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p53 Protocols

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Adenovirus Expressing p53

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1. Introduction

Mutation in the *p53* gene is the most frequently found genetic abnormality in human tumors, resulting in increased resistance to chemo- and radiotherapy ***(1,2)***. The underlying rationale for *p53*-mediated cancer gene therapy is to restore the ability of *p53* mutant tumors to undergo apoptosis. When the wild-type *p53* gene is delivered into tumor cells of various origins by adenoviral vector, the cells become more sensitive to cisplatin and radiation therapy ***(3)***. In addition, adenovirus expressing p53 is a great laboratory tool for the determination of function, for conveniently producing large quantities of p53 protein, and for determining the effect of p53 on cellular gene expression using microarray technology ***(4)***.

The technology for making recombinant adenovirus was developed in the early 1980s by several groups ***(5,6)***. Although the principles for making recombinant adenovirus remain the same today, sophisticated and clever ways of conveniently making these viruses are continuously evolving. The most commonly used virus DNA backbone has the *EIA*/*E1B* genes deleted, which makes this virus replication incompetent, unless these protein functions are provided in *trans*. Stably transfected cell lines that express the E1A and E1B proteins can support replication of virus ***(7,8)***. The *E3* region, which is not essential for the adenovirus life cycle, has also been used as an integration site for *trans*-genes in $E1A^+$ adenovirus, in which case the virus is able to replicate in a variety of different cell types ***(9)***. The maximum DNA packaging capacity of an adenovirus is 105% of the approx 36-kb adenovirus genome, making it possible to insert DNA fragments of 7.5–8 kb in the first and second generation

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adenovirus vectors ***(10)***, which is usually more than sufficient for typical applications. More recent vector designs have taken advantage of removing either the *E2* or *E4* or both regions ***(11,12)***, making it possible to insert even larger pieces of DNA. Conditionally replicating adenoviruses have been developed that only replicate in p53 mutant cells or in tumor cells using a tumor-specific promoter driving E1A/E1B expression, and are expected to improve the spread of virus within the tumor ***(13,14)***.

A number of different ways for constructing recombinant adenovirus have been reported ***(5,6)***. More recently, methods have been developed that rely entirely on making virus in bacteria ***(15–17)***. A plasmid having the entire adenovirus genome with the desired mutations and/or deletions is used. This plasmid is linearized at the desired site of *trans*-gene insertion, e.g., either in the *E1A* or *E3* regions, and mixed with a DNA fragment having the *trans*-gene flanked by DNA sequences homologous to the flanking sequences in the linearized plasmid. These DNAs are then introduced by standard bacterial transformation into an *Escherichia coli* strain with mutations in the *recB* and *recC* genes ***(16)***. As a result, these cells are void of any exonuclease RecB/C activity and are highly prone to undergo homologous recombination of the linearized DNAs. A large proportion of the resulting plasmid clones will have the desired *trans*-gene inserted into the larger plasmid. The adenovirus DNA, including the *trans*-gene, is then released from this plasmid by digestion with a restriction endonuclease. Subsequently, adenovirus is made by transfecting human 293 cells, which express the E1A/E1B proteins, allowing for replication and maturation of virus. This bacterial approach for generating recombinant adenovirus is very attractive, not only because of the ease and speed by which one can generate new virus, but also because the resulting adenovirus is clonal even before it passes through the 293 cells. Therefore, only one or a few plasmid clones may have to be transfected into 293 cells and tested for *trans*-gene function without the tedious and repeated plaque purification that is needed using the more traditional methods for making virus.

This chapter describes in detail our protocol for making adenoviruses in bacteria expressing various p53 derivatives, including wild-type and mutant proteins (R175H, N239S, R248W, R273H, D281G, $CTD_{305–392}$), that we have used to investigate ways of increasing radiosensitization of human glioma cells ***(3)*** (Valerie et al., in preparation).

2. Materials

1. pTGCMV, pZeroTGCMV, p53 cDNA plasmids.
2. *E. coli* strains BJ5183, XL1-Blue (Stratagene, La Jolla, CA, USA).
3. Restriction endonucleases (New England Biolabs, Beverly, MA, USA), *Pac*I, *Pme*I, etc.

4. HEK293 (for making virus), A549, or other suitable cell line that can easily be transduced by adenovirus.
5. Tissue culture media and fetal bovine serum (FBS), antibiotics, dishes and flasks, Noble agar (Difco, Detroit, MI, USA), etc.
6. Standard molecular biology supplies: CsCl, Tris buffer, NaCl, polythylene glycol (PEG)-8000 (Fisher Scientific, Pittsburgh, PA, USA), etc.
7. Supplies for growing *E. coli*: T broth, agar, Petri dishes, etc.
8. Ampicillin, kanamycin, carbenicillin (Sigma, St. Louis, MO, USA).
9. Centrifuges and rotors: Sorvall® (GS-3) (NEN® Life Science Products, Boston, MA, USA); Beckman Instruments (Fullerton, CA, USA) (SW28, SW41), or equivalent.
10. Superfect (Qiagen, Valencia, CA, USA; cat. no. 301307), or equivalent transfection reagent.
11. Agarose electrophoresis equipment.
12. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) equipment and gels.
13. Anti-p53 antibody (Ab-1; Calbiochem-Novabiochem, San Diego, CA, USA).
14. Western blot equipment, e.g., Bio-Rad (Hercules, CA, USA) electrophoresis and transfer units.
15. Dialysis cassettes (Pierce Chemical, Rockford, IL, USA)

2.1. Solutions

1. LB agar (500 mL): 5 g bacto-tryptone, 2.5 g bacto-yeast extract, 5 g NaCl, 7.5 g bacto-agar. Adjust the pH by adding 150 µL of 10 N NaOH. Bring vol to 500 mL with deionized water. Autoclave.
   To pour plates:
   Melt agar in microwave or use directly from autoclave.
   Cool to 55°C.
   To 500 mL of agar, add 500 µL filter-sterilized ampicillin (100 mg/mL stock) or carbenicillin (50 mg/mL stock) to 100 or 50 µg/mL final concentration or 500 µL kanamycin (30 mg/mL stock) to 30 µg/mL final concentration.
   Add 5 mL filter-sterilized 20% glucose.
   Makes 20–25 plates.
2. SOB (500 mL): 10 g bacto-tryptone, 2.5 g bacto-yeast extract, 0.25 g NaCl. Bring vol to 500 mL with deionized water. Autoclave.
3. T broth (4 L): 48 g bacto-tryptone, 96 g bacto-yeast extract, 16 mL glycerol. Bring vol to 4 L with deioinized water. Autoclave.
4. SOC (25 mL): 25 mL SOB, 250 µL 1 *M* $MgCl_2$, 250 µL 1 *M* $MgSO_4$, 500 µL 20% glucose. Filter-sterilize (0.22 µm).
5. TB salt (100 mL): 2.31 g KH_2PO_4, 12.54 g K_2HP0_4. Bring vol to 100 mL with deionized water. Autoclave. Add 50 mL TB salt per 500 mL TB broth before use.
6. 1.8% Noble agar: 0.45 g Noble agar (Difco; cat. no. 0142-15-2). Put into 125-mL bottle. Bring vol to 25 mL with distilled water. Autoclave.

7. 10 m*M* Tris-HCl (500 mL): 0.6 g Tris-base. Bring vol to 450 mL. Use concentrated HCl to adjust the pH to 8.0. Bring vol to 500 mL with distilled water. Filter-sterilize (0.22 μm) and store refrigerated.
8. Light CsCl (100 mL): 28.85 g CsCl. Bring vol to 100 mL with 10 m*M* Tris-HCl (pH 8.0). Filter-sterilize (0.22 μm) and store refrigerated.
9. Heavy CsCl (100 mL): 73.44 g CsCl. Bring vol to 100 mL of 10 m*M* Tris-HCl (pH 8.0). Filter-sterilize (0.22 μm) and store refrigerated.
10. Dialysis buffer: 13% glycerol in 1X phosphate-buffered saline (PBS) with 2 m*M* $MgCl_2$ (4 L): 520 mL glycerol, 38.2 g PBS (Sigma), 4 mL 2 *M* $MgCl_2$ (sterile). Bring vol to 4 L with distilled water. Filter-sterilize (0.22 μm) into 500-mL sterile glass bottles (store refrigerated).
11. 20% PEG-8000 with 2.5 *M* NaCl (1 L): 200 g of PEG-8000, 146.1 g NaCl. Bring vol to 1 L with distilled water. Heat gently to dissolve. Autoclave.
12. 2X Dulbecco's modified Eagle's medium (DMEM) (500 mL): 13.5 g DMEM (Sigma; cat. no. D-7777), 3.7 g sodium bicarbonate. Bring vol to 500 mL with distilled water. Filter-sterilize (0.22 μm) and dispense 100 mL in sterile 125-mL glass bottles. Store refrigerated. Add 1 mL FBS and 0.5 mL penicillin–streptomycin (pen/strep) per 25 mL when used.

3. Methods

In the following sections, we describe how to make a first-generation adenovirus expressing human p53. The steps involve: (*i*) cloning of the p53 cDNA in the adenovirus transfer plasmid, pZeroTGCMV; (*ii*) recombination of the p53 expression cassette into the adenovirus plasmid, pTGCMV; (*iii*) generation of adenovirus in HEK293 cells; (*iv*) characterization of p53 adenovirus; and (*v*) purification of a large-scale preparation of p53 adenovirus.

3.1. Transformation and Recombination in Bacteria

The first step to generate an adenovirus expressing p53 is to clone the cDNA into pZeroTGCMV, a modified version of the plasmid pTG9539 ***(16)***, which confers kanamycin resistance rather than ampicillin. The wild-type and R273H p53 cDNAs were isolated from pLp53BRHL and pLp53ERNL (kindly provided by Wen-Hwa Lee, University of Texas, San Antonio, TX) on an agarose gel and cloned into pZeroTGCMV ***(18)***. Once the "transfer plasmid" has been made (**Fig. 1**), follow the procedure below.

3.1.1. Preparation of Adenovirus Transfer Plasmid

1. Digest 5–10 μg of pZeroTGCMV-p53 plasmid DNA overnight with *Pac*I, followed by digestion with a second enzyme that cuts downstream of the insert (but does not cut within the insert). We generally use *Bgl*II or *Bsu*36I (*see* **Note 1**). Sequential digestion may be necessary if the enzymes are not compatible with the same buffer.
2. Run 5 μL (1/20 total vol) on 1% agarose gel to ensure complete digestion.

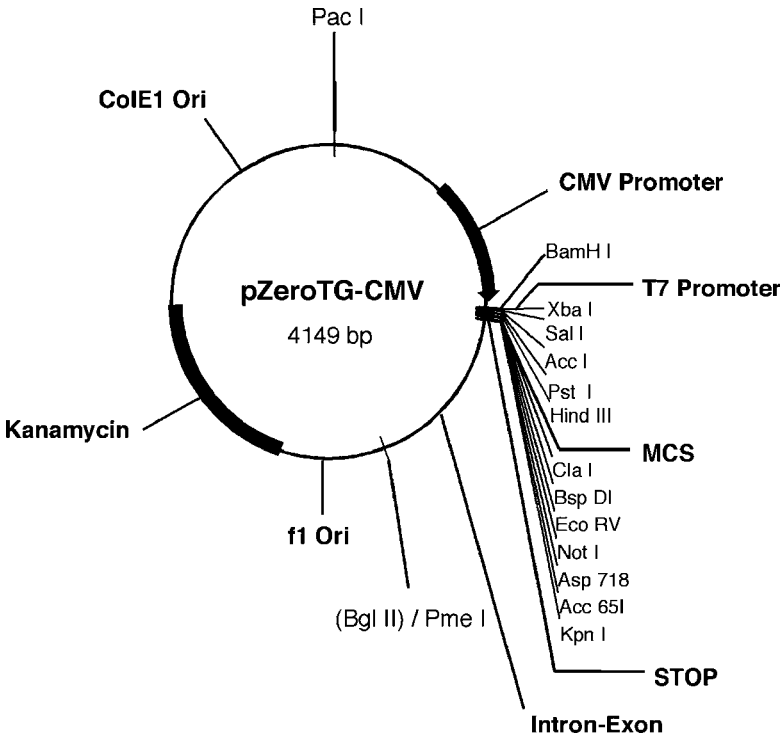

Fig. 1. Advenovirus transfer plasmid.

3. Phenol extract and ethanol precipitate DNA.
4. Resuspend in 10 µL TE buffer and run 1 µL on an agarose gel to quantify by visualization.

3.1.2. Transformation of BJ5183 Cells

In this step, the released Ad-cytomegalovirus (CMV)-p53-Ad DNA fragment is recombined into *Cla*I-digested pTGCMV (**Fig. 2**). Co-transform 3–5 µg of digested pZeroTGCMV-p53 with 0.5 µg of *Cla*I-digested pTGCMV, as follows:

1. Thaw competent BJ5183 cells (*see* **Note 2**) on ice. Aliquot 50 µL of cells for each transformation. Include in separate tubes; negative controls (no DNA and pTGCMV digested with *Cla*I [0.5 µg]) and positive control (uncut pTGCMV [0.5 µg]). The pTGCMV/*Cla*I control transformation should produce significantly fewer colonies on agar plates than co-transformation with pZero-TGCMV-p53.
2. Add DNA to competent cells, and incubate on ice for 30 min. Keep DNA to 10% or less of cell vol (e.g., 5 µL of DNA to 50 µL of cells).
3. Heat shock at 42°C degrees for 80 s.

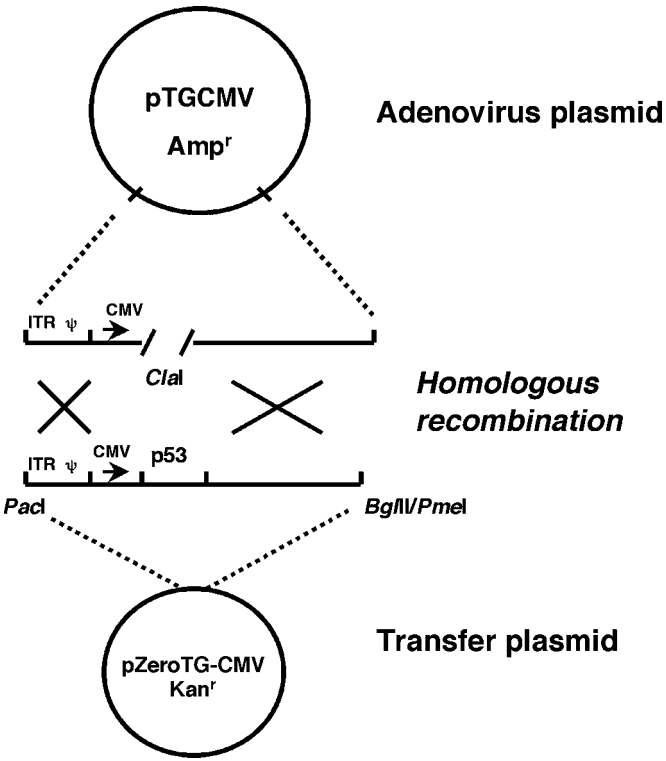

Fig. 2. Recombination in *E. coli* for generating adenovirus.

4. Incubate on ice for 2 min.
5. Add 400 μL SOC broth to each tube.
6. Incubate at 37°C for 1–1.5 h.
7. Spread 150 μL from each transformation reaction onto a LB-carbenicillin plate and incubate plate overnight at 37°C. We use carbenicillin rather than ampicillin, because "satellites" form on ampicillin plates.
8. The following morning, check negative and positive control plates. There should be no colonies on the "No DNA" plate, many on the "uncut pTGCMV" plate, and no to few colonies on the "pTGCMV/*Cla*I" plate. There should be more colonies on the plate that received both pZeroTGCMV-p53 and pTGCMV/*Cla*I. Pick four colonies from this plate, and grow 10-mL cultures in T Broth supplemented with ampicillin (100 μg/mL).
9. Shake at 37°C for 6–8 h or overnight (*see* **Note 3**).
10. Centrifuge at 5000 rpm (4400*g*) for 5 min, pour off supernatant, freeze bacterial cell pellet on dry ice, and store at –20°C until ready to proceed.

3.1.3. Purification of Plasmid DNA and Transformation into XL-1 Blue Cells

The quality of the plasmid DNA from BJ5183 cells is generally very poor. Therefore, we find it more convenient to pick a handful of colonies, transfer the plasmid DNA into XL-1 Blue cells, and perform restriction digest analysis rather than using polymerase chain reaction (PCR) to identify positive clones. The recombination in BJ5183 cells occurs with high probability, so one does not need to examine many clones to get the desired one.

1. Purify plasmid DNA using Bio-Rad's Plasmid Miniprep Kit or similar method. Elute DNA in 100 μL.
2. Transform 5 μL of DNA (1/20 of miniprep) with 50 μL of competent cells. Set up one reaction for each BJ5183 colony pick (should be four reactions total) and one positive control using 0.5 μL of pUC18 control from Stratagene (provided with competent cells).
3. Add 0.85 μL of 2-mercaptoethanol per reaction and incubate on ice for 10 min.
4. Add DNA to tubes and incubate on ice for 30 min.
5. Heat shock at 42°C for 45 s.
6. Incubate on ice for 2 min.
7. Add 400 μL TB broth to each tube.
8. Incubate at 37°C for 1.5 h.
9. Spread 50–150 μL from each transformation reaction onto LB-ampicillin plates and incubate overnight at 37°C.
10. The following day, pick one colony from each plate and grow 10-mL cultures in T Broth supplemented with ampicillin.
11. Shake at 37°C overnight.
12. Remove 1-mL of bacterial culture from each tube, and store at 4°C in microfuge tube until positive clones have been identified. Upon positive confirmation that the *p53* gene has been successfully recombined into the pTGCMV plasmid, freeze cells in 20% sterile glycerol at –80°C in a screw-cap tube.
13. Centrifuge remaining 9 mL of bacterial culture at 5000 rpm (4400*g*) for 5 min, pour off supernatant, and purify plasmid DNA using Bio-Rad's Plasmid Miniprep Kit.

3.1.4. Confirmation of Successful Transfer of the p53 cDNA into Adenoviral Plasmid and Preparation of DNA for Transfection

1. Digest 4 μL of DNA (1/25 total miniprep elution) with *Eco*RI. Digest 1 μg of pTGCMV as a control. Run an aliquot from each digest on a 1% agarose gel.
2. Determine if plasmids are positive for the *trans*-gene by comparing *Eco*RI-digested pTGCMV and pTGCMV-p53. The smallest fragment of 1.7 kb should change in size, since this is the fragment into which the p53 cassette is transferred.
3. Choose one positive plasmid, and digest 50 μL of DNA (1/2 total miniprep elution) with *Pac*I overnight.

4. The next day, check for completion of digest by agarose gel electrophoresis. A fragment corresponding to the plasmid backbone (approx 3 kbp) should be visible on the gel. Phenol/$CHCl_3$ extract and EtOH precipitate. Dry pellet, and resuspend in 20 µL of TE buffer. Check quantity of DNA on agarose gel.
5. Proceed with transfection.

3.2. Transfection and Generation of Adenovirus

3.2.1. Transfection of p53 Adenoviral Plasmid into HEK293 Cells

1. Grow 293 cells to approx 90% confluence on 6-cm dishes. Dishes should be seeded 2 d prior to transfection to allow for sufficient cell attachment.
2. Use 3–5 µg of DNA (pTGCMV-p53/*Pac*I) and 2.5 µg of control DNA (pTGCMV/*Pac*I). Also use one dish as a negative control. Add DNA to a sterile 5-mL polypropylene tube.
3. Add 150 µL of DMEM (without FBS and pen/strep) to each tube and vortex mix.
4. Add 30 µL of Superfect transfection reagent to each tube and vortex mix. Spin at 2000 rpm (700*g*) for 15–20 s.
5. Incubate 5–10 min at room temperature.
6. Immediately before adding DNA/Superfect complex to cells, remove all media from dishes.
7. Add 1 mL complete DMEM (10% FBS, pen/strep) to each tube, mix by pipeting, and add to cells.
8. Rock 2 h on a rocker (Bellco) placed in a tissue culture incubator.
9. Add 4 mL of fresh media to each dish and incubate overnight.
10. Remove the media the following morning and overlay (as when titering virus; *see* **Subheading 3.4.**) with a 1.8% Noble agar/DMEM solution. Add 5 mL of overlay solution to each dish. Let cool in hood for 10 min and return dishes to incubator.
11. Wait for plaques to show, which generally takes 1 to 2 wk (*see* **Note 4**).

