1 h at 37°C.
3. Heat inactivate at 65°C for 10 min, then cool on ice.
4. Use immediately in PCR reaction or store at –20°C.

3.4.2. Two-Stage Nested PCR Amplification

1. cDNA of 4 μL is mixed with 35 ng of each outside segment one primer, 2.5 U of Ampli*Taq*, and appropriate buffer containing dNTPs in a 20 μL PCR reaction according to Bind-Aid amplification kit manufacturer's instructions.
2. Thermocycle for 10 cycles of: 95°C for 30 s, 55°C for 2 min, 70°C for 2 min, then 70°C for 5 min.
3. For the second stage: 30 μL of an additional mix containing 350 ng of internal primers for segment one, 3.75 U of Ampli*Taq*, and appropriate Bind-Aid amplification kit buffer components including dNTPs are added to the 20 μL of first stage reaction.

4. Thermocycle the 50 μL PCR reaction for 30 cycles of: 95°C for 30 s, 62.5°C for 2 min, 70°C for 2 min, then 70°C for 5 min.
5. PCR products can be used immediately in the TnT reaction or stored at –20°C

3.5. Amplification of APC Segments 2–5 (PCR)

1. Five to ten microliters of chelex treated blood from **Subheading 3.2., step 5** (approx 100 ng of genomic DNA) is mixed with 350 ng of each appropriate primer pair, 5 U of Ampli*Taq*, and appropriate Bind-Aid amplification kit buffer components including dNTPs in 50 μL PCR reactions according to manufacturer's instructions with the addition of Ampliwax gem beads according to manufacturer's instructions for a form of "hot" start amplification (*see* **Note 7**).
2. Thermocycle for 35 cycles of 95°C for 30 s, anneal temp (*see* **Note 8**) for 90 s, 70°C for 90 s, then 70°C for 5 min.
3. PCR products can be used immediately or stored at –20°C.

3.6. Coupled Transcription/Translation Reaction

1. Thaw components of Promega's coupled TnT (T7 polymerase) kit and keep on ice throughout.
2. Mix 3 μL of PCR product from **Subheading 3.4.2., step 5** or **Subheading 3.5., step 3** with 40 μCi of ^{35}S-methionine translabel, 10 U of RNasin, and appropriate components of the TNT kit in a 25 μL reaction according to manufacturer's instructions.
3. Incubate reaction at 30°C for 1 h (*see* **Note 9**).
4. Add 25 μL of protein sample buffer to the reaction tube, then boil for 5 min and give quick spin to bring condensate down off lid (*see* **Note 10**).

3.7. Gel Electrophoresis and Fluorography

1. Rinse precasted 10–20% gradient gel (ISI) and place in casting mount.
2. After adding APS and TEMED, pour in top stacking gel layering over gradient gel.
3. Add comb and let polymerize for approx 45 min.
4. Remove comb and set up gel in electrophoresis apparatus in appropriate buffer.
5. Load samples (5 μL from **Subheading 3.6., step 5**) and electrophores at constant current (approx 30 mAmps) till dye at bottom of gel (approx 2.5 h).
6. Take down apparatus and place gel in fixative solution with gentle shaking for 30 min.
7. Place gel in ENHANCE solution for 60 min.
8. Place gel in water for 30 min.
9. Place gel on Whatman paper, cover with plastic wrap, and dry on gel dryer till dry (*see* **Note 11**).
10. Place dried gel on Whatman paper in film cassette and expose overnight at room temperature or at –80°C, then develop film.