3.2.2. Isolation of Primary Plaque

1. Grow 293 cells to confluence in a 24-well plate.
2. Add 300 µL of fresh DMEM (10% FBS, pen/strep) to sterile microfuge tubes.
3. Using sterile Pasteur pipets, pick isolated virus plaques by penetrating the agar overlay with the pipet tip. Remove the agar piece and transfer into a microfuge tube containing DMEM. Pipet up and down to wash the tip and drop a small amount of media onto the plaque to wash as well. Pick five plaques per plate. Vortex mix each tube and incubate on ice for 30 min to 1 h. Centrifuge at 14,000 rpm (16,000*g*) for 1 min.
4. Remove media from wells of 24-well plate, leaving approx 150 µL per well. Add 100 µL of virus to each well. Freeze primary pick plaques and store frozen at –20°C.
5. Rock plate for 2 h in tissue culture incubator.
6. Add 1 mL of fresh media to each well of infected cells.

7. Incubate and wait for cells to lift from plate. Monitor daily for cytopathic effects, which could take up to 1 wk.
8. After evidence of good infection, pipet up and down to detach all cells and remove media from each well into a sterile 2-mL screw-cap tube. Freeze and store –20°C (*see* **Note 5**).

3.3. Large-Scale Virus Preparation and CsCl Purification

3.3.1. Infection of 293 Cells

1. Start with 50 15-cm dishes of 293 cells grown to confluence grown in DMEM supplemented with 10% FBS and pen/strep. Add 2 mL of Fungizone (Life Technologies, Rockville, MO, USA) per liter DMEM to avoid fungal contamination.
2. Thaw secondary virus stock at 37°C and place on ice.
3. Dilute appropriate vol of crude virus (based on known titer and multiplicity of infection [MOI] equal to 1) in enough conditioned media to achieve final vol of 55 mL.
4. Remove media from the 15-cm dishes, leaving 10 mL of media on each dish, and add 1 mL of virus suspension to each dish.
5. Rock plates for 2 h on rocker in tissue culture incubator.
6. Add back 9 mL of fresh media (supplemented only with pen/strep, no FBS) to each dish.
7. Incubate until all cells have lifted from surface of plate, which takes 5–7 d with an MOI of 1.

3.3.2. Harvesting of Cells

1. Harvest by scraping cells with media still on dish. Be careful to avoid contaminated dishes (add Chlorox to contaminated dishes [10% final concentration] and autoclave infectious waste).
2. Remove the cell-containing media from each dish with a 25-mL pipet, and transfer into autoclaved 200-mL centrifugation buckets (Sorvall).
3. After all dishes have been harvested, spin at 5000 rpm (2100*g*) for 15 min to remove cell debris (*see* **Note 6**).
4. Transfer virus-containing lysate into autoclaved 500-mL centrifugation buckets.
5. Resuspend cell pellets in 10–20 mL of the supernatant and transfer to a 50-mL conical tube. For CsCl gradient the following day, store buckets on ice at 4°C overnight. Otherwise, freeze virus-containing supernatant and resuspended cell pellet on dry ice and store at –20°C (thaw overnight at 4°C when ready for CsCl gradient).

3.3.3. Washing of Cell Pellet

1. Resuspended cell pellet must go through 3 freeze–thaw cycles (freeze on dry ice and thaw at 37°C).
2. After thawing cell suspension for the third time (only proceed with this step on the day of CsCl purification), spin at 3000 rpm (1600*g*) for 5 min.

3. Transfer supernatant into a new tube and wash remaining cell pellet with 5 mL of sterile 10 m*M* Tris-HCl (pH 8.0). Spin again at 3000 rpm (1600*g*) for 5 min.
4. Transfer supernatant to previously collected supernatant and wash again with 5 mL Tris-HCl. Spin at 5000 rpm (2100*g*) for 5 min. Transfer supernatant again, and wash once more (third wash) with 5 mL Tris-HCl. Spin at 5000 rpm for 5 min and transfer supernatant. Now take collected supernatant and spin at 5000 rpm for 5 min. Distribute supernatant equally among virus-containing lysate already in 500-mL buckets. Discard cell pellet.

3.3.4. Precipitation of Virus with PEG

1. Add 0.5 vol 20% PEG-8000/2.5*M*NaCl to each bucket. Incubate on ice for 1 h to precipitate virus particles.
2. Centrifuge buckets in a Sorvall Superlite GS-3 rotor at 8000 rpm (10,800*g*) for 30 min at 4°C. Remove supernatant, autoclave, and dispose. Resuspend viral pellet in 25 mL of 10 m*M* Tris-HCl, using a 10-mL plastic pipet. Be sure to resuspend precipitate thoroughly. Keep virus suspension on ice while working. Transfer virus suspension to a sterile 50-mL tube.

3.3.5. First CsCl Gradient

1. In each of two 30-mL polyallomer tubes, aliquot 8 mL of light CsCl. Underlay light CsCl with 8 mL of heavy CsCl. Carefully add the virus suspension onto the top of the light CsCl layer. Distribute virus equally between the two tubes. Add no more than 13 mL of virus suspension to each tube.
2. Spin at 20,000 rpm (72,000*g*) for 2 h at 4°C in Beckman SW28 rotor. If necessary, balance with 10 m*M* Tris-HCl. Disable brake after centrifuge has started spinning. This is a "timed" spin.
3. Carefully collect virus band (in a sterile 15-mL tube) by piercing the bottom of each tube with a sterile 20-gage needle. Collect waste below, and above virus band in 50-mL tubes.
4. Quantify the collected vol of virus (limit to 3 mL) and slowly add an equal vol of 10 m*M* Tris-HCl. Add Tris-HCl in a drop-wise fashion, mixing while adding.

3.3.6. Second CsCl Gradient

1. In each of two 12-mL polyallomer tubes, aliquot 3.3 mL of light CsCl. Underlay light CsCl with 3.3 mL of heavy CsCl, using a Pasteur pipet. Evenly distribute virus among the two tubes by carefully adding it onto the layer of light CsCl (as in the preparation of the first CsCl gradient). Add no more than 3 mL of virus to each tube.
2. Spin overnight (approx 16 h) at 20,000 rpm (68,500*g*) and 4°C. Use Beckman SW41 or equivalent rotor.

3.3.7. Dialysis

1. The following morning, stop the centrifuge without using the brake. Collect the virus band as before, and again limit collection to 3 mL total vol.
2. Using a 3-mL syringe and 1.5-in. 20-gage needle, withdraw the virus, and carefully inject it into a 3-mL dialysis cassette (Pierce Chemical). We use dialysis cassettes, but regular dialysis tubing could be used instead. However, it is critical to keep the virus sterile, which is easier when using the dialysis cassette.
3. Attach a styrofoam floater to the top of the cassette where the virus suspension was injected, and place into a sterile 250-mL beaker (with magnetic stir bar). Fill beaker with dialysis buffer and place it on a stir plate at 4°C in a cold room. After 30 min, change dialysis buffer. Continue to change dialysis buffer four more times at 1-h intervals. Entire dialysis should take 5 to 6 h.
4. Remove the cassette from the beaker. Using another 3-mL syringe with needle, remove the virus from the cassette, and transfer purified virus into a sterile 2-mL tube on ice. Be sure to insert the needle through the top of the cassette to avoid contamination.
5. Aliquot purified virus into sterile 500-µL microtubes in appropriate sizes, snap-freeze in liquid nitrogen, and store in –80°C freezer.
6. Autoclave and clean reusable items, such as centrifuge buckets, etc. (*see* **Note 7**).

3.4. Quantification of Virus

3.4.1. Titering

For each virus to be titered, grow five 6-cm dishes of 293 cells to confluence. For crude virus, thaw at 37°C. For CsCl purified virus, thaw on ice.

3.4.2. Serial Dilutions

1. Serially 10-fold dilute virus in microfuge tubes containing fresh DMEM (10% FBS and pen/strep), starting with 1:100 dilution of virus. Use aerosol-resistant pipet tips, changing them between each dilution. For the first dilution, add 5 µL of virus to 495 µL of media. Vortex mix for 5 s, and be careful not to splash media onto cap of microfuge tube.
2. Next, remove 50 µL from the first tube and add it to 450 µL of media in the second tube. Vortex mix. Continue diluting 1:10 until end dilution is reached (typically 10^{-10} for CsCl-purified virus) (*see* **Note 8**).
   Example:
   Tube 1 = 10^{-2}dilution: add 5 µL of virus to 495 µL of media. Vortex mix.
   Tube 2 = 10^{-3}dilution: remove 50 µL from Tube 1 and add to 450 µL of media. Vortex mix.

Tube 3 = 10^{-4}dilution: remove 50 µL from Tube 2 and add to 450 µL of media. Vortex mix, etc.

3. After dilutions are completed, remove media from 6-cm dishes, leaving 2 mL/dish. Beginning with the highest dilution, add 200 µL of virus dilution per dish. One dish per dilution for four dilutions. Rock plates for 2 h on rocker in tissue culture incubator.

3.4.3. Agar Overlay

1. Approximately 30 min before overlaying dishes, prepare overlay solutions: melt previously autoclaved 25 mL of 1.8% Noble agar solution in 125-mL glass bottle in microwave and keep at 42°C in water bath. Aliquot 25 mL of 2X DMEM into a sterile 50-mL tube, add 1 mL of FBS and 0.5 mL of pen/strep, and warm to 37°C.
2. Immediately before overlaying, combine 25 mL of Noble agar with 25 mL of 2X DMEM in glass bottle to make overlay solution. Swirl to obtain a homogenous mixture. It is critical that the final vol is 50 mL. In case water has evaporated from the bottle with the autoclaved agar, which happens occasionally when stored, add sterile distilled water to 50 mL final.
3. Beginning with the dish with the highest virus dilution, remove all of the virus-containing media from each of the four dishes, and discard in a virus waste container (bleach added to make a concentration of 10% of final waste vol) using a 10-mL plastic pipet. Use the same pipet to speed up the process. It is critical that the cell monolayer is not left to dry for more than 1 min.
4. Again, beginning with the dish of highest dilution (least virus), slowly add 5 mL of overlay solution to each dish from a 25-mL plastic pipet. Add the overlay solution drop-wise with the tip close to the edge of the dish while gently tilting the dish. Be careful not to break the cell monolayer.
5. After adding overlay to a dish, immediately rock back and forth gently a few times to allow for any remaining medium left on the dish to mix fully with the overlay. If this is not done, there may be a liquid layer between the cells and the overlay. This causes the cells and virus to move freely, and no distinct plaques will be obtained.
6. Allow dishes to cool in hood for 10 min and then return to incubator. It is advantageous to leave the dishes on the cool metal surface, since this dissipates the heat more quickly than if left on a more heat-buffering surface.
7. Plaques should show over the next 2 wk.

3.4.4. Neutral Red Agar Overlay

1. Thirteen days after infection, overlay the existing overlay with a 3-mL overlay solution containing neutral red (Sigma). Prepare overlay solution as before, but add 500 µL of 1% neutral red (100X) to the 50 mL of Noble agar/DMEM solution. Swirl to mix. Add 3 mL of overlay solution (now containing neutral red) to each plate over the existing overlay.

2. Allow plates to cool in hood and return to incubator. Count plaques the following day, when cells are stained red, and plaques appear as opaque holes.

3.4.5. Counting Virus Plaques and Calculating the Titer

1. The day following the neutral red overlay, plaques should be counted on each plate. This is exactly 14 d after infection. A 10-fold difference in plaque numbers between plates should be observed if dilutions were done accurately. Calculate titer by multiplying number of plaques by appropriate dilution factor (*see* **Note 9**):

Plate	*Dilution factor*
10^{-5}dilution	no. plaques multiplied by 5×10^5
10^{-6}dilution	no. plaques multiplied by 5×10^6, etc.

Example:
Eight plaques counted on a 10^{-6} dish = $8 \times 5 \times 10^6 = 4 \times 10^7$ plaque-forming unit (pfu)/mL

3.4.6. Viral Particle Number

Because the conditions for determining viral titers vary tremendously from laboratory to laboratory, a more objective way of estimating the titer is to determine the DNA concentration of SDS-treated CsCl-purified virus by determining the absorbance using 260/280 readings on any standard UV spectrometer. The relationship between virus particle (vp) number and absorbance is: 1 A_{260} unit = 1.1×10^{12} vp ***(19)***. If a pure virus preparation is obtained from the second CsCl gradient without any contamination of cellular DNA, RNA, or protein, then the A_{260}/A_{280} ratio should be between 1.2–1.3 ***(20)***. Under our conditions for titering virus on 6-cm dishes (slowly rocking in tissue culture incubator for 2 h) and reading the plates after 14 d, we see a vp:pfu ratio of 20, i.e., for each infectious virus there are 20 vp.

3.5. Characterization of p53 Virus

To determine whether the virus expresses p53, we perform Western blot analysis of extracts from appropriate cells transduced at different MOI. The cells used should be those planned for future experiments. When we check for expression of the *trans*-gene, we often use 293 cells. The signal on Western blots will be amplified due to virus replication. It is then important to harvest the cells within 24 h after transduction, since they would otherwise lyse, and the proteins would then get lost into the tissue culture medium. In case of p53 adenovirus, infection and expression of p53 in 293 cells is not possible, since p53 is highly unstable in 293 cells due to the presence of E1A. In the example shown here, we use mouse astrocytoma p53 (–/–) knock-out cells or human U87 (p53+) glioma cells transduced with viruses expressing either wild-type or R273H mutant p53 at MOIs of 10 or 100 (**Fig. 3**).

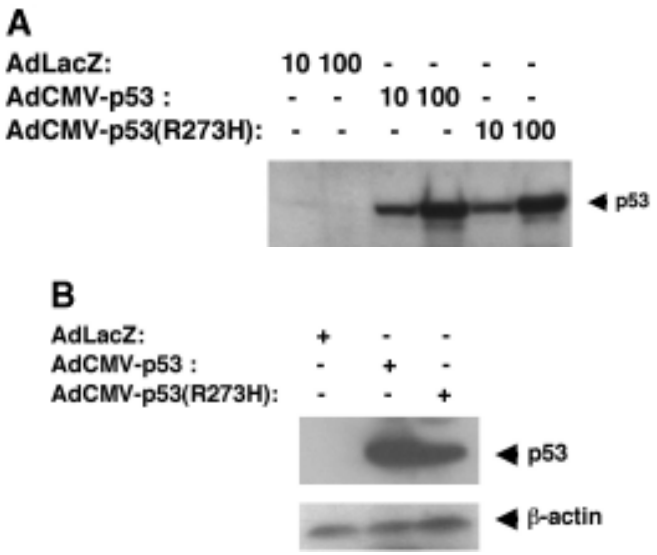

Fig. 3. Transduction of (**A**) mouse Ast. 11.9-2 (p53-/-) and (**B**) human U87 glioma cells with adenovirus expressing p53.

4. Notes

1. We have modified the pZeroTGCMV plasmid to have a *Pme*I site instead of the *Bgl*II site, thus making it more convenient to release the *trans*-gene cassette. *Pme*I is a rare cutting restriction enzyme that is unlikely to cut the DNA insert.
2. We usually prepare competent BJ5183 by the method of Hanahan ***(21)*** and keep frozen aliquots in the –80°C freezer.
3. We find that if the BJ5183 culture is overgrown, then plasmid deletions occur at higher frequency. Therefore, we are careful not to overgrow these cultures.
4. To speed up the process of generating adenovirus, we usually pool the content from one transfected dish that has not been overlaid with Noble agar. Thus, the supernatant contains a mixture of clones, but since the plasmid they were generated from was cloned in bacteria, they should all be the same.
5. Secondary stocks are prepared by infecting 10- or 15-cm dishes confluent with 293 cells. Upon complete detachment of cells from the dish, collect cells in medium, freeze-thaw 3X, and centrifuge to obtain a clear supernatant that is aliquoted and snap-frozen for future use.
6. Save the equivalent of two dishes to make crude virus stocks to be used for future large-scale infections and virus purification. Harvest by scraping, but transfer media from these dishes separately into a sterile 50-mL tube. Proceed as in crude virus production with three freeze–thaw cycles. Centrifuge and transfer supernatant to sterile 15-mL tubes. Save a small aliquot for titering. Freeze all tubes on dry ice and store at –20°C.

7. For small and large collection bottles, add tap water until one-fourth full. Autoclave on liquid cycle for 30 min. Discard liquid waste and rinse with deionized water. Allow to dry, and then autoclave on dry cycle (30 min to sterilize and 10 min to steam dry) for the next time. For SW28 and SW41 swinging buckets, let soak in a warm detergent solution (use Alconox) for several hours. Rinse with deionized water to remove detergent residue, allow to dry, and return buckets to storage at 4°C.
8. When titering crude virus, end dilution will be 10^{-8}. Plate dilutions 10^{-5} through 10^{-8}. When titering CsCl-purified virus, the end dilution will be 10^{-10}. Plate dilutions 10^{-7} through 10^{-10}.
9. Crude stocks usually range in titer from 10^{7}–10^{9} pfu/mL. CsCl-purified stocks range in titer from 10^{10}–10^{12} pfu/mL.

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2

Purification of Recombinant p53 from Sf9 Insect Cells

Xiu Zhu Sun, John Nguyen, and Jamil Momand

Summary

We describe a method for purifying recombinant p53 from baculovirus infected cells in one step by anion exchange chromatography. The p53 is full-length with no flanking sequences and its expression is driven by the baculovirus polyhedron promoter. We also describe how to concentrate the p53 up to 0.9 mg/mL. By gel filtration analysis, we demonstrate that 20% of the p53 forms a tetramer, and 80% forms a monomer. In a DNA binding assay known as the electromobility shift assay, the purified p53/DNA complex forms a single band the gel. This simple procedure should be useful for investigations into the biochemistry of the p53 protein.

Key Words

anion exchange chromatography, gel filtration chromatography, virus titer

1. Introduction

Human p53 is a protein with a theoretical molecular weight of 43,653 Da, based on its amino acid sequence. p53 is conserved within a wide variety of eukaryotes ***(1)***. The term p53 was coined because it has an apparent molecular weight of 53,000 Da when compared to proteins of known size upon analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After cellular DNA damage, p53 up-regulates the expression of genes that repair DNA, prevent cell cycle progression, and initiate apoptosis. These activities are essential for human tumor suppression and, hence, mutations in the *p53* gene are prevalent in a wide variety of tumors ***(2,3)***. The full-length polypeptide of the gene product can form a homotetramer that has an unusual shape. The tetramer has an increased Stokes' radius when compared to globular proteins of known molecular weight ***(4)***. The homotetramer binds to p53 responsive sequences in the promoters of several genes. Perturbation of the C terminus by acetylation, phosphorylation, or noncovalent interaction with other macromol-

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ecules often increases the affinity of p53 for its responsive element. This communication describes the method we use to routinely generate high levels of human p53 from *Spodoptera frugiperda* 9 (Sf9) insect cells infected with recombinant baculovirus. This method is a modification of the one originally used by Delphin and his colleagues ***(5)***. We also describe an analysis of the purified p53 by gel filtration chromatography and electromobility shift assay.

The methods described below outlines: (*i*) infection of cells for virus production; (*ii*) the virus plaque assay; (*iii*) the titer calculation; (*iv*) the optimization of protein expression; (*v*) the infection of Sf9 cells for p53 production; (*vi*) the harvesting of cells and preparation of soluble nuclear lysate; (*vii*) the anion exchange column purification; (*viii*) the concentration of p53; (*ix*) the analysis by gel filtration chromatography; and (*x*) the electromobility shift assay.

2. Materials

2.1. Commercial Products

1. Sf9 cells (Invitrogen, Carlsbad, CA, USA; cat. no. B825-01).
2. Complete insect media: 90% Grace's insect medium, supplemented (Invitrogen; cat. no. 11605-094), 10% heat-inactivated fetal bovine serum (Cellgro-Mediatech, Herndon, VA, USA; cat. no. 35-010-CV) (*see* **Note 1**), 10 μg/mL gentamycin (Invitrogen; cat. no. 15710-064), 0.25 μg/mL Fungizone (also known as amphotericin B; Invitrogen; cat. no. 15290-018), 100 U/mL penicillin/100 μg/mL streptomycin (Bio Whittaker, Walkersville, MD, USA; cat. no. 17-602E).
3. Recombinant full-length human p53 baculovirus (with no flanking coding sequences). Our recombinant virus was originally created by the Prives laboratory ***(6)***.
4. 4% Baculovirus agar (Invitrogen; cat. no. 18300-012).
5. 2X Complete Grace's insect media: 90% 2X Grace's insect media (Invitrogen; cat. no. 11667-037), 20% heat-inactivated fetal bovine serum, 20 μg/mL gentamycin, 0.50 μg/mL Fungizone.
6. Agarose–media overlay: a 1:3 mixture of 4% agarose gel to 2X complete Grace's insect medium. Prepare just prior to use. To prepare, heat agarose to 70°C in a water bath to melt. Cool to 37°C (*see* **Note 2**). Heat 2X complete Grace's insect media to 37°C. Then mix at the appropriate ratio.
7. Q Sepharose® Fast Flow (Amersham Pharmacia Biotech, Piscataway, NJ, USA; cat. no. 17-0510-01).
8. Open column (1.5 × 20 cm) (Bio-Rad, Hercules, CA, USA; cat. no. 737-1521).
9. γ-^{32}P-ATP (6000 Ci/mmol at 150 mCi/mL) (Perkin Elmer Life Science, Gaithersburg, MD, USA; cat. no. NEG-035C).
10. *mdm2* promoter oligonucleotide sequence: 5'-AAAGGAGTTAAGTCCTGAC TTGTCTCCAGCT-3' and its complement (Integrated DNA Technologies, Coralville, IA, USA).