4. Notes

1. Duplicate samples are usually made at this step since blood samples are not always easily obtained.

2. A moppet can be used to slowly add the Histopaque to the bottom of the tube below the blood that rises above the histopaque, being less dense.
3. If a clear layer of mononuclear cells is not formed after the first spin, repeating centrifugation can sometimes help form a better layer to pipet out. Blood that has been in tubes longer than 2 d can be hard to layer out the cells.
4. An aliquot can be extracted immediately for nucleic acids (RNA or DNA) or this solution can be stored frozen for later use. Syringe aspiration with a 23-gage needle can be used to help decrease viscous solutions.
5. This supernatant contains genomic DNA and can be used immediately in a PCR amplification reaction or stored frozen for later use with repeat vortexing and centrifugation.
6. A mock reverse transcription reaction including everything except the enzyme is performed in parallel and further used as template in PCR amplification to serve as a control to identify any contamination problems.
7. Reaction (40 μL) was first mixed including everything except Ampli*Taq* and Bind-Aid. An Ampliwax gem bead was then added to the tube with a sterile needle and heated at 70°C for several minutes to melt the wax to form a barrier; 10 μL of top mix containing Ampli*Taq* and Bind-Aid was then pipeted onto the solid wax barrier for subsequent thermocyling.
8. Annealing temperatures used for PCR amplification of the various segments of APC included: segment 2—65°C, segment 3—60°C, segment 4—62.5°C, segment 5—60°C.
9. Incubating this reaction longer can lead to increased protein degradation products. Protease inhibitors added little to prevent protein degradation.
10. This sample can be loaded for gel electrophoresis immediately or stored at –80°C for subsequent loading and analysis after thawing and reboiling. One tenth of the reaction usually gives adequate signals on fluorography.
11. Gel cracking can be a problem with high-percentage acrylamide gels (i.e., 20%). Therefore, steady heat and vacuum applied to the gel is critical in drying, often requiring several hours.

Acknowledgments

This work was supported in part by a Foundation AGA Research Scholarship award and NIH grant 1R29CA67900-01. I would like to thank Melissa Schmitt for help in preparing this chapter.

References

1. Powell, S. M., Petersen, G. M., and Krush, A. J. (1993) Molecular diagnosis of familial adenomatous polyposis. *N. Engl. J. Med.* **329,** 1982–1987.
2. van der Luijt, R., Khan, P. M., Vasen, H., van Lecuwen, C., Tops, C., Roest, P., den Dunen, J., and Fodde, R. (1994) Rapid detection of translationterminating mutations at the adenomatous polyposis cold *(APC)* gene by direct protein truncation test. *Genomics* **20,** 1–4.
3. Cotton, R. G. H. (1993) Current methods of mutation detection. *Mutat. Res.* **285,** 125–144.

4. Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., and Sekiya, T. (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single strand conformation polymorphisms. *Proc. Natl. Acad. Sci. USA* **86,** 2766–2770.
5. Myers, R. M., Maniatis, T., and Lerman, L. S. (1987) Detection and localization of single base changes by denaturing gradient gei electrophoresis. *Meth. Enzymol.* **155,** 501–527.
6. Landegran, U., Kaiser, R., Sanders, J., and Hood, L. (1988) A ligase mediated gene detection technique. *Science* **241,** 1077–1080.
7. Schad, C. R., Jalal, S. M., and Thibodeau, S. N. (1995) Genetic testing for Prader-Willi and Angelman syndromes. *Mayo Clin. Proc.* **70,** 1195,1196.
8. Kinzler, K. W., Nilber, M. C., Su, L. K., Vogelstein, B., Bryan, T. M., Levy, D. B., Smith, K. J., Preisinger, A. C., Hedge, P., McKechnie, D., Finniear, R., Markham, A., Groffen, J., Boguski, M. S., Altschul, S. F., Horii, A., Ando, H., Miyoshi, Y., Miki, Y., Nishisho, I., and Nakamura, Y. (1991) Identification of FAP locus genes from chromosome *5q21. Science* **253,** 661–665.
9 Groden, J., Thilveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., Sargeant, L., Krapcho, K., Wolff, E., Burt, R., Hughes, J. P., Warrington, J., McPherson, J., Wasmuth, J., LePaslier, D., Abderrahim, H., Cohen, D., Leppert, M., and White, R. (1991) Identification and characterization of the familial adenomatous polyposis cold gene. *Cell* **66,** 589–600.
10. Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S., Hedge, P., Markham, A., Krush, A. J., Petersen, G., Hamilton, S. R., Nilbert, M. C., Levy, D. B, Bryan, T. M., Preisinger, A. C., Smith, K. J., Su, L. K., Kinzler, K. W., and Vogelstein, B. (1991) Mutations of chromosome *5q21* genes in FAP and colorectal cancer patients. *Science* **253,** 665–669.
11. Joslyn, G., Carlson, M., Thiliveris, A., Albertsen, H., Gelbert, L., Samowitz, W., Groden, J., Stevens, J., Spirio, L., Robertson, M., Sargeant, L., Krapcho, K., Wolff, E., Burt, R., Hughes, J. P., Warrington, J., McPherson, J., Wasmuth, J., LePaslier, D., Abderrahim, H., Cohen, D., Leppert, M., and White, R. (1991) Identification of deletion mutations and three new genes at the familial polyposis locus. *Cell* **66,** 601–613.
12. Bussey, H. J. R. (1990) Historical clevelopments in familial adenomatous polyposis, in *Familial Adenomatous Polyposis* (Herrera, L., ed.), Liss, New York, pp. 1–7.
13. Miyoshi, Y. Ando, H., Nagase, H., Nishisho, I., Horii, A., Miki, Y., Mori, T., Utsunomiya, J., Baba, S., Petersen, G., Hamilton, S. R., Kinzler, K. W., Vogelstein, B., and Nakamura, Y. (1992) Germ-line mutations of the *APC* gene in 53 familial adenomatous polyposis patients. *Proc. Natl. Acad. Sci. USA* **89,** 4452–4456.
14. Groden, J., Gelbert, L., Thliveris, A., Nelson, L., Robertson, M., Joslyn, G., Samowitz, W., Spirio, L., Carlson, M., Burt, R., Leppert, M., and White, R. (1993) Mutational analysis of patients with adenomatous polyposis: identical inactivating mutations in unrelated individuals. *Am. J. Hum. Genet.* **52,** 263–272.
15. Nagase, H., Miyoshi, Y., Horii, A., Aoki, T., Petersen, G. M., Vogelstein, B., Maher, E., Ogawa, M., Maruyama, M., Utsunomiya, J., Baba, S., and Nakamura, Y. (1992) Screening for germ-line mutations in familial adenomatous polyposis patients: 61 new patients and a summary of 150 unrelated patients. *Hum. Mutat.* **1,** 467–473.

16. Nagase, H. and Nakamura, Y. (1993) Mutations of the *APC* (adenomatous polyposis coli) gene. *Hum. Mutat.* **2,** 425–434.
17. Jen, J., Powell, S. M., Papadopoulos, N., Smith, K. J., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. (1994) Molecular determinants of cysplasia in colorecal lesions. *Cancer Res.* **54,** 5523–5526.
18. Papadopoulos, N., Leach, F. S., Kinzler, K. W., and Vogelstein, B. (1995) Monoalleli mutation (MAMA) for identifying germline mutations. *Nature Genet.* **11,** 99–102.
19. Petersen, G. M. (1995) Genetic counseling and predictive genetic testing in familial adenomatous polyposis. *Sem. Colon Rectal Surg.* **6/1,** 55–60.
20. Lynch, H. T., Smyrk, T. C., Watson, P., Lanspa, S. J., Lynch, J. F., Lynch, P. M., Cavalieri, R. J., and Boland, C. R. (1993) Genetics, natural history, tumor spectrum, and pathology of hereditary nonpolyposis colorectal cancer. *Gastroenterology* **104,** 1535–1549.
21. Leach, F. S., Nicolaides, N. C., Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomaki, P., Sistonen, P., Aaltonen, L. A., Nystrom-Lahti, M., Guan, X. Y., Zhang, J., Meltzer, P. S., Yu, J. W., Kao, F. T., Chen, D.J., Cerosaletti, K. M., Rournier, R. E. K., Todd, S., Lewis, T., Leach, R. J., Naylor, S. L., Weissenbach, J., Mecklin, J. P., Jarvinen, H., Petersen, G. M., Hamilton, S. R., Green, J., Jass, J., Watson, P., Lynch, H. T., Trent, J. M., de la Chapelle, A., Kinzler, K. W., and Vogelstein, B. (1993) Mutations of a *mutS* homolog in hereditary non-polyposis colorectal cancer. *Cell* **75,** 1215–1225.
22. Fishel, R., Lescoe, M. K., Rao, M. R. S., Copeland, N. G., Jenkins, N. A., Garber, J., and Kolodner, R. (1994) The human mutator gene homolog *MSH2* and its association with hereditary nonpolyposis colon cancer. *Cell* **75,** 1027–1038.
23. Papadopoulos, N., Nicolaides, N. C., Wei, Y. F., Ruben, S. M., Carter, K. C., Rosen, C. A., Haseltine, W. A., Fleischmann, R. D., Fraser, C. M., Adams, M. D., Venter, J. C., Hamilton, S. R., Petersen, G. M., Watson, P., Lynch, H. T., Peltomiki, P., Mecklin, J. P., de la Chapelle, A., Kinzler, K. W., and Vogelstein, B. (1994) Mutation of a *mutL* homolog in hereditary colon cancer. *Science* **263,** 1625–1629.
24. Bronner, C. E., Baker, S. M., Morrison, P. T., Warren, G., Smith, L. G., Lescoe, M. K., Kane, M., Earabino, C., Lipford, J., Lindblom., A., Tannergard, P., Bollag, R. J., Godwin, A. R., Ward, D. C., Nordenskjold, M., Fishel, R., Kolodner, R., and Liskay, R. M. (1994) Mutation in the DNA mismatch repair gene homologue *hMLH1* is associated with hereditary non-polyposis colon cancer. *Nature* **368,** 258–261.
25. Nicolaides, N. C., Papadopoulos, N., Liu, B., Wei, Y. F., Carter, K. C., Ruben, S. M., Rosen, C. A., Haseitine, W. A., Fleischmann, R. D., Fraser, C. M., Adams, M. D., Venter, J. C., Dunlop, M. G., Hamilton, S. R., Petersen, G. M., de la Chapelle, A., Vogelstein, B., Kinzler, K. W. (1994) Mutations of two *PMS* homologues in hereditary nonpolyposis colon cancer. *Nature* **371,** 75–80.
26. Liu, B., Parsons, R., Papadopoulos, N., Nicolaides, N., Lynch, H. T., Wastson, P., Jass, J. R., Dunlop, M., Wyllie, A., Peltomaki, P., De La Chapelle, A., Hamilton,