2.2. Buffers

1. Isotonic buffer: 10 m*M* Na_2HPO_4, pH 7.2, 130 m*M* NaCl, 1 m*M* diethylenetriaminepentaacetic acid dianhydride (DTPA) (Sigma, St. Louis, MO, USA; cat. no. D 6518).
2. Cell lysis buffer: 50 m*M* Tris-HCl, pH 8.0, 5 m*M* ethylenediamine tetraacetic acid (EDTA), 150 m*M* NaCl, 0.5% (v/v) Nonidet® P-40.
3. Phosphate-buffered saline (PBS): 8 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 137 m*M* NaCl, 2.7 m*M* KCl.
4. Buffer A: 20 m*M* Tris-HCl, pH 8.0, 12% sucrose, 2 m*M* ethyleneglycol-bis-(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 2 m*M* phenylmethylsulfonylfluoride (PMSF) (Fisher Scientific, Tustin, CA; cat. no. A 270184G005) (*see* **Note 3**), 5 m*M* dithiothreitol (DTT), 1 m*M* DTPA.
5. Buffer B: 20 m*M* Tris-HCl, pH 8.0, 2 m*M* EGTA, 2 m*M* PMSF, 10 m*M* DTT, 50 μg/mL leupeptin (Roche Molecular Biochemicals, Indianapolis, IN, USA; cat. no. 1017128), 10 μg/mL pepstatin (Roche Molecular Biochemicals; cat. no. 1524488), 10 μg/mL E-64 (Roche Molecular Biochemicals; cat. no. 1585673), 1 m*M* DTPA.
6. Buffer C: 40 m*M* Tris-HCl, pH 8.0, 10 m*M* DTT, 1 m*M* DTPA.
7. Laemmli protein denaturation buffer: 1.25 *M* Tris-HCl, pH 6.8, 20% glycerol (v/v), 0.02% bromphenol blue (w/v), 5% SDS (w/v), 10% β-mercaptoethanol (v/v).
8. 5X DNA binding buffer: 50 m*M* Tris-HCl, pH 7.5, 250 m*M* NaCl, 5 m*M* $MgCl_2$, 2.5 m*M* EDTA, 2.5 m*M* DTT, 20% (v/v) glycerol, 0.1 mg/mL poly(dI-dC)·poly(dI-dC) (Amersham Pharmacia Biotech; cat. no. 27-7880-02)
9. 10X Electrophoretic mobility shift assay (EMSA) gel loading buffer: 250 m*M* Tris-HCl, pH 7.5, 40% (v/v) glycerol, 0.2% (w/v) bromphenol blue, 0.2% (w/v) xylene cyanol.
10. TE: 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA.

3. Methods

3.1. Infection for Virus Production

1. Seed each of two T-25 flasks with 2×10^6 Sf9 cells (in the log-phase of growth) in 5 mL of complete Grace's insect media and incubate at 27°C in a nonhumidifying incubator with no added CO_2. Wait 2 to 3 h for the cells to attach to the bottom of the flask. Cells must be at least 50% confluent prior to the next step.
2. Transfer 20 μL of P1 (passage 1 virus) from virus stock stored at 4°C to each flask. Briefly rock flasks to ensure even distribution of virus.
3. At 8 d postinfection, remove the media containing the P2 virus to a 15-mL conical plastic tube and centrifuge (2500*g* for 5 min) to remove cell debris.
4. Transfer 2 mL of the supernatant (P2 virus) to a new tube and store at –80°C for long-term storage. Store the remaining portion of the P2 virus at 4°C.
5. To generate P3 virus, seed 2×10^7 Sf9 cells in each of four T-175 flasks with 25 mL of complete Grace's insect media.

6. At 12 h postseeding, add 500 μL of P2 virus from 4°C stock to each flask to infect cells.
7. At 9 d postinfection, harvest the P3 virus as described above.
8. Store the majority of the P3 virus at 4°C for use within 2 yr, and store a small aliquot at –80°C for long-term storage. The P3 virus will be used to determine the titer.

3.2. Plaque Assay

1. Prewet the 10-cm tissue culture dishes (Falcon 353003) with complete Grace's insect media and seed with 5×10^6 Sf9 cells that are in the log phase of growth. Prepare two dishes for each virus dilution you plan to use (we usually use six dilutions), plus an extra pair for mock infection.
2. Rock dishes for 10 min at room temperature (8 side-to-side motions/min) on a platform rocker.
3. Stop rocker and ensure the platform is level to produce an evenly distributed monolayer of cells. After 10 min, use a microscope to inspect a few dishes to ensure that the cells are evenly distributed.
4. Remove dishes to a level area, and allow cells to attach to bottom surface (approx 30 min). Cells should be approx 50% confluent on the dish bottom.
5. Prepare 10-fold serial dilutions of virus inoculum in 1-mL total vol of complete Grace's insect media. The dilutions should range from 100-fold to 10^7-fold.
6. Aspirate all but 2 mL of the medium in pre-seeded dishes.
7. Carefully add 1 mL of the diluted virus dropwise over the course of 90 s, taking special care not to disturb the monolayer of cells.
8. Incubate plates at room temperature on a rocking platform (approx 2 side-to-side motions/min) for 1 h.
9. Aspirate remaining media and overlay the infected cells with 10 mL of agarose–media overlay.
10. Incubate cells at 27°C in the incubator until plaques are formed (approx 5 to 6 d).
11. View cells at 30× magnification with a dissecting microscope and count plaques as distinct opaque white dots.

3.3. Titer Calculation

The titer is the number of plaque-forming units (pfu)/mL of virus. Use the following formula to calculate titer:

pfu/mL = (1/dilution) × number of plaques produced by the inoculum on dish.

The pfu/mL for the p53 virus is typically 10^8. To determine the amount of inoculum needed for p53 protein production, we use the following formula:

$$\text{mL of inoculum} = \frac{(\text{multiplicity of infection [MOI]} \times \text{total number of cells to be infected})}{\text{titer of virus (pfu/mL)}}$$

We typically use an MOI of 6 for large-scale p53 production.

3.4. Optimization of Protein Expression

The optimal time for p53 expression post-virus infection is obtained from a simple timecourse experiment followed by SDS-PAGE and Coomassie® staining.

1. Seed seven T-25 flasks with 3×10^6 Sf9 cells in 5 mL of complete Grace's insect media.
2. Infect six of the seven flasks with 125 µL (approx 4 MOI) of virus inoculum.
3. Collect cells on day 2, 3, 4, 5, 6 and 10 postinfection.
4. Collect the uninfected cells on day 2.
5. Remove cells by scraping the surface of the flasks with disposable cell scrapers (Falcon®; cat. no. 353086).
6. Pour the cell–medium mixture into 15-mL plastic tubes with screwtops. Centrifuge the cells (3200*g* for 5 min) at 4°C in a swinging bucket rotor. In all subsequent steps, maintain cells at 0°C by keeping the tubes on wet ice.
7. Remove supernatant and add 5 mL of ice-cold PBS. Gently resuspend cells by flicking the bottom of the tube with a finger, then centrifuge, and remove supernatant.
8. Add 300 µL of cell lysis buffer and resuspend cells by vortex mixing.
9. Sonicate cells in a bath sonicator (Fisher Scientific 550 Sonic Dismembrator, setting 5) in continuous mode for 10 s with 1 min rest intervals. Sonicate five times, and centrifuge the lysed cells (3200*g* for 5 min) to remove particulate material.
10. Add 10 µL of supernatant to 10 µL of Laemmli protein denaturation buffer. Vortex mix, boil for 5 min, and load onto a 10% Laemmli gel. After electrophoresis, stain gel with Coomassie Blue dye.

One typically finds that 3 d postinfection is optimal for p53 expression (**Fig. 1**). The p53 protein can be detected by eye as a protein with an apparent molecular weight of 53 kDa (*see* **Note 4**).

3.5. Infection of Sf9 Cells for p53 Purification

1. Seed 5×10^6 Sf9 cells in the log phase of growth into a T-75 flask containing 15 mL of complete Grace's insect media. Incubate overnight at 27°C.
2. Mix 2 mL of complete Grace's insect media with 300 µL of P3 p53 virus stock (6 MOI) to create inoculum.
3. Remove old media from cells and replace with 2.3 mL inoculum.
4. Rock flasks at a rate of 4 side-by-side motions/min for 1 h at room temperature.
5. Add 13 mL of complete Grace's insect media and incubate at 27°C in nonhumidified incubator, 0% CO_2 for 72 h.

In a typical preparation, p53 is purified from 14 T-75 flasks of infected cells.

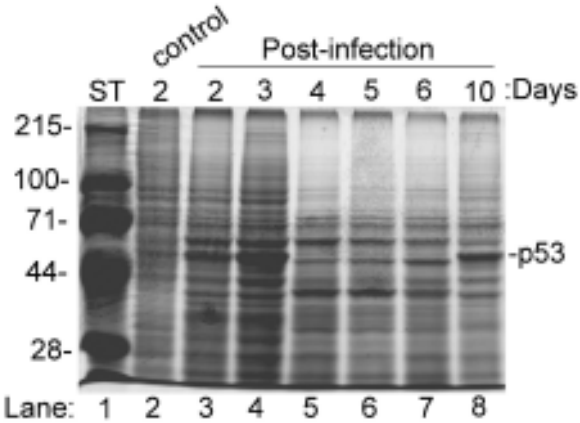

Fig. 1. Equal vol of soluble cell lysate was loaded onto a 10% polyacrylamide gel. Lane 1, molecular weight standards; lane 2, lysate from uninfected cells; lanes 3–8, lysate from infected cells obtained at indicated days postinfection.

3.6. Harvesting Cells and Preparation of Soluble Nuclear Lysate

1. Scrape cells from flasks, without draining the media, using cell scrapers. Pour cell–media mixture into 50-mL plastic tubes with screwtops.
2. Centrifuge at 4°C (3200*g* for 5 min), remove media, and wash cell pellet 2× with ice-cold isotonic buffer.
3. To lyse cells, resuspend the cell pellet in 100 mL of Buffer A plus 0.2% (v/v) Triton® X-100 by gentle inversion. Transfer cell lysate to appropriate centrifuge tubes (*see* **Note 5**). Centrifuge the nuclei at 5600*g* for 8 min and discard the supernatant.
4. Resuspend the pellet in 100 mL of Buffer A plus 0.1% (v/v) Triton X-100 by gentle inversion. Centrifuge the nuclei at 5600*g* for 8 min and discard the supernatant.
5. Lyse nuclei by adding 30 mL of Buffer B plus 0.5 *M* NaCl and vortex vigorously. Allow the mixture to remain on ice for 20 min.
6. Transfer the lysate to centrifuge tubes (Ultra-Clear™, 1 × 3.5 in.; Beckman-Coulter, Fullerton, CA, USA) and add more Buffer B plus 0.5 *M* NaCl to top off if necessary. Centrifuge mixture at 100,000*g* for 60 min at 4°C in a swinging bucket rotor.
7. Remove supernatant and dilute five-fold with Buffer B plus 0.1 *M* NaCl. Gently mix, and centrifuge the diluted mixture at 20,000*g* for 30 min at 4°C.
8. Remove soluble nuclear lysate (supernatant) and aliquot to new tubes. These may be stored at –80°C indefinitely at this point (*see* **Note 6**).

3.7. Anion Exchange Column Purification of p53

1. Rinse Q Sepharose Fast Flow beads with water.
2. Equilibrate Q Sepharose Fast Flow beads with Buffer C plus 0.1 *M* NaCl, according to the manufacturer's instructions.
3. Pour equilibrated beads into an open 1.5 × 20 cm column up to a final column vol of 15 mL.
4. Wash the column with 50 mL of Buffer C plus 0.1 *M* NaCl.
5. Thaw soluble lysate on ice and add 75 mL to column. The flow rate of the column should be approx 0.625 mL/min. Allow lysate to completely pass through column.
6. Wash column with 50 mL Buffer C plus 0.1 *M* NaCl.
7. Wash column with 50 mL of Buffer C plus 0.2 *M* NaCl.
8. Elute p53 with Buffer C plus 0.4 *M* NaCl in three successive 15-mL fractions. The second fraction contains the highest level of p53.

3.8. Concentrating p53

p53 tends to bind irreversibly to ultrafiltration membranes during concentration. We have found that pretreating the membranes with bovine serum albumin (BSA) helps to prevent some of the p53 loss during ultrafiltration.

1. Pretreat YM30 Centricon® (Millipore, Bedford, MA, USA) with 2 mL of 0.01 mg/mL (w/v) BSA (Sigma; cat. no. A-7906) dissolved in water.
2. Centrifuge for 10 min at 5000*g* and remove collected filtrate.
3. Rinse the Centricon 12X with water.
4. Centrifuge 2 mL of water through the Centricon for 10 min at 5000*g*.
5. Place purified p53 into pretreated Centricon and centrifuge at 5000*g* per manufacturer's instructions.
6. Determine the final p53 protein concentration by Bradford assay *(7)* or by comparison of p53 protein band intensity to the intensity of known amounts of BSA separated by 10% SDS-PAGE (we usually perform both tests).

Figure 2 shows the unconcentrated and concentrated p53 compared to BSA standards on a gel stained with Coomassie Blue dye. In the second fraction from the column, we obtain an initial concentration of 0.2 mg/mL of p53. After the Centricon step, the p53 concentration is between 0.6–0.9 mg/mL. However, approx 50% of the p53 is lost due to its nonspecific binding to the Centricon during the concentration step.

3.9. Analysis by Gel Filtration Chromatography

p53 can form a tetramer through an oligomerization domain in its C terminus. One method to demonstrate that p53 forms a tetramer is gel filtration analy-

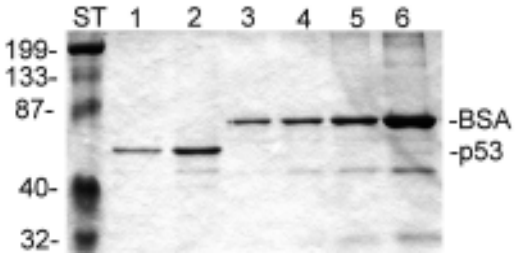

Fig. 2. Purification of p53 through a Q Sepharose Fast Flow column. ST, MW standards; lane 1, unconcentrated p53; lane 2, p53 after ultrafiltration-mediated concentration; lane 3, BSA protein standard (1 μg); lane 4, BSA protein standard (2 μg); lane 5, BSA protein standard (4 μg); lane 6, BSA protein standard (10 μg).

sis. The p53 that we purify contains approx 20% tetramer and 80% monomer by comparison to protein standards of known MW.

Concentrated p53 (94 μL, 0.5 mg/mL) was injected onto a 300 × 7.8 mm Bio-Sil SEC 250-5 high-performance liquid chromatography (HPLC) column, 5-μm particle size (Bio-Rad) equilibrated with 100 m*M* $NaHPO_4$, pH 7.06, 1 m*M* EDTA. The HPLC components were a Varian 9050 UV/*VIS* detector, a Varian 9012 pump, and a Varian 9100 autosampler. The flow rate was 1 mL/min, and the p53 elution profile was monitored at a wavelength of 280 nm. **Figure 3** shows a chromatograph of p53 protein. Comparison to molecular weight (MW) standards indicates that the monomer has a MW of 69.3 kDa, and the tetramer has a MW of 328 kDa. The ratio of monomer to tetramer was 4:1.

3.10. Electromobility Gel Shift Assay

We use a standard DNA binding assay called the EMSA to determine whether p53 is functional. Briefly, p53 is incubated with radiolabeled synthetic double-stranded DNA containing a p53-responsive element. The p53/DNA complex is separated from the nonbound DNA by electrophoresis. The gel is dried and exposed to X-ray film to visualize the DNA bound to p53. To ensure specificity of the binding, we usually run a number of controls. Negative controls include nonaddition of p53 to the reaction mixture, excess nonradiolabeled DNA (competitive inhibitor), and excess poly(dI-dC)·poly(dI-dC) (nonspecific inhibitor).

3.10.1. Annealing Complementary Strands

1. Dissolve complementary single-stranded oligonucleotides containing consensus sequence in TE at a concentration of 1 μg/μL. We used the second p53 response element within the P2 promoter of human *mdm2*.

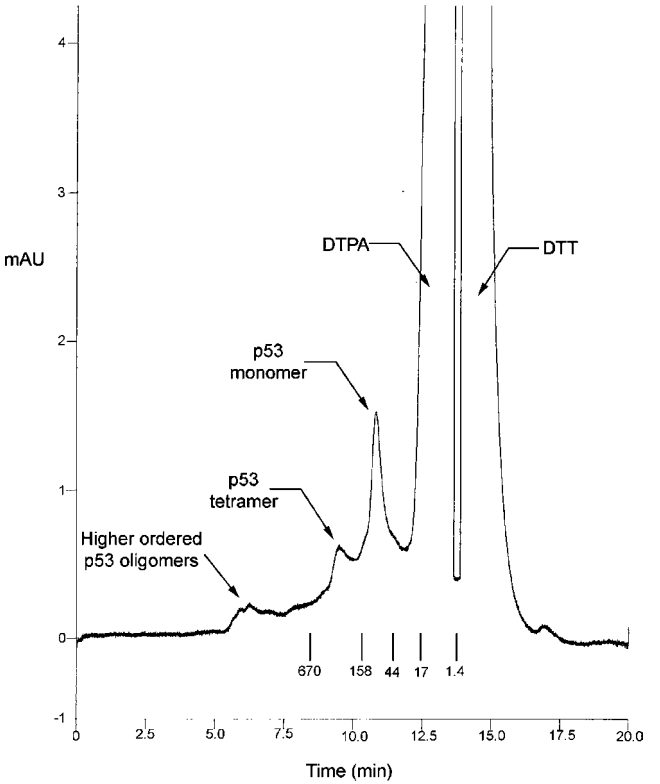

Fig. 3. Gel filtration analysis of purified p53. Chromatogram of concentrated p53 eluted from a Bio-Sil SEC 250-5 column monitored at 280 nm. Elution positions of protein standards are depicted within the chromatogram and their MWs (in kDa) are shown.

2. Add 20 µL of each oligonucleotide to a polylethylene tube and heat to 85°C for 5 min.
3. Remove from heat source and allow sample to cool down to room temperature on benchtop for 3 h.

3.10.2. End-Labeling Double-Stranded Oligonucleotide with γ-^{32}P-ATP

1. Add the following reagents together in the following order: (*i*) 5 µL T4 polynucleotide kinase 10X buffer (Roche Molecular Biochemicals); (*ii*) 2 µL T4 polynucleotide kinase (Roche Molecular Biochemicals); (*iii*) 1 µL DNA probe (1 µg/µL); (*iv*) 3 µL γ-^{32}P-ATP (6000 Ci/mmol at 150 mCi/mL); and (*v*) 39 µL water for a total of 50 µL.
2. Mix and incubate at 37°C for 10 min. Stop the reaction by adding 1 µL of 0.5 *M* EDTA, pH 8.0.

3.10.3. Removal of Unreacted γ-^{32}P-ATP from Radiolabeled DNA

Gel filtration is used to remove unreacted γ-^{32}P-ATP from DNA. The radiolabeled DNA will be excluded from the column beads, and the γ-^{32}P-ATP will be trapped within the column beads.

1. Centrifuge G-50 spin column (Roche Molecular Biochemicals; cat. no. 1 273 965) with its collection tube at 1100*g* for 2 min at room temperature.
2. Discard the collection tube and eluted buffer. Keep the column in the upright position and add 50 μL of the quenched reaction mixture to the center of the column bed (it is essential that the mixture not contact the sides of the column during pipeting).
3. Place the column into a new collection tube, while maintaining it in an upright position. Centrifuge on a swinging bucket rotor at 1100*g* for 4 min at room temperature (*see* **Note 7**).
4. Recover the labeled DNA in the collection tube, and remove 1 μL for scintillation cocktail counting. We typically achieve a specific radioactivity in the range of 0.7–3 × 10^7 counts per minute $\left(\frac{\text{cpm}}{\mu\text{g}}\right)$.
5. Dilute radiolabeled DNA with TE to a final concentration of 6000 cpm/μL (*see* **Note 8**).