S. R., Vogelstein, B., and Kinzler, K. W. (1996) Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. *Nature Med.* **2,** 169–174.

27. Luce, M. C., Marra, G., Chauhan, D. P., Laghi, L., Carethers, J. M., Cherian, S. P., Hawn, M., Binnie, C. G., Kam-Morgan, L. N. W., Cayouette, M. C., Koi, M., and Boland, C. R. (1995) *In vitro* transcription/translation assay for the screening of *hM4H1* and *hMSH2* mutations in familial colon cancer. *Gastroenterology* **109,** 1368–1374.
28. Liu, B., Farrington, S. M., Petersen, G. M., Hamilton, S. R., Parsons, R., Papadopoulos, N., Fijiwara, T., Jen, J., Kinzler, K. W., Wyllie, A. H., Vogelstein, B., and Dunlop, M. G. (1995) Genetic instability occurs in the majority of young patients with colorectal cancer. *Nature Med.* **1/4,** 348–352.
29. Trofatter, J. A., MacCollin, M. M., Rutter, J. L., Murrell, J. R., Duyao, M. P., Parry, D. M., Eldridge, R., Kley, N., Menon, A. G., Pulaski, K., Haase, V. H., Ambrose, C. M., Munroe, D., Bove, C., Haines, J. L., Martuza, R. L., MacDonald, M. E., Seizinger, B. R., Short, M. P., Buckler, A. J., and Gusella, J. F. (1993) A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. *Cell* **72,** 791–800.
30. Rouleau, G. A., Merel, P., Lutchrnan, M., Sanson, M., Zucman, J., Marineau, C., Hoang-Xuan, K., Demczuk, S., Desmiaze, C., Plougastel, B., Pulst, S. M., Lenoir, G., Bijlsma, E., Fashold, R., Dumanski, J., de Jong, P., Parry, D., Eldrige, R., Aurias, A., Delattre, O., Thomas, G. (1993) Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. *Nature* **363,** 515–521.
31. Latif, F., Tory, K., Gnarra, J., Yao, M., Duh, F. M., Orcutt, M. L., Stackhouse, T., Kuzmin, I., Modi, W., Geil, L., Schmidt, L., Zhou, F., Li, H., Wei, M. H., Chen, F., Glenn, G., Choyke, P., Walther, M. M., Weng, Y., Duan, D. S. R., Dean, M., Glavac, D., Richards, F. M., Crossey, P. A., Ferguson-Smith, M. A., Le Paslier, D., Chumakov, L., Cohen, O., Chinault, A. C., Maher, E. R., Linehan, W. M., Zbar, B., and Lerman, M. I. (1993) Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* **260,** 1317–1320.
32. Malhotra, S. B., Hart, K. A., Klamut, H. J., Thomas, N. S. T., Bodrug, S. E., Burghes, A. H. M., Bobrow, M., Harper, P. S., Thompson, M. W., Ray, P. N., and Worton, R. G. (1988) Frame-shift deletions in patients with Duchenne and Becker muscular dystrophy. *Science* **242,** 755–759.
33. Collins, F. S. (1996) *BRCA1*–Lots of mutations, lots of dilemmas. *N. Engl. J. Med.* **334/3,** 186–188.
34. Kuivaniemi, H., Tromp, G., and Prockop, D. J. (1991) Mutations in collagen genes: causes of rare and some common diseases in humans. *FASEB J* **5,** 2052–2060.
35. Yandell, D. W., Campbell, T. A., Dayton, S. H., Petersen, R., Walton, D., Little, J. B., McConkie-Rosell, A., Buckley, E. G., and Dryja, T. P. (1989) Oncogenic point mutations in the human retinoblastoma gene: their application to genetic counseling. *N. Engl. J. Med.* **321/25,** 1689–1695.
36. Kazazian, H. H. Jr., Dowlin, C. E., Boehm, C. D, Warren, T. C., Economou, E. P., Katz, J., and Antonarakis, S. E. (1990) Gene defects in beta-thalassemia and their prenatal diagnosis. *Ann. NY Acad. Sci.* **612,** 1–14.