3.10.4. DNA Binding Reaction

1. Add the following in a polyethylene tube:
   (*i*) 3.5 μL 5X DNA binding buffer; (*ii*) 1 μL poly(dI-dC)·poly(dI-dC) (1 μg/μL); (*iii*) 6 μL water; and (*iv*) 3 μL p53 protein (0.2–0.5 μg/μL) for a total of 13.5 μL.
2. Mix and leave at room temperature for 10 min.
3. Add 2 μL of radiolabeled DNA (6000 cpm/μL) and incubate at room temperature for 20 min.
4. Add 2 μL of 10X EMSA gel loading buffer to quench the DNA binding reaction. Mix.
5. Load quenched samples onto 6% nondenaturing polyacrylamide gel and electrophorese with 0.5X TBE running buffer. **Figure 4** shows an autoradiogram of a p53 EMSA.

4. Notes

1. To heat inactivate fetal bovine serum, first thaw at 4°C. Then incubate in water bath at 56°C for 30 min.
2. Use extra caution to ensure that temperature is no higher than 37°C and no lower than 35°C. A higher temperature will kill the insect cells, and a lower temperature will cause the agarose–media overlay to form lumps.
3. Create PMSF stock solution by preparing a 0.1 *M* solution in 100% ethanol. Be careful not to inhale the PMSF powder during handling.
4. Sometimes it is difficult to distinguish between p53 and virus proteins in the Coomassie-stained gel. To ensure that p53 is expressed, perform an immunoblot

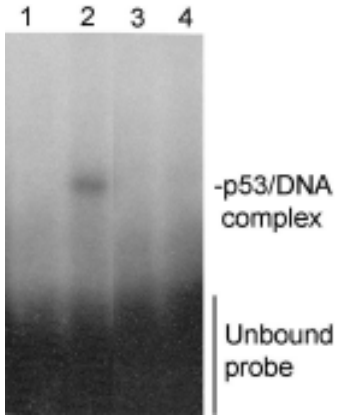

Fig. 4. EMSA assay to detect p53 binding to DNA. An autoradiograph of a gel demonstrating p53 binding to DNA. *mdm2* p53 responsive element-2 was incubated with p53 under various conditions to demonstrate specificity of p53 binding activity. Lane 1, absence of p53 from the reaction mixture; lane 2, complete reaction mixture; lane 3, complete reaction mixture plus excess unlabeled *mdm2* p53 responsive element-2; lane 4 complete reaction mixture plus excess poly(dI-dC)·poly(dI-dC).

analysis with a commercially available antibody. After the immunoblot image is recorded, stain the membrane with Coomassie Blue dye for 15 min and air dry. Within 1 h, many abundant proteins can be detected on the membrane. Comparison of the immunoblot image with the Coomassie-stained membrane will indicate which stained protein is p53.

5. We use 50 mL Oak Ridge polycarbonate centrifuge tubes (Nalge, Rochester, NY, USA) for centrifugation at *g* forces between 4000 and 20,000*g*.
6. Delphin et al. reported that p53 undergoes oxidation when p53 is stored in the absence of DTT ***(5)***. It has been our experience that p53 is extremely sensitive to oxidation as well. Ensure that DTT is fresh whenever buffers are made, and remake the buffers every 3 mo. We purge our solutions with N_2 gas for 5–10 min to remove dissolved O_2.
7. We use the Centra CL2 IEC centrifuge equipped with a 52212 IEC rotor. One must use some caution in choosing the correct centrifuge–rotor combination. We found that at 1100*g*, some heavy swinging buckets do not achieve the vertical position in a timely manner, the result of which forces the sample to the side of the column prior to its migration to the collection tube. The recovery of purified DNA from these preparations is often very low.
8. Only dilute a portion of the radiolabeled DNA. If an experiment must be repeated later, then use a more concentrated solution of radiolabeled DNA to compensate for the radioactivity decay.

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3

Generation and Characterization of p53 Mutant Mice

Melissa L. Dumble, Lawrence A. Donehower, and Xiongbin Lu

Summary

p53 is one of the most well-characterized members of the tumor suppressor gene family. The role of *p53* in controlling cellular homeostasis has proven critical, with over half of all human tumors having either lost or mutated *p53*. The emergence of technology facilitating the ablation of a gene within an animal's genome allowed great advances in the study of *p53*. The p53 knock-out mouse was one of the first of its kind and provided a powerful tool for the study of p53. Production of the p53 knock-out mouse demonstrated the protein's dispensability during embryogenesis, while highlighting its essential role in controlling tumor formation. A variety of p53 mutant models have emerged since the original p53 knock-out mouse, along with improved techniques for regulating gene targeting. This chapter describes the necessary steps and protocols involved in producing a mutant mouse as well as the characterization that follows.

Key Words

p53, tumor suppressor, mutant, knock-out, gene targeting, ES cells, chimera, phenotype, genotype

1. Introduction

The *p53* tumor suppressor gene encodes a transcriptional regulatory protein. In response to various forms of stress, p53 exerts an antiproliferative effect either via growth arrest, apoptosis, or cellular senescence ***(1–3)***. Highlighting its pivotal role in cancer prevention, the *p53* gene is found mutated or lost in more than half of all human tumors ***(4)***. The development of transgenic and gene targeting technologies enables us to utilize mouse models to investigate the critical functioning of p53. During the last decade, numerous p53 transgenic and knock-out mouse models have been developed, which have greatly improved our understanding of the role of p53 in organismal development, carcinogenesis, and cancer therapy ***(5)***.

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Among the targeted p53 mutant mouse models, the p53 knock-out mouse has been the most widely used for cancer studies. Inactivation of the *p53* gene in the mouse germline has provided a model similar in many respects to the inherited human cancer predisposition, Li-Fraumeni syndrome. Li-Fraumeni is a rare autosomal dominant syndrome characterized by the occurrence of diverse mesenchymal and epithelial neoplasms at multiple sites ***(4,6)***. p53-deficient mice have been particularly valuable in examining the effects of p53 loss on tumor initiation and progression ***(7,8)***. A p53 mutant mouse with an allele encoding changes at Leu25 and Trp26 was generated by Jimenez, Wahl, and colleagues ***(9)***. Since these mutated amino acids are known to be essential for transcriptional transactivation and Mdm2 binding, this mouse is p53 transactivation deficient and provides insights into p53 regulation and function during tumor formation. To understand the relevance of p53 missense mutations in vivo, Liu and Lozano's group generated a mouse containing an Arg-His substitution at codon 172 in the p53 protein ***(10)***. Osteosarcomas and carcinomas that developed in these mutant mice more frequently metastasized. The association between p53 and organismal aging has recently been reported. Our laboratory generated a mouse with a deletion mutation in the first six exons of the p53 gene and a point mutation at codon 245 ***(11)***. This mutant mouse exhibits enhanced resistance to spontaneous tumors, but displays early aging phenotypes.

Regulatable or conditional gene targeting technologies are now utilized to establish correlations between a specific genetic lesion and a particular phenotype. Multiple approaches now exist to switch genes on and off under temporal or spatial control. The most commonly used system employs either the phage P1 Cre recombinase that recognizes 34bp LoxP sites or, alternatively, the *Saccharomyces cerevisiae* Flp recombinase that recognizes the 48bp Frt site. Both the Cre and Flp proteins work as site-specific recombinases, which bind to their respective LoxP or Frt sites and mediate reciprocal recombination. In the presence of Cre or Flp, the two LoxP or Frt sites (inserted into genomic DNA in the same orientation) will recombine and, thus, delete the DNA sequences lying between ***(12)***. When produced, mice carrying conditional alleles lack overt phenotypes. However, when bred to transgenic mice that express Cre or Flp recombinase (from a variety of cellular promoters), these alleles can be excised and inactivated in an organ or cell lineage-specific way. The Berns laboratory used this Cre-LoxP system to generate mice carrying conditional *Brca2* and/or *Trp53* alleles and a *cre* transgene under control of the human K14 gene promoter, which is only active in dividing cells of the stratified epithelia. This mouse model was used to study the role of BRCA2 in mammary tumorigenesis ***(13)***.

Inducible transcription systems are also widely used to create mice in which expression of the targeted allele is induced by agents such as doxycycline or

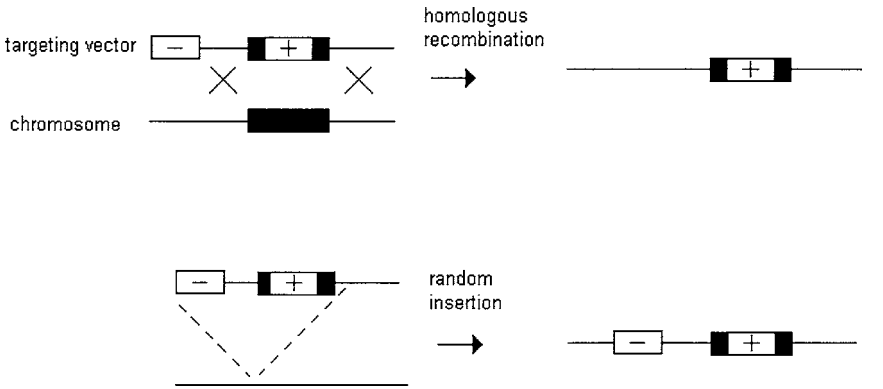

Fig. 1. Integration patterns for a replacement target vector. The "+" and "–" represent positive and negative selection markers respectively. The black box represents coding sequences of a target gene that is disrupted by a positive selection cassette in the targeting vector. The horizontal lines in the targeting vector represent regions of homology with the targeted allele. Vectors that recombine with the endogenous targeted allele will lose the negative selection cassette. Cells with the properly targeted allele will be resistant to negative selection, whereas ES cells containing random insertions of the targeting vector will retain the negative selection cassette and be sensitive to the negative selection drugs.

tamoxifen. The tetracycline operon (tTA) and the tetracycline transcriptional activators (rtTA) have shown great utility in the inducible transcription systems. Mice bitransgenic for the *tet*-operon gene of interest and rtTA will express the gene of interest when the drinking water is supplemented with the tetracycline analogue, doxycycline. However, bitransgenic mice for the *tet*-operon gene of interest and tTA will constitutively express the gene of interest unless doxycycline is supplied ***(14)***. This approach has yielded several inducible mouse models, including the *Hras-G12V* mouse model of melanoma and the *c-Myc* model of T cell lymphoma and acute myeloid leukemia ***(15,16)***. The conditional control of gene expression in these mouse models allows the investigation of complex and multistaged biological processes, such as cancer and embryogenesis.

This chapter attempts to provide experimental design and protocols to generate and characterize p53 targeted mutant mice. We have described various gene targeting strategies and methods for constructing p53 gene targeting vectors, culturing of embryonic stem (ES) cells, introducing targeting vector DNA to ES cells, utilizing targeted ES cells to generate p53 mutant mice, genotyping, and characterizing p53 mutant mice. Given the large array of protocols, it was not possible to include all methods for generating and analyzing mouse mod-

Table 1
Commonly Used Positive and Negative Selection Markers

Selection markers	Selection drug
Positive selection	
Neomycin phosphotransferease (*neo*)	G418
Puromycin N-acetyltransferase (*puro*)	Puromycin
Hygromycin B phosphotransferease (*hph*)	Hygromycin B
Blasticidin S deaminase (*bsr*)	Blasticidin S
Xanthine–guanine phosphoribosyl transferase (*gpt*)	Mycophenolic acid
Negative selection	
Herpes simplex thymidine kinase (HSV-tk)	GANC, FIAU
Mammalian thymidine kinase (tk)	5BdU
Hypoxanthine phosphoribosyl transferase (hprt)	6TG
Xanthine/guanine phosphoribosyl transferase (gpt)	6TG

GANC, gancyclovir; FIAU, 1(1-2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil; 6TG, 6-thioguanine; and 5BdU, 5-bromodeoxyuridine

els, so for further and more detailed information, other reviews, databases, and books should be consulted ***(17–19)***.

2. Materials

1. Targeting vector sequences.
2. Restriction enzymes and DNA modifying enzymes.
3. Agarose and DNA purification kit (Qiagen, Valencia, CA, USA).
4. ES and feeder cell line.
5. Cell medium, ES qualified fetal bovine serum (FBS), trypsin, sterile plasticware, laminar flow hood, tissue culture humidified incubator (37°C, 5% CO_2).
6. Electroporator.
7. Selection drugs (*see* **Table 1**).
8. Stable platform mounted micromanipulation apparatus and inverted phase microscope.
9. Mice: donor females, stud males, pseudopregnant surrogate mothers, vasectomized males, and wild-type mice for breeding to chimeras.
10. Hormones: human chorionic gonadotrophin (hCG) and pregnant mare serum (PMS).
11. Oligonucleotide primers (polymerase chain reaction [PCR] genotyping), *Taq* DNA polymerase.
12. cDNA probe (Southerns), nylon membrane (Zeta-Probe® GT; Bio-Rad, Hercules, CA, USA).

3. Methods

3.1. Targeting Vectors

The majority of knock-out mice and standard lines of p53 mutant mice have been generated by targeting in ES cells using an allelic replacement strategy. A replacement vector is typically composed of homologous regions flanking the portion of the gene to be targeted, along with positive and/or negative selection markers (*see* **Fig. 1**). Expression of selection markers is usually driven by a strong mammalian gene promoter; this may also influence expression of the targeted gene. To avoid this problem, many take advantage of Cre-loxP technology to excise the selection marker from the recombinant locus after the targeted ES cell clone is identified (*see* **Figs. 2A** and **2B**).

The desired genetic recombination is that vector sequences effectively replace the homologous region in the chromosome (*see* **Note 1**). Positive selection markers can be used to select colonies that contain integrated vector DNA. However, most colonies are produced from random integration, because the frequency of random integration is generally several orders of magnitude higher (*see* **Fig. 1**). To eliminate undesirable randomly integrated clones, negative selection markers are attached to the end of the vector. Homologous recombination occurring correctly between the targeting vector and the endogenous gene should result in the loss of the negative selection cassette. One of the more common negative selection cassettes is the herpes simplex virus thymidine kinase (HSV-*tk*) gene. Correct homologous recombination yields clones that are *tk* minus and survive the negative drug selection after gancyclovir is added to the cell culture medium. The commonly used positive and negative selection markers are listed in **Table 1**. Adequate amounts of targeting DNA is amplified and purified in preparation for electroporation (*see* **Note 2**).

3.2 ES Cells: Maintenance, Gene Delivery, Microinjection, and Transplantation

ES cells were first isolated over 20 yr ago and were derived from the inner cell mass (ICM) of a d 3.5 mouse blastocyst, and specific conditions were defined for their maintenance and growth in vitro ***(20,21)***. ES cells are defined as pluripotent cells that resemble cells of the ICM in their ability to differentiate into any functional cell of the adult organism. Conditions for ES cell culture are outlined in this section, along with procedures for ES cell transduction, selection, screening, microinjection, transfer, and finally, the generation of the chimeric mice carrying a germline mutation. **Figure 3** illustrates the sequence of events involved in mutant mouse production; as well, there are texts available describing these methods in precise detail and should be referred to ***(18,19)***.

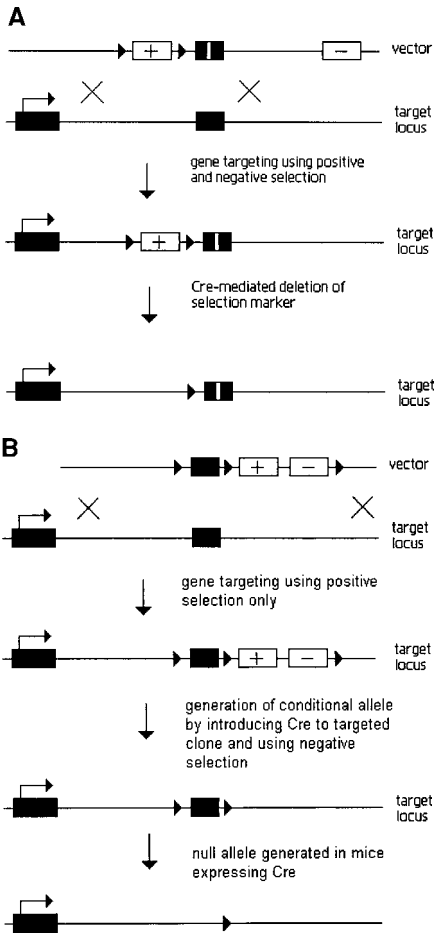

Fig. 2. Creating subtle mutations or a conditional allele by gene targeting and use of Cre-loxP technology. The "+" and "–" boxes represent positive and negative selection markers. The black box represents exons of a target gene. In panel **A**, a white strip in the black box represents the subtle mutation(s) of the target gene. Two black arrows flanking the selection markers and exons represent a pair of loxP sites inserted in the same orientation. The arrow line above the black rectangular box represents the transcriptional direction of the target gene. Following transfection with the linerized replacement vector, ES cells undergo positive and negative selection. The proper integration of the targeting vector by homologous recombination should result in loss of the negative selection cassette, yielding clones that are resistant to both positive and negative selection. Finally, the selection markers are removed by introducing Cre into the properly targeted ES cells. In panel **B**, an endogenous gene is targeted in a manner that leaves the coding sequences intact but flanked by loxP. Following positive drug selection

3.2.1. Maintenance and Propagation of ES Cells

Many different ES cell lines exist, and each has recommended growth media and culture conditions. All culture procedures are carried out using aseptic techniques, sterile plasticware, and medium (warmed to 37°C prior to use, stored at 4°C, and supplemented with glutamine every 4 wk). ES cell lines are cultured on a feeder layer, which is either made from preexisting cell lines or freshly isolated embryonic fibroblasts with the addition of leukemia inhibitory factor (LIF) to the medium. More commonly, many use feeder cells transduced with a LIF construct and, therefore, constitutively express LIF, eliminating the need for an exogenous supply. These conditions ensure the maintenance of ES cell pluripotency ***(22,23)***. The p53 knock-out mouse ***(7)*** was created using the AB1 ES cell line ***(24)***, so the conditions for their culture will be described in this section.

The AB1 cell line was derived from a 129/Sv mouse using standard procedures ***(24)***. AB1 cells are grown on a feeder layer of SNL76/7 cells made mitotically inactive by exposure to mitomycin C. The SNL76/7 cell line was clonally derived from STO embryonic fibroblast cells transfected with both the G418 resistance and LIF expression constructs. The AB1 cells are grown in Dulbecco's modified essential medium (DMEM) with glucose, sodium pyruvate, and FBS added. The ES cells are maintained at subconfluency (just until individual colonies are touching) and passaged regularly (every 2 to 3 d), each time splitting 1:5 to 1:7 (*see* **Note 3**). ES cells are passaged by exposure to trypsin-ethylenediamine tetraacetic acid (EDTA) diluted in calcium- and magnesium-free buffer to detach cells from the plate.

3.2.2. ES Cell Transduction and Selection of Positive Clones

Introduction of a gene targeting vector to ES cells can be performed by either electroporation or transfection. Electroporation is the favored technique, since cells do not need to be highly proliferative; an electrical current is passed

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Fig. 2 *(continued from page 6)*, identification and expansion of properly targeted clones, Cre is introduced into the ES cells to excise the drug selection markers. Negative selection will result in loss of either the drug selection cassettes only, or loss of both the drug selection cassettes and the nearby exon of the target gene. The first event creates a conditional allele, whereas the second event would generate a loss of function allele. Genotyping of ES cell clones by PCR or Southern analysis can be used to distinguish these two different events. The conditional allele has two loxP sites flanking the exon and thus will excise the exon upon subsequent transduction of Cre in the cells of tissues of mice.