37. Giannelli, F., Green, P. M., High, K. A., Lozier, J. N., Lillicrap, D. P., Ludwig, M., Olek, K., Reitsma, P. H., Goossens, M., Yoshioka, A., Sommer, S., and Brownlee, G. G. (1990) Haemophilia B: database of point mutations and short additions and deletions. *Nucleic Acids Res.* **18,** 4053–4058.
38. Tsui, L. C. (1992) Mutations and sequence variations, detected in the cystic fibrosis transmembrane conductance regulator (CFTR) gene: a report from the cystic fibrosis genetic analysis consortium. *Hum. Mutat.* **1,** 197–203.
39. Tsui, L. C. (1992) The spectrum of cystic fibrosis mutations. *Trends Genet.* **8/11,** 392–398.
40. Collins, F. S. (1992) Cystic fibrosis: molecular biology and therapeutic implications. *Science* **256,** 775–779.
41. Heim, R. A., Silverman, L. M., Farber, R. A., Kam-Morgan, L. N. W., and Luce, M. C. (1994) Screening for truncated *NF1* proteins. *Nature Genet.* **8,** 218, 219.
42. Roest, P. A. M., Roberts, R. G., Sugino, S., van Ommen, G. B., and Dunnen, J. T. (1993) Protein truncation test (PTT) for rapid detection of translation-terminating mutations. *Hum. Mol. Genet.* **2,** 1719–1721.
43. Hogervorst, F. B. L., Cornelis, R. S., Bout, M., van Vliet, M., Oosterwijk, J. C. Olmer, R., Bakker, B., Klijn, J. G. M., Vasen, H. F. A., Meijers-Heijboer, H., Menko, F. H., Cornelisse, C. J., den Dunnen, J. T., Devilee, P., and van Ommen, G. J. B. (1995) Rapid detection of *BRCA1* mutations by the protein truncation test. *Nat. Genet.* **10,** 208–212.
44. Powell, S. M. (1995) Clinical applications of molecular genetics in colorectal cancer. *Sem. Colon. Rectal Surg.* **6(1),** 2–18.

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PCR Fingerprinting for Detection of Deleted or Amplified Sequences in Human Cancer

Takashi Kohno and Jun Yokota

1. Introduction

Genomic fingerprinting is one of several methods for screening human genome to identify genetic alterations in cancer cells. Arbitrarily primed polymerase chain reaction (AP-PCR) is a PCR-based genomic fingerprinting method ***(1)***. In AP-PCR, a single oligonucleotide is used to initiate DNA synthesis from sites along the template with which it matches only imperfectly. PCR is performed under a low stringent condition in the initial five cycles to permit hybridization of arbitrary primers to various sequences dispersed throughout the human genome. This is followed by 30–35 cycles of PCR under high stringent condition, so that only the best matches of the initial annealing events are further amplified. By controlling the stringency of the initial cycles, 50–100 distinct DNA fragments can be amplified from the human genome by a single PCR. When those fragments are size fractionated by gel electrophoresis, a representative sample of cell genome is visualized by staining or autoradiography as a genomic fingerprint. Because of the arbitrary nature for designing a primer and the low stringent condition for hybridization of a primer, DNA fragments are simultaneously amplified from various chromosomal regions in a single PCR. By comparison of AP-PCR genomic fingerprints of DNA from tumors and normal tissues, deleted and amplified DNA sequences in cancer cells have been detected and cloned ***(2–5)***. Intensities of deleted and amplified DNA fragments are decreased and increased, respectively, by AP-PCR. Therefore, AP-PCR is a simple and effective method to screen quantitative and qualitative differences between normal cell genomes (**Fig. 1**). Theoretically, qualitative changes such as chromosomal rearrangements can also be detected by AP-PCR. However, since only a few kilobases of the human genome can be

From: *Methods in Molecular Biology, Vol. 92: PCR in Bioanalysis*
Edited by: S. J. Meltzer

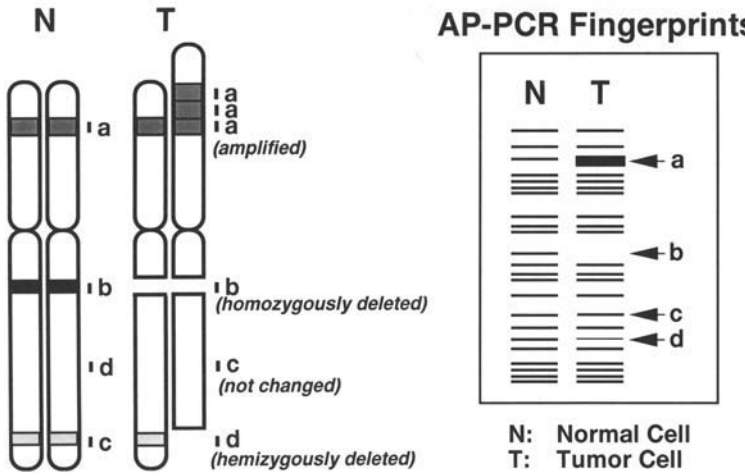

Fig. 1. AP-PCR fingerprinting for detection of deleted and amplified sequences in tumor cells.

screened in a single experiment, it would be a laborious work to detect qualitative changes by this method. The advantage of AP-PCR fingerprinting method is that targeted DNA fragments can be easily reamplified and cloned by reamplification of the target sequence with the same primer as the one used for initial genomic fingerprinting. The disadvantage of AP-PCR is the lack of information about map location of each amplified DNA fragment.

2. Materials

2.1. AP-PCR Amplification

1. Sterile water.
2. 10X AP-PCR buffer: 100 m*M* Tris-HCl (pH 9.0 at room temperature), 20–50 m*M* $MgCl_2$, and 500 m*M* KCl. Prepare by adding 5–35 μL of 1 *M* $MgCl_2$ solution to 1 mL 10X PCR buffer (Pharmacia Biotech, Piscataway, NJ).
3. 10 m*M* stocks of deoxynucleotides dATP, dTTP, dCTP, and dGTP. Prepare by adding 100 μL of each 100 m*M* dNTP stocks (Pharmacia Biotech) to 600 μL sterile water for a total volume of 1 mL.
4. Oligonucleotide primers diluted in sterile water to 0.5 μg/μL (*see* **Note 1**).
5. α-[^{32}P] dCTP (~3000 Ci/mmol, PB10205, Amersham, Cleveland, OH).
6. *Taq* polymerase (5 U/μL).
7. Sterile 0.2-mL PCR reaction tubes.
8. Genomic DNA samples prepared according to the standard method (0.1 μg/μL, *see* **Note 2**).