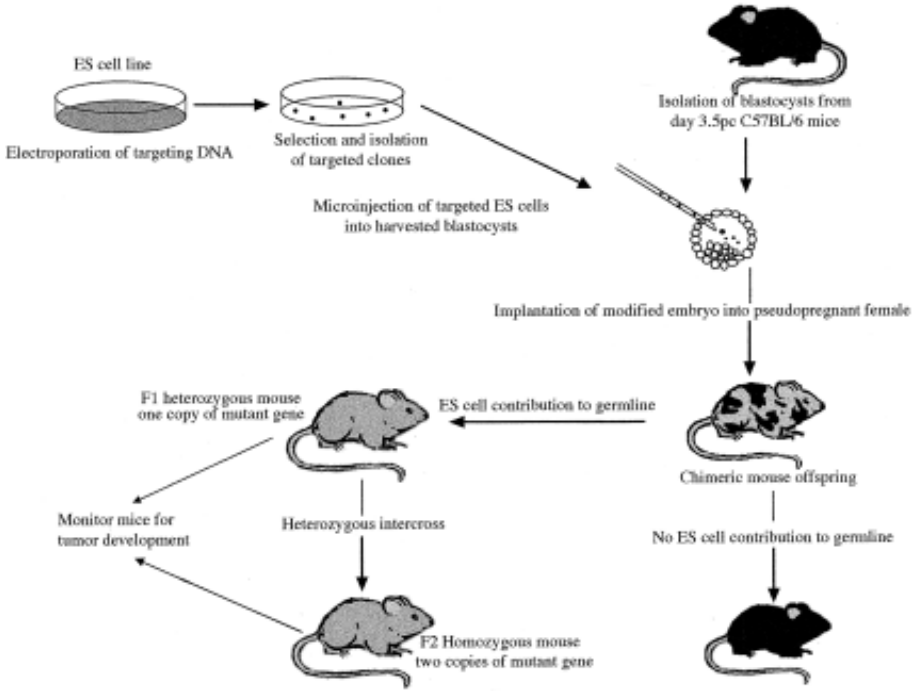

Fig. 3. Generation of a targeted mutant mouse using ES cells. ES cells are grown in culture and electroporated with the targeting vector of choice. Correctly targeted ES cells are selected using positive and negative drug selection. Targeted ES cells are transferred to harvested blastocyts and transplanted back into a pseudopregnant mother. Some offspring are chimeric containing both cells of ES cell origin and cells of maternal blastocyst origin. These chimeric offspring are crossed with wild-type mice, resulting in production of F1 heterozygotes that carry one copy of the mutant allele. These heterozygotes can be crossed to produce homozygote mutant mice. Crossing chimeric mice with wild-type can result in a wild-type offspring, indicating the engineered mutation is not in the germline. The phenotype of the heterozygous and homozygous mutant mice is then determined using the suggested panel of characterizations.

through the cells creating pores in the cell membrane through which the DNA can pass ***(25)*** (*see* **Note 4**).

The following is a brief summary of the electroporation of AB1 ES cells with the vector sequence used to create the p53 knock-out mice ***(7)***. AB1 ES cells (2×10^8) were collected by trypsinizing 3 h following feeding. The cells were centrifuged out of trypsin and resuspended in phosphate-buffered saline (PBS) (void of calcium and magnesium ions) to a cell density of 1.2×10^7 cells/mL. The linearized vector DNA was used at a concentration of 25 μg/mL. Aliquots (0.9 mL) of the AB1 cells were exposed to one round of electroporation at 230 V, 500 μF, followed by another at 240 V, 500 μF. The cells were then plated back onto 90-mm feeder plates and allowed to recover for 24 h prior to selection.

Gene targeting vector sequences usually contain both a positive drug resistance gene (e.g., neomycin) to select for the colonies containing the introduced DNA, as well as a negative selection marker (e.g., HSV-*tk*) to select against colonies with the vector sequence inserted randomly in the DNA. This increases the frequency of clones containing homologously recombined vector sequence. There are numerous agents (*see* **Table 1**) that can be used for these selection purposes. ES cells with the targeting vector used to produce the p53 knock-out mouse were selected in both G418 (positive selection) and FIAU (negative selection). Selection begins 24 h after electroporation and proceeds for 9–12 d thereafter without subculture.

3.2.3. Screening of Resistant ES Cell Clones

Once the ES cells have been selected and resistant clones remain, colonies are picked, expanded, and analyzed for correct transgene incorporation. Colonies can be picked around 10–12 d following electroporation; by this time the colonies will be approx 1.5–2 mm in diameter (*see* **Note 5**). Single ES cell colonies are grown, some are frozen down, and the remainder are expanded and screened to isolate clones that have integrated the vector sequence appropriately. Screening of ES cell colonies can be done by both Southern analysis and PCR. These techniques are described in **Subheading 3.3.** of this chapter.

3.2.4. Microinjection of ES Cells to Blastocyst and Transfer to Mouse

The correctly targeted ES cells are then transferred back into the blastocyst. Blastocysts are isolated on the day of transfer from female mice who are 3.5 d postcoitum (pc). C57BL/6 mice are commonly used for blastocyst injection and were used in the production of the p53 knock-out mouse. The C57BL/6 strain is preferred, as they differ from 129/sv mice (from which most ES cell lines are derived) in coat color (black, nonagouti) and other useful markers. Furthermore, the C57BL/6 blastocysts have been shown to be compatible hosts

for the 129/sv-derived ES cells, as chimera formation and germline transmission is easily obtainable.

The protocols for blastocyst recovery, manipulation, and targeted ES cell injection are very well-established and can be found in many texts (e.g., *see* **refs.** ***18***,***19***. Thus, these will not be discussed in detail. In brief, an average of 10–15 targeted ES cells are transferred to each blastocyst injected. Targeted ES cells in the exponential phase of growth are fed 1 h before being trypsinized to create a single cell suspension, and resuspended in medium. ES cells (10–15) are drawn into the injection pipet while the blastocyst is held by suction to the holding pipet, so that the ICM can be clearly seen. The ES cells are then injected into the blastocoelic cavity, and the injected blastocysts are allowed to recover for 30–60 min prior to being transferred back into the mouse. These micromanipulations are performed using a standard inverted phase microscope–micromanipulator setup mounted on a stable platform.

Once the harvested blastocysts have been injected, they are then transferred back into the oviducts of a pseudopregnant host mouse until they are delivered (*see* **Note 6**).

3.2.5. Generation of Chimeric Mice

Pups are generally delivered 17 d after transfer into the pseudopregnant female and can be visually identified as being chimeric 10 d later by the agouti pigmentation (from the AB1 agouti ES cells) of their coat. Chimeric males can then be set up to breed 6 wk later with C57BL/6 females. Germline transmission of the targeted gene can be determined by the agouti coat of the F1 offspring, as well as by allele-specific PCR on blastocyst outgrowths after 3 d of in vitro culture.

3.3. Genotyping of Targeted Mice and ES Cell Colonies

Protocols 1 and 2 describe the isolation of DNA from ES cell colonies and mouse tails, respectively. To screen targeted ES cell colonies and genotype targeted mice, usually Southern (Protocol 3) or PCR-based analysis of genomic DNA (Protocol 4) is performed (*see* **Note 7**).

To eliminate the need for conventional genotyping, genes that affect coat color have been cloned and used to facilitate genotyping visually ***(26–28)***. For example, Bradley and colleagues introduced a tyrosinase minigene to the vector for targeting a *p53* gene knock-out ***(28)***. The tyrosinase gene has been shown to give pigment to an albino mouse. Thus, p53–/– mice, with two copies of the tyrosinase gene, exhibit a darker coat color (brown) than p53 +/– mice (beige), and wild-type mice (white) have no coat color. This visual approach avoids the need for laborious and error-prone genotyping methods.

3.4. Characterization of p53 Mutant Mice

3.4.1. Assessment of Mutant Mouse Development

Germline mutation of genes has often been shown to cause embryonic lethality. Many tumor suppressor gene knock-outs (e.g., Rb –/– ***[29,30]*** and Brca1 –/– ***[31,32]***) are prone to lethality because of the pivotal role they play in cell cycle control and apoptosis, which are two elements critical to embryonic development. Thus, it was initially surprising that the p53 knock-out and other mutant mice were shown to be embryonically viable ***(7,33,34)***. Even though live homozygous knock-out births are obtained, this does not exclude the possibility that a subset of knock-out mice die *in utero*.

One test to determine this is to set up matings of heterozygous mice and determine whether the genotypes of the resulting offspring give the expected Mendelian ratio. That is, you would expect approx 25% of the offspring to be homozygous null. In fact, this was not the case for the p53 knock-out mouse, and it has been reported that approx 16% of the female p53 null mice exhibit exencephaly, resulting from failure of the neural tube to completely close ***(35)***. Any significant reduction in the number of mutant mice from what is expected should be investigated to determine the stage at which normal embryonic development ceases. If a developmental problem is suspected, embryos should be analyzed at mid-gestation (10.5–12.5 pc) and early gestation (6.5–8.5pc). The embryos can be sectioned and examined for developmental defects.

The reproductive capacity of both the male and female adult mutant mice should also be assessed. Often the adult homozygous mutant mice have difficulties with either reduced or absent fertility or maternal instinct. For example, a fraction of male p53 null mice do not reproduce. p53 is reported to play a role in spermatogenesis ***(36–38)***. Lack of p53 in the testes results in development of a degenerative syndrome in the spermatogenic pathway. The female homozygous knock-out mice are found to have a poor maternal instinct and often cannibalize offspring shortly after birth. Also, in some cases, female p53 null mice gestate fetuses to term but fail to deliver the litter (unpublished observations). p53 null and heterozygous knock-out mice are produced by crossing a homozygous male (with a reproductive history) with a heterozygous female mouse. Breeding pairs should be organized and monitored for any reproductive difficulties.

3.4.2. Assessment of Tumor Development

Since p53 has been so heavily implicated in the development of many tumors (from various animals and tissue types), analyzing the incidence and spectrum of tumors is one of the more important characterizations. Generally, we take a

fairly large sample size of wild-type, heterozygous, and homozygous mutant mice (50–80 mice of each genotype) from both sexes and observe them for 18–24 mo to record time for tumors to develop, as well as the types of tumors that arise. The large sample size is important to generate data reflecting the range of the tumor spectrum and also to generate comprehensive survival plots. The monitoring time of 18–24 mo is suggested, since we have noticed some weakly tumor-susceptible lines that will develop the odd tumor before 18 mo, but increase in frequency between 18 and 24 mo (L. Donehower, unpublished data). In our colony, wild-type C57BL/6 × 129/Sv mice are susceptible to tumor development after 21 mo ***(39,40)***, so a large enough population of these controls is necessary to establish a background tumor incidence. Generally, we monitor mice that are 3 to 4 backcrosses into the C57BL/6 strain background, so in most cases the mice are predominately C57BL/6 strain with some 129/Sv. Because each strain's background can affect tumor incidence and spectra, the ideal situation would be to backcross each mutation into a pure inbred background, such as C57BL/6. The Jackson Laboratory (Bar Harbor, ME, USA) has backcrossed the p53 null allele into several different inbred backgrounds.

The colony of mice should be monitored through daily checks by animal care technicians and once a week by the scientist in charge of the project. There are a number of characteristics of a mouse that may indicate the growth of a tumor. These include: obvious growths, swelling of the abdomen or other areas (possible internal tumor), overall reduction in body size or wasting, sluggishness, difficulty moving, ruffled and thinning fur, pale ears and tail, heavy breathing, and hind limb paralysis (in the p53 +/– mouse this indicates possible osteosarcoma). When the animal displays such signs of tumor development, it is sacrificed immediately to limit pain and suffering and subjected to a complete examination and necropsy.

Necropsies should ideally be carried out in collaboration with a veterinary pathologist. However, if one is not available, there are many texts detailing the procedure ***(41–44)***. Results of the examination should be thoroughly recorded, noting the type, size, and position of the primary tumor, any other tissue abnormalities, and smaller secondary tumors. The primary tumor and some unaffected organs should be excised, and a portion fixed in formalin (or the best fixative for later analyses) for histology, while the remainder should be snap-frozen in liquid nitrogen for subsequent molecular analysis (RNA, DNA, and protein). The tumor tissue should be cleaned free of any skin, connective tissue, blood, or other tissue types prior to freezing to minimize contamination of future results by nontumorigenic tissue. Also, at this stage, blood and urine can be collected and passed through routine, automated biochemical tests common

to veterinary pathology laboratories. The fixed tissues can be passed through graded ethanols and embedded into paraffin. The tissues are sectioned at 4 μm and stained with hematoxylin and eosin (H & E) to determine tissue architecture and cell composition. The stained sections should be examined to determine the tumor type. This should be performed by a veterinary pathologist or a clinical pathologist who is familiar with mouse tissues.

When tumor monitoring is completed, our laboratory then charts the spectrum of tumors and plots the survival of the various mutant mice. Tumor spectrum is represented in a simple table listing the type of primary tumor and the number of mice of each genotype to succumb to such a lesion. To simplify this information, the incidence of the most common tumors can be represented in a pie graph for each mouse genotype, and the remaining tumor types grouped together as "others." The tumor incidences are best displayed by a Kaplan-Meier graph, which plots the percentage of tumor-free mice in each genotype against the age of the mice. Details of the construction of this graph can be obtained from any biostatistics book ***(45,46)***. Our laboratory uses the StatView (SAS Institute, Cary, NC, USA) software to construct these plots.

3.4.3. Assessment of the Aging Process

Recently, our laboratory has documented a p53 mutant mouse (p53 +/m) that is resistant to tumor formation. However, this mutant line displayed early-aging-associated phenotypes at 16–18 mo ***(11)***. The p53 +/m mouse has exons 1–6 deleted and a mutation at codon 245. This resulted in a C-terminal p53 fragment being produced that is thought to activate wild-type p53. This aging phenotype was identified as the p53 +/m lifespan was significantly reduced with no signs of overt neoplasms and by the "aged appearance" of the mouse. The p53 +/m mouse appears smaller (reduced adipose tissue and muscle atrophy), with a hunched spine, ruffled fur, and sluggish movements. Many tests were used to characterize this accelerated aging phenotype, a few of which are described below.

Some of the more simple tests to perform on these animals is to weigh both the whole body as well as individual organs. Body weight is reported to decline with the aging process, as is the weights of organs such as the liver, spleen, kidney, and muscle. Bone density is known to decrease with age, and so simple X-rays of the whole animal can reveal any changes to bone mass. If differences are seen between the mutant and wild-type mouse, histological examination of the affected organs can be performed to assess cellular changes. Organs should be excised, fixed in formalin, paraffin-embedded, sectioned to 4 μm, and stained with a routine histological stain, such as H & E, to assess cellularity. In

collaboration with a pathologist experienced in mouse histology, it is possible to assess morphological changes to organ architecture and any aberrations in cellular composition.

Three organ systems that can be simply studied to assess aging include the skin, hair, and hematopoietic system. Our laboratory uses a wound-healing assay to assess the re-epithelialization ability of skin from various p53 mutant mice ***(47)***. This assay involves anesthetizing the mouse (2.5% Avertin; *see* **ref. *18***) and shaving a small dorsal region of the mouse. Two 3-mm biopsy punches are made in the shaved area, and the skin is excised using curved scissors. The mouse is allowed to recover for 4 d, after which it is sacrificed, the diameter of the holes measured, and the area sampled and prepared for histological cross-section. Routine staining with H&E will reveal the degree of re-epithelialization, and comparisons can be made between the various genotypes. Assessment of the rate of hair regrowth after shaving is another good indicator of age ***(48)***. Our laboratory conducts studies whereby the dorsal region on the mouse is again shaved, and the length of the hair 20 d after shaving is recorded and compared. With age, the rate of hair regrowth is dramatically reduced.

Finally, the use of 5-fluorouracil (5-FU) to ablate rapidly diving mature hematopoietic cells is another simple way to assess age. The rate at which precursor hematopoietic cells reconstitute the adult population is reduced in an older animal. An intraperitoneal injection of 5-FU (100 mg/kg body weight) is given to the test and control mice. Prior to injection, and on d 6 and 11 after injection, a small amount of peripheral blood is taken from the tail vein, and this blood can be analyzed for hemoglobin amounts as well as blood cell counts. We express the white blood counts as a function of time to generate a graph displaying the number of cells over this time period. Cells from an older or aging mouse exhibit a reduced ability to reconstitute the adult blood cell population compared to the control.

3.5. Protocol 1. Genomic DNA Purification from ES Cells in 96-Well Plate (49)

1. When ES cells are confluent, remove medium from plate. Wash 2× with PBS.
2. Add 50 µL of lysis buffer (10 m*M* Tris-HCl, pH 7.5, 10 m*M* EDTA, 10 m*M* NaCl, 1 mg/mL proteinase K, 0.5% sodium dodecyl sulfate [SDS]) to each well.
3. Seal plate with tape and incubate overnight at 55°C.
4. Remove tape and add 100 µL of fresh ice-cold NaCl/ethanol (15 µL of 5 *M* NaCl per 1 mL of cold 100% ethanol) to each well. Shake plate gently.
5. Incubate at room temperature for > 1 h. DNA precipitate should be visible.
6. Cover the plate with several layers of paper towels. Invert the plate with paper towel, and blot away liquid from wells. DNA precipitate is attached to the bottom of well.

7. Add 200 µL of 70% ethanol into each well; then remove the DNA with a blunt-end pipet tip into a microcentrifuge tube. Wash the DNA 2× with 70% ethanol.
8. Air-dry DNA for 1 to 2 min; then resuspend in 20 µL of TE buffer (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0). Leave at room temperature for several hours to allow the DNA to dissolve completely.

3.6. Protocol 2. Preparation of Genomic DNA from Mouse Tails

1. Cut approx 1.5 cm of tail from each 3- to 6-wk-old mouse, and put the tail into a 1.5-mL microcentrifuge tube. Store tails at –20 °C if not used immediately.
2. Add 0.5 mL of fresh tail lysis buffer (50 m*M* Tris-HCl, pH 7.5, 50 m*M* EDTA, 100 m*M* NaCl, 1% SDS, 5 m*M* dithiothreitol [DTT]) plus 2 µL of 50 mg/mL proteinase K to each tube. Incubate tails overnight at 60°C.
3. Invert tubes several times to ensure the tail is completely digested. Usually only small pieces of bone and hair are visible inside the tube. Add 0.5 mL of phenol:chlorofrom (1:1) to each tail lysate. Invert tube vigorously for 30 s. Do not vortex mix.
4. Spin lysate at 10,000*g* for 2 to 3 min. Transfer aqueous phase to a fresh tube with a wide-bore 1-mL pipet tip. Avoid the interface.
5. Add 0.8 mL of 100% ethanol to each tube. Invert tube gently until cloudy DNA precipitate forms. Spool out DNA with a pipet tip into a clean tube. Wash DNA with 70% ethanol once and air-dry the DNA for 1 to 2 min.
6. Add 100 µL of TE buffer (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0) and incubate at 37°C for 1 to 2 h to dissolve completely.

3.7. Protocol 3. PCR Analysis of p53 Genotype: for p53 Mutant Mice Engineered by Donehower and Bradley (7)

1. In a 200-µL or 500-µL thin-wall PCR tube, mix in the following order: 5.0 µL 10X PCR buffer with 1.5 m*M* $MgCl_2$, 5.0 µL 10 m*M* dNTPs, 1.0 µL forward primer (1 µg/µL), 1.0 µL reverse primer (1 µg/µL), 1.0 µL *Taq* DNA polymerse (0.5 U/µL), 33.0 µL sterile double-distilled water, and 1.0 µL genomic DNA (250 ng/µL).
   Primer sequences: forward (WT p53) 5'-GTG TTT CAT TAG TTC CCC ACC TTG AC-3'; reverse (WT p53) 5'-ATG GGA GGC TGC CAG TCC TAA CCC-3'; forward (mutant p53) 5'-CTT GGG TGG AGA GGC TAT TC-3'; reverse (mutant p53) 5'-CCT GAT GCT CTT CGT CCA G-3'.
2. If thermal cycler does not have a heated lid, overlay the reaction mixtures with 50 µL of mineral oil to prevent evaporation. Place tubes in the thermal cycler.
3. Set PCR cycles as follows: 1 cycle of 1 min at 94°C, then 30 cycles of 30 s at 94°C, 30 s annealing at 55°C, 30 s at 72°C, then 1 cycle of 2 min at 72°C. Annealing temperature depends on the melting temperature of the primers used. For genotyping PCR, amplified PCR products are usually shorter than 1 kb, therefore, 1 min of elongation is sufficient. If longer PCR products are expected, we recommend elongation times calculated as 1 min/1 kb.

4. Prepare 2.0% agarose gel with 0.5 μg/μL ethidium bromide (EtBr). Electrophorese 20 μL of product, and visualize under UV light. The wild-type p53 primers generate a 320-bp product, while the primers specific to the neomycin cassette in the mutant allele produce a 450-bp product.

3.8. Protocol 4. Genotyping p53-Deficient Mice (Donehower–Bradley Strain) by Southern Analysis

1. Digest tail DNA with appropriate restriction enzyme overnight. For genotyping p53 –/–, +/–, and +/+, *Bam*HI is used. Mix 21 μL double-distilled water (DDW), 3 μL 10X enzyme buffer, 5 μL tail DNA (Protocol 2), 1 μL restriction enzyme, total vol 30 μL.
2. Southern blot protocol is adapted from that described by Reed and Mann ***(50)***. Prepare 0.7% agarose gel including 0.5 μg/mL of ethidium bromide. Load and electrophorese digested DNA samples. Following electrophoresis, soak gel in 0.25 *M* HCl for 10 min, and rinse 2X with double-deionized water.
3. In a deep dish, invert a gel tray, and place a wick made of filter paper and soaked with 0.4 M NaOH over the platform. Eliminate all air bubbles. Place gel upside down on wick, and then place presoaked nitrocellulose membrane on gel. Wet four filter papers and place on top of membrane followed by 2 in. of paper towels. Add 200–400 g weight and allow to transfer by capillary overnight.
4. Disassemble the transfer, and rinse the membrane once in wash Solution 1. Bake membrane at 70°C for 1 h to immobilize the DNA to the membrane.
5. Slide the membrane into a Teche hybridization tube (22 × 8 cm). Add 20 mL of prehybridization buffer to the tube and then prehybridize the membrane in the Teche HB-1D hybridization oven for 1 h at 68°C. Prehybridization solution (20 mL): 1 mL 10% milk, 2 mL 10% SDS, 1.5 mL 20X SSPE (3.0 *M* NaCl, 0.2 *M* NaH_2PO_4, 0.02 *M* EDTA, pH 7.4), and 14.5 mL DDW. Heat this solution to 70°C. Boil 1 mL of 2.5 μg/μL salmon sperm DNA for 5 min; then add to prehybridization solution just before use.
6. Prepare radiolabeled probe from murine p53 cDNA comprising exons 2–6. In a microcentrifuge tube, add 10 μL of DDW and 1 μL of DNA template (approx 20 ng). Boil for 5 min and cool immediately on ice. Add 5 μL of isotope (^{32}P α-dCTP, 10 μci/μL) and 4 μL of High Prime solution (Roche Molecular Biochemicals, Indianapolis, IN, USA; cat. no. 1585592). Mix well and incubate the reaction tube at 37°C for 10–20 min.
7. Purify radiolabeled probe with MicroSpin G-50 spin column (Amersham Pharmacia Biotech , Piscataway, NJ, USA; cat. no. 27-5330-01).
8. Heat purified probe at 100°C for 5 min. Prepare hybridization solution. Hybridization solution (20 mL): 7.5 mL DDW, 1.5 mL 20X SSPE, 2 mL 10% SDS, 1 mL 10% fat-free milk, 8 mL 25% dextran sulfate. Heat this solution to 70°C, immediately add denatured probe, and hybridize the membrane overnight at 68 °C.
9. Following hybridization, rinse membrane with Wash Solution 1 briefly. Wash membrane for 10 min in each of Wash Solution 2, 3, 4, and 5 (heated to 50°C) sequentially. Rinse membrane with Wash Solution 6. Air-dry membrane for 2 to

3 min. Wrap it in plastic wrap, and expose to X-ray film in cassette at –80°C for 12 h to 1 wk, depending on radioactive counts per minute recorded from the blot.
Wash Solution 1: 2X SSC (20X: 3.0 M NaCl and 0.3 M sodium acetate, pH 7.0.
Wash Solution 2: 2X SSC with 0.1% (w/v) SDS.
Wash Solution 3: 0.5X SSC with 0.1% (w/v) SDS.
Wash Solution 4: 0.1X SSC with 0.1% (w/v) SDS.
Wash Solution 5: 0.1X SSC with 0.1% (w/v) SDS.
Wash Solution 6: 0.1X SSC.

10. For wild-type mice, the autoradiograph will exhibit a band at 10 kb (pseudogene) and at 5 kb (wild-type allele). For heterozygote mice, the film will show bands at 10 k, 5 kb, and 6.5 kb (mutant allele). For homozygous mutant mice, only 10 and 6.5-kb bands will appear.

4. Notes

1. The length and sequence of the homologous sequences in the vector will affect the targeting efficiency. For most vectors, the length of homology ranges from 2–8 kb.
2. Recombination-deficient bacterial strains, such as DH5α and HB101, should be used to amplify vector DNA. A large-scale preparation of vector DNA is usually prepared and purified over a CsCl gradient. Sometimes DNA isolated using commercial columns can be toxic to ES cells. After restriction enzyme digestion to linearize the vector DNA, the DNA should be extracted 2× with phenol–chloroform, ethanol-precipitated, washed 2× with 70% ethanol, and resusupended in sterile PBS. The DNA concentration for electroporation should be optimized to acquire enough positive colonies after selection, but avoid a high number of nonspecific insertions of vector DNA into chromosomal DNA.
3. DMEM is best bought ready-made to eliminate the introduction of endotoxins; the FBS should be batch-tested to ensure support of optimal growth. Extreme care is essential culturing ES cells, so they should not be allowed to overgrow and should not be split at too low a density, minimizing the number of populations the cells pass through.
4. Electroporation should be optimized to determine the best conditions for a balance between a high transfection efficiency and cell viability (in general, electroporation causes 50% of cells to die). Conditions that influence these two parameters include the voltage, ion concentration, the concentration of DNA, and the ES cell confluency.
5. It is easiest to pick the colony using a drawn out pasteur pipet or yellow tip and transfer the cells to a small vol of trypsin to be broken up. Following disaggregation, the trypsin is diluted by addition of growth media, and the cells are replated on gelatin-coated plastic.
6. The foster mother is usually chosen for her reproductive performance. High fecundity and good maternal instinct are found in the commercially available B6D2 line, although any random-bred mice with these maternal characteristics will work. Likewise, the same strain vasectomized males (reproductively viru-

lent) can be used to produce the pseduopregnant females. Ideally, a coat color marker could be incorporated to distinguish any offspring that result from the vasectomized male instead of the transferred blastocysts.

7. The PCR method has many advantages: (*i*) lower amount of DNA is needed; (*ii*) the PCR analysis can be done on pools of DNA samples; (*iii*) isotopes are not used for the PCR analysis; and (*iv*) the PCR analysis only takes a few hours to get a result, while Southern analysis takes a few days to a week. However, significant numbers of false positives and negatives can appear in the PCR results, which reduces the reliability of the technique. Our laboratory prefers Southern analysis, despite the time and labor, since misgenotyping by PCR can have devastating effects further along. Actually, many other investigators prefer Southern analysis to genotype when they have sufficient genomic DNA available from ES cell colonies or mouse tails.

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4

Identification of p53 Target Genes by Fluorescent Differential Display

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Summary

Differential display (DD) is a method used worldwide for identifying differentially expressed genes in eukaryotic cells. The mRNA DD technology works by systematic amplification of the 3' terminal regions of mRNAs. Using anchored primers designed to bind 5' boundary of the polyA tails for reverse transcription, followed by polymerase chain reaction (PCR) amplification with additional upstream primers of arbitrary sequences, mRNA subpopulations are separated by denaturing polyacrylamide electrophoresis. This allows direct side-by-side comparison of most of the mRNAs between or among related cells. Because of its simplicity, sensitivity, and reproducibility, the mRNA DD method is finding wide-ranging and rapid applications in developmental biology, cancer research, neuroscience, pathology, endocrinology, plant physiology, and many other areas. Since the recent development of the fluorescent differential display (FDD), the first nonradioactive DD system with equivalent sensitivity to the original ^{33}P isotopic labeling method, it is now possible with this technology to automate, which can greatly increase the throughput and accuracy of mRNA DD.

Key Words

mRNA differential display, fluorescent differential display, differential gene expression, tumor suppressor gene p53, p53 target genes

1. Introduction

Cancer is a disease state caused by multiple genetic alterations, which lead to unregulated cell proliferation. The most frequently mutated gene among all genes known to be involved in human cancers is the tumor suppressor *p53*. A major outcome of such mutations is inactivation of the biochemical and biological functions of the wild-type (wt) p53 protein. Among the biological effects elicited by wt p53, the best documented are cell cycle arrest and pro-

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grammed cell death (apoptosis). p53 is a transcription factor that can mediate many downstream effects by the activation or repression of target genes. The tumor suppressor p53 is activated by a variety of cellular stresses such as heat shock, hypoxia, osmotic shock, and DNA damage (e.g., UV), which in turn leads to growth arrest and/or apoptosis. Apoptosis is likely to be the most important function of p53 in suppressing tumor formation. However, the mechanism by which p53 actually induces apoptosis remains to be determined. p53 can induce expression of proteins that target both the mitochondrial and the death receptor-induced apoptotic pathways. Presently, particular interest has focused on identifying target genes that mediate p53-induced apoptosis, because the induction of programmed cell death appears to be a critical component of p53-mediated tumor suppression and because of the therapeutic potential of reactivation of this response in tumors. Recently, several p53 target genes were reported, which appear to contribute to p53-dependent apoptosis pathways ***(1–4)***. Remarkably, many of these p53 target genes were found by the differential display (DD) method. However, the identification of additional, if not all, p53 target genes remains to be of great importance, which could provide the missing link between p53-mediated apoptosis and tumor suppression.

In this chapter, we present the procedure of DD and also discuss some critical factors affecting the accuracy of the method. DD methodology was invented in 1992 ***(5)***. Traditionally, DD is based on ^{33}P radioactive labeling of cDNA bands. This is the most commonly used DD technology because of its sensitivity, simplicity, versatility, and reproducibility. Since its description, numerous differentially expressed genes have been successfully identified in diverse biological fields ranging from cancer research, developmental biology, neuroscience, plant physiology, and many other areas. Recently, a very similar sensitive DD method was established by fluorescent labeling ***(6)***. This is the first nonradioactive DD system with the equivalent sensitivity to the ^{33}P isotopic labeling method. Fluorescent labeling is optimal for automation, which can greatly increase the throughput and accuracy of DD. The general strategy for fluorescence differential display (FDD), which is very similar to the radioactive DD, is outlined in **Fig. 1**. Total or poly(A) RNA can be used for DD technique. Removal of all chromosomal DNA from the RNA samples with DNase I (with addition of RNase inhibitor) is essential before carrying out DD. The principle of the DD and FDD methods is to detect different gene expression patterns by reverse transcription polymerase chain reaction (RT-PCR). The RT used one of the three individual one-base anchored oligo(dT) primers, which anneals to the beginning of a subpopulation of the poly(A) tails of mRNAs. This anchored oligo(dT) (H-T_{11}V) primer consists of 11 T's (T_{11}) with a 5' *Hin*dIII (AAGCTT) site plus one additional 3' base V (V may be dG, dA, or dC), which provide specificity. For FDD, fluorescent (rhodamine [R],

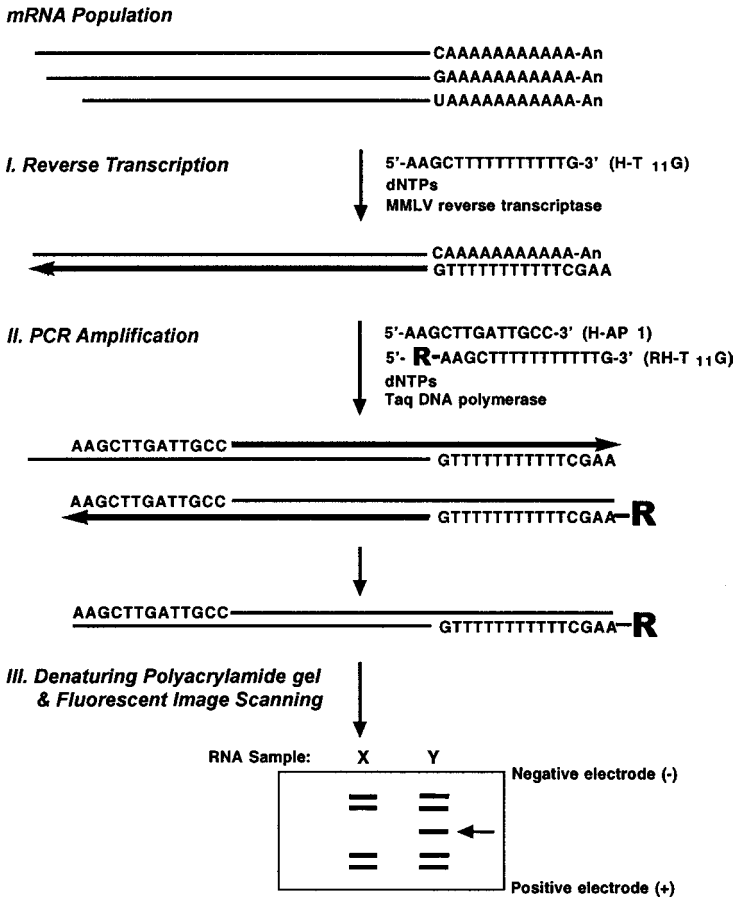

Fig. 1. Schematic representation of FDD. Illustration courtesy of GenHunter Corporation.

red)-labeled anchored R-H-T_{11}V primers are combined with various arbitrary primers (AP) (13-mer, also containing a 5′ *Hin*dIII site, H-AP primers) in PCR steps. The amplified PCR products up to 700 bp can be separated on a denaturing polyacrylamide gel. FDD image can be obtained using a fluorescent laser scanner. Side-by-side comparisons of cDNA patterns between or among relevant RNA samples would reveal differences in gene expression. The cDNA fragments of interest can be retrieved from the gel, purified, and reamplified with the same set of primers (but without fluorescent labeling of H-T_{11}V primer) under the same PCR conditions as in the initial FDD-PCRs. For further molecular characterization, the obtained reamplified PCR fragments are cloned and sequenced. DNA sequence analysis of these cDNA fragments by Basic

Local Alignment Search Tool (BLAST) Search of the GenBank® (http://www.ncbi.nlm.nih.gov/BLAST/) may provide information whether a gene identified by DD is a known, homologous to known, or a novel gene. The final step of the DD procedure is to confirm the differential expression of the obtained partial cDNAs by Northern blotting. The result of this analysis provides not only confirmation by a method independent of DD, but also information about the size of the gene. After confirmation by Northern blot, the cloned cDNA probe can be used to screen a cDNA library for a full-length clone, which is helpful for the functional characterization of the gene.

2. Materials

2.1. RNA Isolation and RNA Purification

1. RNApure™ Reagent (GenHunter®, Nashville, TN, USA).
2. MessageClean® kit (GenHunter), 10X reaction buffer: 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$.
3. Diethyl pyrocarbonate (DEPC)-treated distilled water (dH_2O) (GenHunter).
4. RNA loading dye (GenHunter).
5. 10X 4-Morpholinepropanesulfonic acid (MOPS) buffer: 0.4 *M* MOPS, pH 7.0, 0.1 *M* sodium acetate, 0.01 *M* ethylenediamine tetraacetic acid (EDTA), store at room temperature in the dark.

2.2. RT Reaction and FDD-PCR

1. 5X RT buffer: 125 m*M* Tris-HCl, pH 8.3, 188 m*M* KCl, 7.5 m*M* $MgCl_2$, 25 m*M* dithiothreitol (DTT) (GenHunter) (*see* **Note 1**).
2. Moloney murine leukemia virus (MMLV) reverse transcriptase (100 U/μL) (GenHunter) (*see* **Note 1**).
3. dNTP mixture (2.5 m*M*) (GenHunter) (*see* **Note 1**).
4. H-T_{11}V anchor primer (V = A, C, G) (2 μ*M*) (GenHunter) (*see* **Note 1**).
5. R-H-T_{11}V anchor primer (V = A, C, G) (2 μ*M*) (GenHunter) (*see* **Note 1**) (rhodamine [R]-labeled primers are light sensitive).
6. H-AP 13-mer primers (1–160) with 50–70% GC content (2 μ*M*) (GenHunter) (*see* **Note 1**).
7. 10X PCR buffer: 100 m*M* Tris-HCl, pH 8.4, 500 m*M* KCl, 15 m*M* $MgCl_2$, 0.01% gelatin (GenHunter) (*see* **Note 1**).
8. FDD loading dye: 99% formamide, 1 m*M* EDTA, pH 8.0, 0.009% xylene cyanol FF, 0.009% bromophenol blue (GenHunter) (*see* **Note 1**).
9. Rhodamine locator dye (GenHunter) (*see* **Note 1**).
10. 10X TBE buffer (for 1 L): 108 g Trizma base, 55 g boric acid, 3.7 g EDTA.

11. Autoclaved double-distilled water (ddH_2O) (dH_2O; GenHunter).
12. *Taq* DNA polymerase (5 U/μL) (Qiagen, Valencia, CA, USA).

2.3. Northern Blot Analysis

1. 20X SSC buffer: 3 *M* sodium chloride, 0.3 *M* sodium citrate, pH 7.0.
2. 100X Denhardt's solution (for 500 mL): 10 g Ficoll® 400, 10 g polyvinylpyrrolidone molecular weight (MW) 360,000, 10 g bovine serum albumin (BSA) fraction V (store at –20°C).
3. Hybridization buffer: 5X SSC, 50% formamide, 5X Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and fresh 100 μg/mL heat-denatured sheared nonhomologous salmon sperm DNA (store at –20°C).
4. HotPrime™ DNA labeling kit (GenHunter).

3. Methods

3.1. RNA Isolation from Cell Cultures

Total RNA can be isolated with one-step acid-phenol extraction method by using RNApure™ Reagent.

1. After removal of the cell culture medium and wash step with 10–20 mL of cold phosphate-buffered saline (PBS), set the flask or plate on ice. Add 2 mL of RNApure Reagent per culture flask to lyse the cells; spread the solution by shaking the plate, and let sit on ice for 10 min. Pipet the cell lysate (using a sterile cell scraper) into sterile Eppendorf® tubes and add 150 μL of chloroform/mL cell lysate; finally, vortex mix well for about 10 s. Freeze the tubes at –80°C or proceed to **step 2**.
2. Spin the tubes in an Eppendorf centrifuge at 4°C for 10 min with maximal speed (16,000*g*).
3. Carefully remove the upper (aqueous) phase and save this phase, which contained the RNA, into a clean new sterile tube.
4. For RNA precipitation, add an equal vol of isopropanol to the aqueous phase, vortex mix well, and let sit on ice for 10 min. Spin down the RNA pellet for 10 min at 4°C at maximal speed (16,000*g*). Rinse the RNA pellet with 0.5–1 mL of cold 70% ethanol (treated with DEPC-dH_2O). Spin down again for 10 min at 4°C at maximal speed (16,000*g*). Remove the ethanol and resuspend the RNA pellet in 20–50 μL of DEPC-dH_2O. Make RNA aliquots and store the RNA at –80°C.
5. Before treatment with DNase I (*see* **Subheading 3.2.**), measure the RNA concentration at OD_{260} with a spectrometer, and check the integrity (18S and 28S rRNA bands) of the RNA samples by running about 2 μg each RNA on a 1% agarose gel with 7% formaldehyde.

3.2. DNase I Treatment of Total RNA

Removal of all contaminating chromosomal DNA from the RNA sample is absolutely essential for successful DD. The MessageClean kit is specifically designed for the complete digestion of single- and double-stranded DNA.

1. Incubate 50 µL (10–50 µg) of total cellular RNA (use DEPC-dH_2O when diluting RNA) with 10 U (1 µL) of DNase I (RNase-free) in 5.7 µL of 10X reaction buffer for 30 min at 37°C.
2. Inactivate DNase I by adding an equal vol of phenol:chloroform (3:1) to the sample. Vortex mix and leave the sample on ice for 10 min. Centrifuge the sample for 5 min at 4°C in an Eppendorf centrifuge at maximal speed (16,000*g*).
3. Save the supernatant and ethanol precipitate the RNA by adding 3 vol of ethanol in the presence of 0.3 *M* sodium acetate.
4. After incubation at –80°C for 1 h (overnight to a few days at –80°C is recommended), pellet the RNA by centrifuging at 4°C for 10 min at maximal speed (16,000*g*). Rinse the RNA pellet with 0.5 mL of 70% ethanol (made with DEPC-dH_2O) and dissolve the RNA in 20 µL of DEPC-treated dH_2O.
5. Measure the RNA concentration at OD_{260} with a spectrophotometer. Check the integrity of the RNA samples before and after cleaning with DNase I by running 2 to 3 µg of each RNA on a 1% agarose gel with 7% formaldehyde. It is recommended to store the RNA samples as 1- to 2-µg aliquots at –80°C before using for DD.

3.3. RT of mRNA

The success of the DD technique is dependent on the integrity of the RNA and that it is free of chromosomal DNA contamination (*see* **Subheading 3.2.** and **Note 1**).

1. Set up 3 RT reactions for each RNA sample in 3 PCR tubes (0.2–0.5 mL size); each should contain one of the 3 different one-base-anchored H-T_{11}V primers (where V may be A, C, or G), for 20 µL final vol: 9.4 µL dH_2O, buffer 4 µL 5X RT, 1.6 µL dNTP mixture (2.5 m*M*), total 2µL RNA (0.1 µg/µL freshly diluted in dH_2O), and 2 µL H-T_{11}V primer (2µ*M*). It is recommended to minimize the pipeting errors that a core mixture without an RNA template is made for each anchored oligo(dT) primer if two or more RNA samples are to be compared.
2. Program the thermal cycler to 65°C for 5 min, 37°C for 60 min, 75°C for 5 min, and 4°C for 5 min.
3. After 10 min, add 1 µL of MMLV reverse transcriptase to each tube, incubating at 37°C to initiate the RT reaction. At the end of the reaction, spin the tube briefly to collect condensation. Set tubes on ice for FDD-RT-PCR or store at –20°C for later use.

3.4. FDD

The RNAspectra™ Red kit (GenHunter) can be used for this step as well as the above RT reactions (*see* **Notes 1–3**). This step can be automated with robotic liquid handling workstation such as BioMek 2000 (Beckman Coulter, Fullerton, CA, USA), which can greatly increase the throughput and accuracy.