2.2. Denaturing Polyacrylamide Gel Electrophoresis (see *Note 3*)

1. 5% polyacrylamide/8% urea sequencing gel.
2. 1X TBE buffer.

3. Denaturing loading buffer: 95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 10 m*M* EDTA (pH 8.0).
4. Autoradiogram markers (Glogos II, Stratagene, La Jolla, CA).
5. X-ray films and exposure cassettes.

2.3. Purification and Reamplification of the Band

1. 10X PCR buffer (Pharmacia Biotech): 100 m*M* Tris-HCl (pH 9.0 at room temperature), 15 m*M* $MgCl_2$, and 500 m*M* KCl.

2.4. Characterization of the Reamplified Band

1. QIAquick PCR Purification Kit (QIAGEN, Santa Clarita, CA).

3. Methods

3.1. PCR Amplification

1. Prepare a master mix of PCR reagents containing 3 μL of 10X AP-PCR buffer (29.5 μL per PCR reaction), 0.6 μL of 10 m*M* stocks of deoxynucleotides, 0.3 μL of a primer, 0.5 μL of α-^{32}P-dCTP (3000 Ci/mL), 0.2 μL of *Taq* polymerase, and sterile water. Make enough for one extra PCR reaction to ensure there is enough for all samples.
2. Add 0.5 μL of sample DNA to the appropriately labeled PCR tubes.
3. Add 29.5 μL of the master mix to each PCR tube and mix thoroughly by pipeting.
4. Amplify the samples according to the following cycling program (*see* **Note 4**):
   Initial denaturation:
   94°C, 5 min
   Followed by 5 cycles of:
   94°C, 40 s
   37–50°C, 40 s
   72°C, 90 s
   Followed by 30–35 cycles of:
   94°C, 40 s
   55°C (annealing temperature, *see* **Note 5**), 40 s
   72°C, 90 s
   Hold: 4°C.
5. Store PCR products at –20°C until electrophoresis.

3.2. Denaturing Polyacrylamide Gel Electrophoresis (see *Note 3*)

1. Dilute 3 μL of PCR products with 27 μL of denaturing loading buffer and incubate at 90°C for 2 min. Chill the solution on ice immediately, and load 2 μL on a 8 *M* urea/5% polyacrylamide sequencing gel.
2. Electrophorese the samples at 1500 V for 3–5 h.
3. Dry the gel under vacuum at 80°C and expose to an X-ray film with autoradiogram markers.
4. Compare AP-PCR fingerprints of DNA from tumors and normal tissues (*see* **Note 5**).

3.3. Purification and Reamplification of the Band

1. Align autoradiogram markers on the gel with their exposed images. Mark the exact position of the band in the dried gel using a needle. Excise the gel with a scalpel. Re-exposure of the gel will confirm the accuracy in the excision of the band.
2. Place the excised portion of the gel (0.5–1 × 3–5 mm) in 50–100 μL of water.
3. Incubate at 100°C for 15 min to elute the DNA.
4. Vortex for 5 min.
5. Repeat steps 3–4 twice.
6. Prepare a master mix of PCR reagents containing (29 μL per PCR reaction) 3 μL of 10X PCR buffer, 0.6 μL of 10 m*M* stocks of deoxynucleotides, 0.3 μL of a primer, 0.2 μL of *Taq* polymerase, and sterile water.
7. Add 1 μL of sample DNA to the appropriately labeled PCR tubes.
8. Add 29 μL of the master mix to each PCR tube and mix thoroughly by pipeting.
9. Amplify the samples according to the following cycling program:
   Initial denaturation:
   94°C, 5 min
   Followed by 40–45 cycles of:
   94°C, 40 s
   55°C (annealing temperature, *see* **Note 6**), 40 s
   72°C, 90 s
   Hold: 4°C.
10. Store PCR products at –20 °C.