1. Set up on ice (in dim light) a 20 µL PCR in thin-walled reaction tubes. For each primer set combination, use the following formula: 4.2 µL dH_2O, 2 µL 10X PCR buffer, 1.6 µL dNTP mixture (2.5 m*M*), 8 µL H-AP-primer (2 µ*M*), 2 µL R-H-T_{11}V (2 µ*M*), 0.2 µL RT mixture from **Subheading 3.3.** (it has to contain the same H-T_{11}V primer used for FDD-PCR), and 0.2 µL *Taq* DNA polymerase. Make as much of the core mixes as possible to avoid pipeting errors.
2. Mix well by pipeting up and down. PCR conditions for 40 cycles are following: 94°C for 20 s, 40°C for 2 min, 72°C for 1 min, followed by 72°C for 5 min, and 4°C for 5 min. Keep samples in the dark after running the PCR, and store at –20°C until the gel run.
3. Prepare a 6% denaturing polyacrylamide gel in 1X TBE buffer. Let the gel polymerize for about 2 h before using. It is recommended that one glass plate be treated with Sigmacote® (Sigma, St. Louis, MO, USA) to facilitate the separation of the plates after running. Prerun the gel for 30 min. Urea in the wells is very critical and must be completely flushed just before loading of the samples.
4. Mix each PCR with 8 µL of FDD loading dye, and incubate at 80°C for 2 to 3 min immediately before loading onto the 6% DNA polyacrylamide gel.
5. Electrophorese for 2 h at 60 W constant power until the xylene dye (the slower moving dye) reaches the bottom.
6. After the gel run, clean the outsides of the plates very well with dH_2O and ethanol.
7. Scan the gel on a Fluorescence Imager using a 585 nm filter (for rhodamine).
8. Cut out the bands of interest after careful separation of both glass plates. For orientation of the lanes, a very helpful hint is to use the rhodamine locator dye.

3.5. Purification and Reamplification of cDNA Bands from FDD

1. Soak the gel slice (from **step 8**, *see* **Subheading 3.4.**) in 1 mL of dH_2O for 30 min, mixing gently by finger-tipping.
2. Remove the water without taking the gel slice and add new 200 µL of dH_2O. Boil the tube with a tightly closed cap for 15 min to elute the DNA from gel. Spin for 2 min at maximal speed (16,000*g*) to collect condensation and pellet the gel. Transfer the supernatant to a new tube and keep for the reamplification reaction. The tube with the gel slice can also be saved for the reamplification PCR.
3. Reamplification should be carried out in a total vol of 40 µL using the same primer combination and concentration (4 µL of each 2 µ*M* primer), but without

fluorescent-labeled anchor primer. The PCR conditions should be also the same, except the dNTP concentrations is changed; use 1 μL of a dNTP mixture (250 μ*M*). As DNA template can be used: (*i*) 4 to 5 μL from supernatant in **step 2**; and/or (*ii*) the gel slice, which contains still little traces of the removed DNA (**step 2**).

4. Check 30 μL of each PCR sample on a 1.5–2% agarose gel (dependent on the expected cDNA length) stained with ethidium bromide. Save the remaining PCR samples at –20°C for future experiments (e.g., cloning, Northern blotting). Compare the size of the reamplified PCR products with the originally found length on the FDD gel. Extract the positive reamplified cDNA fragments from the agarose gel with a QIAex® II Gel Extraction Kit (Qiagen).

3.6. Sequencing and Cloning of PCR Products

One crucial advantage of FDD is the rapid identification of the cDNA sequence by direct sequencing of the PCR products without subcloning of these fragments. After gel purification from **step 4** (*see* **Subheading 3.5.**), reamplified cDNA probes can be ligated into various cloning vector systems and then subjected to DNA sequence analysis.

3.7. Confirmation of Different Gene Expression by Northern Blot

The confirmation by Northern blotting of the differential gene expression, which was found by the FDD procedure, is a very important method, because they are extremely sensitive and provide details exactly about the mRNA size of the screened cDNA fragments. Using the HotPrime DNA labeling kit, following the standard procedure *(7)*, a Northern blot can be performed.

One should be reminded that the DD method is not likely to be able to detect mutations at the DNA level directly. For diseases caused by single gene mutations that have a clear genetic component, chromosome mapping of the mutation locus should be a method of choice. It should be emphasized that the method is only a simple screening tool. However, after confirmation by Northern blot, the differentially expressed cDNA probe(s) might release a series of molecular studies in order to understand complex pathways.

3.8. Specific Applications of FDD

The p53 tumor suppressor gene has been implicated in the control of cell proliferation and tumor progression. Mutations in p53 are involved in a variety of human cancers (including breast, ovary, colon, and lung). p53 is not highly expressed in the nuclei of normal cells; however, a variety of cellular insults (DNA damage, hypoxia, heat, starvation, etc.) result in elevated levels of this protein, whereas no dramatic induction of p53 mRNA levels occurred. The expression of the p53 protein protects the genome from accumulating excess mutations. p53 is well known as a cell cycle check point regulator, and promoter of cell cycle arrest and apoptosis in response to DNA damage. The

human p53 contains an N-terminal transcriptional activation domain, a central DNA-binding domain, and a tetramerization domain on the C-terminal region. The central region binds to a consensus DNA sequence and allows p53 to regulate the transcription of a series of genes, the best characterized of which are *mdm2* and $p21^{WAF/CIP}$ ***(8)***. *mdm2* (murine double minute 2) is a proto-oncogene and a negative feedback regulator of the tumor suppressor gene *p53*. *mdm2* binds to the N terminus of *p53*, represses *p53*-dependent transactivation of target genes, and is also able to promote the rapid degradation of *p53* through the ubiquitin–proteosome pathway. The transcriptional activity of *p53* leads to increased expression of $p21^{WAF/CIP}$ (cyclin-dependent kinase inhibitor, a universal cell cycle inhibitor). $p21^{WAF/CIP}$ is a direct p53 target gene, and deletion of this gene significantly reduces the cell cycle arrest response to *p53* ***(9)***. In the last years, an intense search occurred for p53 target genes, which are involved in the p53-dependent apoptosis pathways. Numerous target genes of p53 have been identified that play a role in the p53-dependent, as well as p53-independent, apoptosis. Interestingly, the DD methodology could be used to successfully identify such p53 target genes, which are involved in the apoptosis pathways (e.g., ***1–4***). However, at the moment, there is still considerable uncertainty about how exactly *p53* expression is able to trigger apoptosis, although there is convincing evidence that *p53* is necessary for the induction of apoptosis. Therefore, it is necessary to search for further p53 target genes, which might be integrated in the p53-mediated programmed cell death.

3.8.1. Identification of p53-Induced and p53-Repressed Genes by FDD

In an attempt to identify p53 target genes, we used a tetracycline-regulated ("tet-off" system) p53 inducible colon cancer cell line, DLD-1 ***(10)***. This cell line contains an inactive endogenous *p53* gene. The p53 protein in this system is induced rapidly after removal of tetracycline (tet). The induction of wt p53 stimulates the expression of the two well-known p53 target genes, *p21* and *mdm2*, which were confirmed by Western blot analysis (**Fig. 2**). Some of our FDD results from a comprehensive FDD screening are shown in **Fig. 3**. Total RNA was extracted from the cells at the same time points (8 and 12 h) either with tet (+tet, no p53 induction) or after tet removal (-tet, increased p53 expression). As a positive control for our FDD system, specific arbitrary 13-mer primer (5'-AAA GCT TAG TGT AC-3') recognizing *p21* was used in combination with an A-anchored primer to confirm the induction of this gene by p53 (**Fig. 3**). Our comprehensive FDD screening using hundreds of FDD primer combinations yielded over two dozen p53-regulated genes (to be published elsewhere). Among them are *p53* itself, displayed by R-H-T_{11}G with arbitrary primer H-AP20, and *mdm-2*, displayed by R-H-T_{11}A with arbitrary primer H-AP10 (**Fig. 3**). This provides a strong validation of our nonbiased and

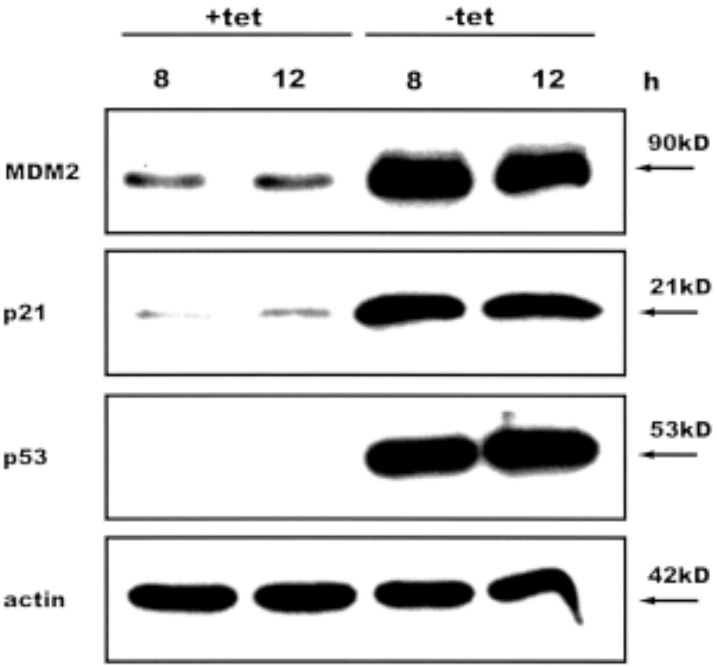

Fig. 2. Western blot analysis of *p53*-regulated protein expression. After removal of tet at different time points (8 h -tet and 12 h -tet), protein levels for p53, p21, and MDM-2 were analyzed in DLD-1 cells. The polyclonal antibody (pAb 1801) against p53 was used. The antibodies against MDM2 (SMP-14) and p21 (C-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). As control for equivalent protein loading, anti-actin antibody was used (Sigma; cat. no. A2066).

exhaustive screening for p53 target genes by DD strategy. Furthermore, FDD also allows the analysis of digital gene expression profiling and precise quantification of gene expression differences (**Fig. 4**). The cell system used in our study showed also clearly apoptotic features approx 16 h after removal of tet. Furthermore, the expression of apoptotic genes, such as *PIGs*, *Bax*, and *GADD45*, could be confirmed in this cell system ***(10)***. We have identified numerous p53 target genes (to be published elsewhere), which are either induced or repressed by p53 using comprehensive FDD screening. Interestingly, over 50% of these genes represent novel and previously uncharacterized genes (to be published elsewhere). This is in contrast to methodology of DNA microarrays, which can generally recognize sample known gene sequences. Other advantages of DD over DNA microarrays are the requirement of much less RNA, the ability to compare more than two different RNA samples simultaneously, and also, the detection of rare mRNAs.

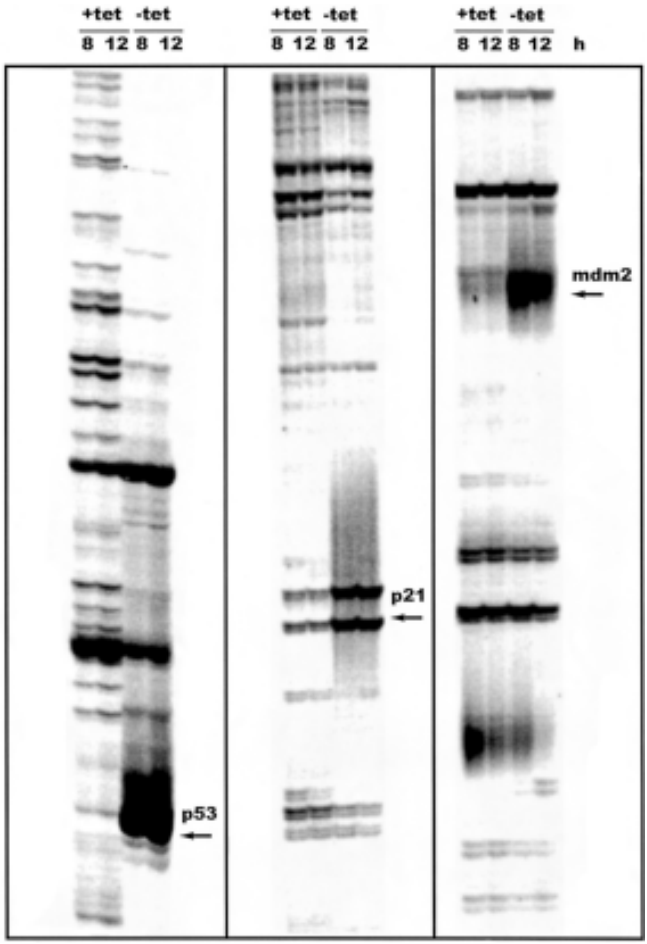

Fig. 3. FDD analysis of *p53*-induced gene expression. Both *p53* and *mdm-2* were among genes identified by comprehensive FDD screening, whereas *p21* was displayed as a positive control using a *p21*-specific 13-mer primer.

4. Notes

1. All materials or products for DD and FDD technology are commercially available from GenHunter Corporation. The company offers also automated FDD services (from RT reactions to FDD results).
2. DD is widely used for the identification and isolation of differentially expressed genes, and now the description of the FDD method can open a big area for automated screening for many more differentially expressed genes. At the moment, some problems may appear in the technical equipment. For example, check the glass plates for the evenness; this is very critical for a correct scanning. The comb

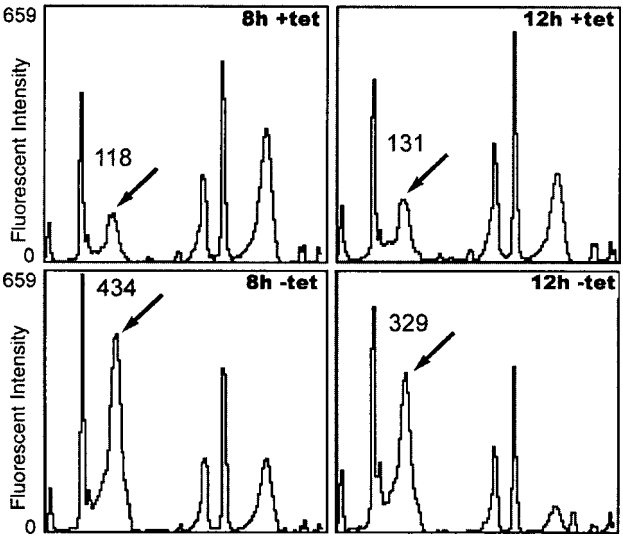

Fig. 4. Quantification of *mdm2*, a p53-induced target gene. The fluorescent intensities (see arrows) from the FDD data (see right panel of **Fig. 3**) were determined and compared using FMBIO® analysis software (Hitachi Genetic Systems, South San Francisco, CA, USA).

must fit well between the two glass plates to avoid leaking among the lanes. The major problem for FDD has to do with that fact that a fluorescent laser scanner is very expensive.

3. Information to minimize extrinsic and intrinsic factors can be found in some recently published papers or reviews ***(11,12)***.

Acknowledgments

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5

p53-Induced Gene Expression Analysis

Joanna K. Sax and Wafik S. El-Deiry

Summary

Researchers in the *p53* field have successfully used many high-throughput screening technologies to analyze and characterize *p53*-induced gene expression. This chapter will focus on one such technology, the Affymetrix GeneChip®. DNA-Chip technology has grown rapidly over the last several years. The ability to hybridize RNA from a sample to thousands to tens of thousands of known and unknown cDNAs spotted on a microarray chip has led to the explosion of information ranging from macro-global expression pattern changes to micro-gene-specific expression changes. The relative ease of making a nonradioactive probe from either total RNA or mRNA to be hybridized to a GeneChip makes microarray technology highly attractive.

Key Words

p53, transcription, microarray, gene expression, DNA-Chip

1. Introduction

Gene chip expression technology has opened the door to explore both global expression pattern differences and to discover potentially new transcriptional targets of a protein of interest. The minimal amount of starting tissue, cell, or RNA material required and the speed of performing the hybridization to the gene chip and receiving high-throughput statistical analysis in a short time make gene chip technology highly attractive for large screens. DNA-Chip technology has been used to monitor changes in global gene expression patterns ***(1–5)***. Moreover, these microarrays have been successfully used to analyze *p53*-regulated gene expression ***(5–7)***. Thousands of oligonuleotides are synthesized on each GeneChip® array (Affymetrix, Santa Clara, CA, USA). Hybridization of RNA probes from both a control and an experimental condition to the GeneChip array allows one to compare which mRNAs are quantita-

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tively increased, decreased, unchanged, or not present. The GeneChip Expression Technical Manual can be used as a guide (with modifications) to prepare samples to be hybridized to GeneChip Expression Probe Arrays. Careful preparation of samples is vital to ensure little to no background on the GeneChip. The following chapter will include a detailed protocol for the preparation of samples from mammalian cells to be hybridized to the GeneChip. This chapter will not include the actual hybridization protocol or the statistical analysis methods.

The basic principle behind the preparation of samples is to make single-stranded biotin-labeled cRNA from total RNA (**Fig. 1**). The cRNA is then hybridized to a GeneChip that is made of cDNA. The advantages of using labeled cRNA over labeled cDNA include: (*i*) high stability of RNA/DNA hybrids; (*ii*) higher target selectivity and avidity; (*iii*) no self-hybridization; and (*iv*) strand selectivity (Enzo® Bioarray™ High Yield™ RNA Transcript Labeling Kit). Briefly, total RNA is made into double-stranded cDNA that is then used as a template to make biotin-labeled cRNA. Once the cRNA is hybridized to the GeneChip, it is stained with fluorescein-labeled steptavidin, and the fluorescent intensity is then measured for each probe set.

2. Materials

1. RNeasy® Total RNA Isolation kit (Qiagen, Valencia, CA, USA).
2. SuperScript™ Choice system (Life Technologies, Rockville, MD, USA).
3. Enzo RNA Trancript Labeling kit (Bioarray; cat. no. 42655).
4. T7-$(dT)_{24}$ primer, high-performance liquid chromatography (HPLC)-purified DNA: 5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$-3'.
5. Phase lock gel (PLG) (Eppendorf Scientific, Westbury, NY, USA).
6. Phenol–chloroform–isoamyl alcohol.
7. 7.5 *M* Ammonium acetate.
8. Absolute ethanol.
9. 80% Ethanol.
10. Water, molecular biology grade.
11. Glycogen, 20 mg/mL.
12. 5X fragmentation buffer: 200 m*M* Tris-acetate, pH 8.1, 500 m*M* KOAc, 150 m*M* MgOAc.
    Note: make with diethyl pyrocarbonate (DEPC)-treated water, filter through a 0.2-μm vacuum filter unit, and store at room temperature.
13. Sterile RNase-free microcentrifuge tubes.
14. Sterile-barrier RNase-free pipet tips.
15. UV spectrophotometer.
16. Heatblock or warm water bath.
17. Cooling water bath.

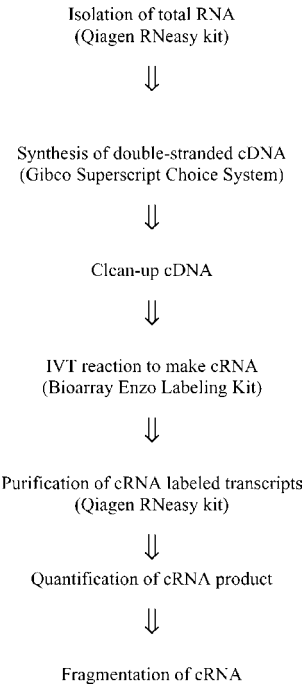

Fig. 1. Flowchart of steps involved to make the probe for the GeneChip. Begin with the isolation of total RNA, followed by the synthesis of double-stranded cDNA. The cDNA is then used as a template to make biotin-labeled cRNA. The labeled cRNA is then used to hybridize to the GeneChip.

3. Methods

The methods described below contain a modified procedure from the Affymetrix GeneChip Expression Technical Manual. The methods will contain: (*i*) isolation of total RNA using RNeasy Total Isolation kit from mammalian cells; (*ii*) synthesis of double-stranded cDNA from purified total RNA; (*iii*) clean up of double-stranded cDNA; (*iv*) in vitro transcription (IVT) reaction to label cDNA; (*v*) clean up of IVT; (*vi*) quantification of IVT product; and (*vii*) fragmentation of cRNA for hybridization.

3.1. Total RNA Isolation of Mammalian Cells

A variety of techniques can be used to isolate total RNA from mammalian cells, and most of these methods are probably reliable ways to obtain RNA. Affymetrix recommends the RNeasy Total Isolation kit. Follow the manufacturer's protocol for total RNA isolation from mammalian cells (*see* **Note 1**).