3.4. Characterization of the Reamplified Band

1. Purify the PCR products using QIAquick PCR, Purification Kit according to suppliers' protocol (*see* **Note 7**).
2. Perform Southern blot hybridization analysis against genomic DNA used for AP-PCR analysis using the reamplified DNA fragments as probes to examine whether the decreased and increased intensities of the PCR bands in tumor DNA represent, respectively, deletion and amplification of the corresponding genomic fragments in cancer cells (*see* **Note 8**).

4. Notes

1. Some oligonucleotides have been reported to generate a reproducible fingerprint of human genome DNA ***(2–5)***. However, any oligonucleotides, including the ones designed for other purposes, can be applied. Although the authors use a single oligonucleotide for AP-PCR, a set of oligonucleotides can be also used ***(3)***.
2. Genomic DNA are extracted from cell lines or frozen tissues using proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation in our laboratory.
3. *See* **ref. *6*** for detailed procedure for the preparation of sequencing gel, electrophoresis, and autoradiography.
4. The authors use an automated Perkin-Elmer model 2400 or model 9600 thermocycler. Annealing temperature in the initial five PCR cycles, which is suit-

able for generating reproducible fingerprint, is different for each oligonucleotide primer used for AP-PCR. The temperature that results in reproducible PCR products needs to be adjusted. The authors usually perform the initial five PCR cycles at an annealing temperature of 42°C.

5. Differences in intensities of the PCR bands in tumor DNA does not always represent deletion and amplification of the corresponding genomic fragments in tumor cells. Some of these differences represent genetic polymorphisms in the human population. It must be checked whether they were present in normal cell samples. Aneuploidy of a tumor cell reflects the differences in the intensities of AP-PCR bands. Chromosome aberrations, such as trisomy and tetrasomy, are frequent in tumor cells. The intensity of DNA fragments in fingerprints could differ in a few fold among cancer cells, if those fragments are located on trisomic or tetrasomic chromosomes. Therefore, we consider that only DNA fragments of more than five times higher intensities in cancer cells are derived from amplified regions ***(4)***.
6. Annealing temperature is determined as 5–7°C below the T_m. T_m for oligonucleotides can be estimated by multiplying the number of A+T residues by 2°C and the number of G+C residues by 4°C and adding the two numbers.
7. Before **step 1, Subheading 3.4.**, it must be checked whether major PCR products are in the appropriated sizes by fractionating 5 μL of PCR products using 3% NuSieve agarose gel stained with ethidium bromide.
8. *See* **ref. *6*** for detailed procedure of Southern blot hybridization analysis against genomic DNA. Since reamplified fragments sometimes contain repetitive sequences, such as *Alu*, it is better to use denatured human placental DNA (Sigma) as a blocking reagent instead of salmon sperm DNA. After **step 2**, the authors usually clone the reamplified fragment using pGEM-T vector systems (Promega) for sequencing and further investigation.

Acknowledgments

We thank Manuel Perucho, Junko Takita, and Tomoko Okazaki for their considerable contributions towards the development and optimization of the AP-PCR method. This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare for the 2nd-term Comprehensive 10-Year Strategy for Cancer Control, Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan, and from the Vehicle Racing Commemorative Foundation.

References

1. Welsh, J. and McClell, M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18,** 7213–7218.
2. Peinado, M. A., Malkhosyan, S., Velazquez, A., and Perucho, M. (1992) Isolation and characterization of allelic losses and amplifications in colorectal tumors by arbitrarily primed polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **89,** 10,065–10,069.

3. Kohno, T., Morishita, K., Takano, H., Shapiro, D. N., and Yokota, J. (1994) Homozygous deletion at chromosome 2q33 in human small-cell lung carcinoma identified by arbitrarily primed PCR genomic fingerprinting. *Oncogene* **9,** 103–108.
4. Okazaki, T., Takita, J., Kohno, T., Handa, H., and Yokota, J. (1996) Detection of amplified genomic sequences in human small-cell lung carcinoma cells by arbitrarily primed-PCR genomic fingerprinting. *Hum. Genet.* **98,** 253–258.
5. Achille, A., Biasi, M. O., Zamboni, G., Bogina, G., Magalini, A. R., Pederzoli, P., Perucho, M., and Scarpa, A. (1996) Chromosome 7q allelic losses in pancreatic carcinoma. *Cancer Res.* **56,** 3808–3813.
6. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

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