3.2. Synthesis of Double-Stranded cDNA from Purified Total RNA

The RNA must first be synthesized into double-stranded cDNA, which will later be used as a template to make the biotin-labeled cRNA (*see* **Subheading 3.4.**). The following protocol is a modified version of the SUPERSCRIPT Choice System from Life Technologies combined with the protocol in the GeneChip Expression Technical Manual.

3.2.1. First Strand Synthesis

1. Begin with 8 μg high-quality total RNA. Combine RNA, DEPC-treated distilled water (dH_2O), and 1 μL T7 primer (*see* **Subheading 2.** for sequence) to a final vol of 12 μL in an RNase-free Eppendorf® tube.
2. Incubate at 70°C for 10 min.
3. Briefly spin down contents of tube and place on ice.
4. Add 4 μL 5X first strand buffer, 2 μL 0.1 *M* dithiothreitol (DTT) and 1 μL 10 m*M* deoxynucleoside triphosphates (dNTPs). Mix gently and incubate at 42°C for 2 min
5. Add 1 μL reverse transcriptase, mix well, and incubate at 42°C for 1 h. After the incubation, spin down the contents briefly, and place the tube on ice.

3.2.2. Second Strand Synthesis

1. Combine the following reagents in the first strand synthesis tube: 91 μL DEPC-treated dH_2O, 30 μL 5X second strand buffer, 3 μL 10 m*M* dNTPs, 1 μL DNA ligase, 4 μL DNA polymerase, and 1 μL Rnase H.
2. Gently tap the tube to mix, briefly spin down the contents, and incubate at 16°C for 2 h.
3. Add 2 μL T4 DNA polymerase and incubate an additional 5 min at 16°C.
4. Add 10 μL 0.5 *M* ethylenediamine tetraacetic acid (EDTA) to stop reaction. Proceed directly to clean up or store at –20°C.

3.3. Clean up of Double-Stranded cDNA

1. Pellet the PLG tube at full speed for 20–30 s.
2. Combine equal vol of phenol–chloroform to final cDNA synthesis preparation and vortex mix.
3. Transfer the mixture to the PLG tube and centrifuge at 13,000 rpm for 2 min (≥ 10,000*g*). **Note:** do not vortex mix once the mixture has been added to the PLG tube.
4. Transfer the upper aqueous phase to a fresh Eppendorf tube. Discard the PLG tube in appropriate phenol–chloroform waste.
5. Add 0.5 vol of 7.5 *M* sodium acetate, 2.5 vol cold 100% ethanol, and 1 μL 20 mg/mL glycogen and vortex mix.
6. Centrifuge the samples at 13,000 rpm (≥ 10,000*g*) at room temperature for 20 min to precipitate the DNA.
7. Remove the supernatant carefully and wash the pellet 2× with 80% ethanol, separated by 5-min spins in the centrifuge.

8. Allow pellet to air-dry, and resuspend in 12 µL DEPC-treated dH2O. Store the samples at –80°C, or continue on the Enzo labeling kit.

3.4. IVT Reaction to Label cDNA

The following protocol can be used to transcribe the cDNA into cRNA with biotin-labeled ribonucletides. The use of RNA probes in GeneChip analysis has several advantages, including high target avidity and specificity, strand specificity, and ease of detection using a fluorophore conjugated to streptavidin (Enzo® BioArray™ Highyield™ RNA Transcript Labeling Kit).

3.4.1. RNA Transcript Labeling Reaction

The following is taken from the Enzo RNA Transcript Labeling kit.

1. Combine 10 µL of the double-stranded cDNA with 12 µL dH_2O, 4 µL 10X HY reaction buffer, 4 µL 10X biotin-labeled ribonucleotides, 4 µL 10X DTT, 4 µL 10X Rnase inhibitor mix, and 2 µL 20X T7 RNA polymerase. Do the previous additions in the order given at room temperature.
2. Carefully mix the reagents and centrifuge the tubes briefly to collect the sample at the bottom of the tube.
3. Incubate the tubes in a 37°C water bath for 4 to 5 h, and gently mix the contents of the tubes every 30–45 min.
4. Store at –20°C, or proceed to the purification of labeled RNA transcripts.

3.5. Purification of RNA-Labeled Transcripts

To clean up the IVT products, use the RNeasy spin columns from Qiagen, and follow the manufacturer's protocol for RNA clean up. Purify the entire reaction at one time, and pass the sample over the column twice before the wash and elution steps (*see* **Note 2**). Use 35 µL elution buffer and wait 1 min before centrifugation.

3.6. Quantification of IVT Products

Dilute the cRNA 1:100 and measure the absorbance using a UV spectrophotometer. Measure the sample at both 260 and 280 nm. The ratio between the measurements should be between 1.9 and 2.1 for pure RNA. For quantification of cRNA, an adjusted yield must be calculated to reflect the carryover of unlabeled RNA (when using total RNA as starting material). As described in the GeneChip Expression Technical Manual, use the following formula to determine the adjusted cRNA yield:

Adjusted cRNA yield = RNA_m – (total RNA_i)(y), where RNA_m = amount of cRNA measured after IVT (µg); total RNA_i = starting amount of total RNA (µg); and Y = fraction of cDNA reaction used in IVT.

For example, starting with 8 µg total RNA, 83% of the cDNA was added to the IVT reaction (i.e., used 10 µL of the 12 µL final vol in **Subheading 3.4.**),

giving a yield of 50 μg cRNA. Therefore, the adjusted yield would equal 50 μg cRNA – (8 μg)(.83) = 43.36 μg.

Approximately 30 μg of cRNA is needed for each GeneChip. The sample should be at a minimal concentration of 0.6 μg/μL. If the sample is too dilute after the Qiagen clean up, then proceed to an ethanol precipitation and resuspend in a smaller vol of RNase-free dH_2O. If the concentration of RNA is appropriate, proceed to fragmentation of cRNA (*see* **Note 3**).

3.7. Fragmentation of cRNA

1. Combine 2 μL of 5X fragmentation buffer for every 8 μL of RNA plus H_2O.
2. Incubate at 94°C for 35 min.
3. Place the reaction on ice following the incubation (*see* **Note 4**).
4. The concentration of cRNA must be adjusted again to take into account the fragmentation buffer.
5. Store samples at –20°C until ready to hybridize on GeneChip.

4. Notes

1. Elute the RNA from the RNeasy spin column in 30 μL total vol. Allow the elution buffer to stay on the column for 1 min before centrifugation. A concentration around 1 μg/μL is optimal.
2. The GeneChip Technical Manual recommends purifying one half of the IVT reaction at a time. However, when beginning with 8 μg RNA as the starting material, it is not necessary to separate the reaction for purification.
3. The GeneChip Technical Manual recommends running a 1% agarose gel with 1% of each sample to visualize the yield and size distribution of the labeled transcripts. This step is optional.
4. The fragmentation reaction should produce RNA products ranging from 35–200 bp. This can be visualized on an agarose gel stained with ethidium bromide.

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6

Real-Time Polymerase Chain Reaction Quantitation of Relative Expression of Genes Modulated by p53 Using SYBR® Green I

Katherine E. Roth Stagliano, Evie Carchman, and Sumitra Deb

Summary

Real-time quantitative polymerase chain reaction (QPCR) using the Roche LightCycler® was used to verify the expression of *asparagine synthetase* (*ASNS*) identified by microarray analysis as a target of p53 transrepression and mutant p53 transactivation. A p53-null cell line derived from lung carcinoma, H1299, was infected with recombinant adenovirus expressing wild-type (WT) p53, mutant p53-D281G, or β-galactosidase as a control. After 24 h of infection, RNA was harvested and used for microarray analysis. *ASNS* was one of several genes whose expression was down-regulated by WT p53 and up-regulated in the presence of mutant p53. Expression levels of *ASNS* were measured relative to an exogenously applied quality-control nucleic acid template. Real-time PCR product accumulation was monitored using the intercalating dye, SYBR® Green I, which exhibits a higher fluorescence upon binding of double-stranded DNA. Relative gene expression was calculated using conditions at the early stages of PCR, when amplification was logarithmic and, thus, could be correlated to initial copy number of gene transcripts. *ASNS* was found to be down-regulated in the presence of WT p53 and up-regulated by mutant p53.

Key Words

p53, real-time PCR, QPCR, SYBR Green I, microarray, asparagine synthetase, transactivation

1. Introduction

As a transcription factor, p53's ability to influence the expression of other genes has been and continues to be a subject of intense scrutiny. A large group of genes has been identified as targets of p53-mediated transactivation or transrepression using techniques, such as microarray analysis or serial analysis

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of gene expression ***(1–4)***. The ability to rapidly analyze the expression levels of genes relevant to p53 is a vital component in understanding p53 function and dysfunction. Real-time quantitative polymerase chain reaction (QPCR) is especially suited to validate the modulation of expression of genes affected by p53.

QPCR can be employed to quantify the expression of genes in essentially any system where traditional methods such as Northern blot hybridizations or RNase protection assays would be used. QPCR is quantitative, rapid, requires as little as 1000-fold less RNA than conventional assays, and is easily adapted to the analyses of new targets ***(5)***. Product accumulation is measured during the log-linear phase of PCR amplification, making QPCR the most accurate method of quantitating gene expression currently available ***(6)***. In this chapter, we describe the application of QPCR to quantitate the differential expression of *asparagine synthetase* (*ASNS*) in the presence of either wild-type (WT) p53 or mutant p53-D281G. These methods have been optimized in our laboratory for use in Roche's LightCycler®. Adjustments to these protocols should adapt them to QPCR machines from other manufacturers.

A p53-null cell line derived from lung carcinoma, H1299, was infected with recombinant adenovirus expressing WT p53, mutant p53-D281G, or β-galactosidase as a control. After 24 h of infection, RNA was harvested and used for microarray analysis. *ASNS* was one of several genes whose expression was down-regulated by WT p53 and up-regulated in the presence of mutant p53. Prior to further investigations, this *in silico* observation was validated using QPCR. Expression levels of *ASNS* were measured relative to an exogenously applied quality-control nucleic acid template. Real-time PCR product accumulation was monitored using the intercalating dye, SYBR® Green I, which exhibits a higher fluorescence upon binding of double-stranded DNA. Relative gene expression was calculated using conditions at the early stages of PCR, when amplification was logarithmic and, thus, could be correlated to initial copy number of gene transcripts.

2. Materials

1. Recombinant adenovirus expressing wild-type p53, mutant p53-D281G, and β-galactosidase.
2. Trizol® reagent (Invitrogen, Carlsbad, CA, USA).
3. Isopropanol for molecular biology (Sigma, St. Louis, MO, USA).
4. RNase-free water (RFW) (Sigma).
5. UV/VIS spectrophotometer (DU® 530; Beckman Coulter, Fullerton, CA, USA).
6. Phenol (Invitrogen).
7. Chloroform for molecular biology (Fisher Scientific, Pittsburgh, PA, USA).
8. Ethanol (ETOH) for molecular biology (Sigma).
9. NaCl Solution for molecular biology (Sigma).
10. 10 m*M* Tris-HCl, pH 8.0.

11. RNase-free Tris-borate-ethylenediamine tetraacetic acid (EDTA) (Eppendorf, Westbury, NY, USA).
12. GeneChip™ System (Affymetrix, Santa Clara, CA, USA).
13. RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA).
14. RiboGreen® RNA quantitation kit (Molecular Probes, Eugene, OR, USA).
15. FLUOstar (BMG LabTechnologies, Durham, NC, USA).
16. Brome mosaic virus (BMV) RNA (Promega).
17. Thermoscript™ reverse transcription (RT)-PCR system (Invitrogen).
18. Oligo 5.0 Software (NBI/Genovus, Plymouth, MN, USA).
19. Oligonucleotide primers (Sigma-Genosys, The Woodlands, TX, USA).
20. LightCycler (Roche Molecular Biochemicals, Indianapolis, IN, USA).
21. LightCycler capillaries (Roche Molecular Biochemicals).
22. Platinum® *Taq* DNA polymerase (Invitrogen).
23. *Taq* DNA polymerase PCR buffer 10X (Invitrogen).
24. PCR-grade water (Invitrogen).
25. 50 m*M* $MgCl_2$ (Invitrogen).
26. 10 m*M* dNTP mixture (Invitrogen).
27. 50 mg/mL Bovine serum albumin (BSA) (Invitrogen).
28. SYBR nucleic acid gel stains (Molecular Probes).
29. Agarose gel electrophoresis equipment.

3. Methods

The methods described below outline: (*i*) preparation of total RNA and microarray analysis; (*ii*) synthesis of cDNA from total RNA for QPCR analysis; (*iii*) design and optimization of QPCR primers; (*iv*) QPCR; and (*v*) interpreting QPCR data.

3.1. Preparation of Total RNA and Microarray Analysis

3.1.1. Adenoviral Infection

H1299 cells, 3×10^6 cells in a 10-cm dish, were infected with recombinant adenoviruses at a ratio of five plaque forming units per cell for the following constructs: WT p53, mutant p53-D281G, and β-galactosidase. These adenoviruses were constructed by Dr. Kristoffer Valerie in the Virus Core Center of the Massey Cancer Center, Richmond, VA. Total RNA was extracted 24 h after infection.

3.1.2. Extraction of Total RNA

Care should be taken when dealing with RNA to protect against degradation by RNases; general precautions have been described by others *(7)*.

1. Extract total RNA from each of the three samples: (*i*) H1299 cells infected with adenovirus expressing WT p53 (H1299 Ad WT p53); (*ii*) H1299 cells infected with adenovirus expressing mutant p53-D281G (H1299 Ad p53-D281G); and (*iii*)

H1299 cells infected with adenovirus expressing β-galactosidase (H1299 Ad βgal). Use Trizol reagent according to manufacturer's instructions. Precipitate RNA by mixing with isopropanol, and store at –80°C for at least 2 h. In our hands, RNA stored at this stage can be recovered successfully more than 1 yr later.
2. Recover RNA from isopropanol as described by the manufacturer, and resuspend in 200 μL of RFW.
3. Determine the concentration of RNA by spectrophotometer. Dissolve RNA (4 μL) in 96 μL of 10 m*M* Tris, pH 8.8. A 260/280 ratio below 1.6 was considered an indication of the presence of protein or other contaminants. Standard phenol–chloroform extraction followed by ETOH precipitation (2.5 vol ETOH with 0.1 vol 2 *M* NaCl) normally enhanced the purity of the sample, as indicated by a 260/280 ratio between 1.7–2.0.
4. Inspect RNA (3–5 μg) by 1.2% Tris-borate-EDTA agarose gel electrophoresis to verify integrity before continuing.

3.1.3. Microarray Analysis

Microarray analysis was performed using the GeneChip System. The GeneChip employs a single color system, in which each sample is hybridized to a separate single use array. The general procedures are described in detail elsewhere ***(8,9)***.

3.2. Synthesis of cDNA from Total RNA for QPCR Analysis

Quantitation of gene expression relies on the analysis of highly labile mRNA. Converting total RNA into cDNA permits extensive analysis without compromising the integrity of the transcript. Care must be taken during cDNA synthesis to maintain the integrity and purity of the sample. Because QPCR is highly sensitive, trace contamination of salts, ETOH, and the like, can alter the efficiency of the reaction and, thus, skew the data.

3.2.1. DNase Treatment

In order to ensure the absence of contaminating genomic DNA, DNase treatment of RNA samples is a standard procedure in our laboratory.

1. Perform RQ1 RNase-free DNase treatment following the manufacturer's protocol, and up-scale the vol to accommodate 25 μg of total RNA for each of the three samples: H1299 Ad βgal, H1299 Ad WT p53, and H1299 Ad p53-D281G.
2. Purify the DNase-treated samples by standard phenol–chloroform extraction and ethanol precipitation as described elsewhere ***(7)***.
3. After recovery from ethanol, suspend RNA in 30 μL of RFW.

3.2.2. Quantitation of Total RNA by RiboGreen

Theoretically, relative quantitation using QPCR does not require that the concentration of control and experimental cDNA be equal. However, the

application of relative quantitation by QPCR usually requires relying on the expression of a "housekeeping gene" whose mRNA levels are thought not to change in relative abundance between samples. Rather than relying solely on the expression of a housekeeping gene, we equalized the amount of RNA in control and experimental samples prior to cDNA synthesis using a fluorescent dye, RiboGreen. RiboGreen is an ultrasensitive fluorescent dye which, when used with a standard curve, can quantitate as little as 1 ng/mL of RNA. RiboGreen fluorescence levels increase linearly with the amount of RNA bound to the dye. The RiboGreen kit includes the RiboGreen reagent, RNase Free Tris-EDTA for dilutions and ribosomal RNA for the creation of a standard curve to allow exact quantification of RNA. Fluorescence was detected using a fluorescence microplate reader (FLUOstar), with excitation at 480 nm and emission at 520 nm.

3.2.3. Exogenously Applied Quality Control Template

Efficiency of reverse transcription of RNA into cDNA can be impacted by many variables, such as contamination by salt or ETOH. In order to take into account efficiency of the RT reaction, we spiked our RNA samples with 50 ng of BMV RNA. BMV infects plants, and its genome contains no homology to mammalian genomes.

3.2.4. cDNA Synthesis

Perform cDNA synthesis using the ThermoScript system, and follow the manufacturer's instructions. Five synthesis reactions were performed per sample of either H1299 Ad βgal, H1299 Ad WT p53, or H1299 Ad p53-D281G using 1 μg RNA per reaction of 20 μL vol. Each sample set also contained a negative control, devoid of reverse transcriptase. We routinely use random primers to generate cDNAs for later QPCR analysis.

3.2.5. Verification of cDNA

After synthesis, test each cDNA reaction using *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) primers. This step ascertains if cDNA was synthesized. Additionally, by testing the negative control, it determines whether contamination by genomic DNA is a problem. Description of QPCR conditions needed to verify cDNA are described in **Subheading 3.4.**

3.2.6. Storage of cDNA

This step allows longer storage of cDNA without compromising its integrity.

1. Combine all five batches of cDNA for the H1299 Ad βgal samples and gently vortex mix.

2. Prepare aliquots (9 µL) in 0.2-mL tubes and then freeze in a –20°C frosty freezer for storage until needed.
3. Repeat this procedure for cDNA of the other samples: H1299 Ad WT p53 and H1299 p53-D281G.

3.3. Design and Optimization of QPCR Primers

3.3.1. Primer Design

1. Software. Primer design is a crucial step in a successful QPCR. Primer design software makes primer design relatively simple and straightforward. We have had a good level of success using Oligo 5.0 Software. A number of primer design programs are also available free on the Web, some of which are listed in **Table 1**. Using the Oligo 5.0 program, QPCR primers were designed for *ASNS*, *GAPDH*, and BMV (**Table 2**). *ASNS* was the target gene of interest. BMV served as a standard, to which *ASNS* expression was normalized, allowing for relative quantitation of gene expression (*see* **Subheading 3.5.**). *GAPDH*, used traditionally as a housekeeping gene, was used to verify the absence of contaminating genomic DNA in cDNA preparations. Normalization to *GAPDH* was also examined (*see* **Note 1**).
2. Specifications of primers. The following specifications, discussed in more detail in the LightCycler Operator's Manual, should be kept in mind during primer design ***(10)***. Roche considers product lengths of 100–150 bp as optimal for amplification using the LightCycler; however, we routinely design our products between 150 and 250 bp. The upper limit for product length is 500 bp. The primers themselves should be approx equal in length, ranging from 18–24 nucleotides. The melting temperature (T_m) of most primers ranges from 55–65°C. Ideally, primers that are used together should have a similar T_m value. The T_m of a primer is the temperature at which 50% of the primer is bound to its target and 50% of the primer is free or single-stranded. T_m is largely affected by GC content; thus, it is advisable to design primers with an equal GC content. GC contents ranging from 40–70% will yield effective primers. In our hands, primers and products with lower GC contents present fewer problems during primer optimization than those with high GC contents. The specifications for the *ASNS* primers developed using Oligo 5.0 Software fall within these guidelines (**Table 3**).
3. Primer dimers. Careful primer design can lessen the occurrence of "primer dimmers," which prevent quantification during QPCR analysis (**Fig. 1B**). Primer dimers are the result of nonspecific annealing and elongation. Primer dimers can result from primers annealing with other primers or with nonspecific sequences. To avoid primer dimers, we design primers in which the 3' end is free of secondary structures, repetitive sequences, palindromes, and degenerate sequences. Likewise, we avoid primer sets that complement each other, specifically at their 3' end. The 3' end is most important, because amplification starts at this end. Further discussions about the occurrence of primer dimers can be found elsewhere ***(10)***.

Table 1
Primer Design Software Available Free on the World Wide Web

Software	Source
NetPrimer	http://www.PremierBiosoft.com/netprimer/netprimer.html
Primer 3	http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi
Primer Selection (USC)	http://norp5424b.hsc.usc.edu/genetools.html
Primer Selection (VGC)	http://alces.med.umn.edu/VGC.html

Table 2
Primer Sequences Designed for Use in QPCR

Primer	Primer sequence 5'-3'	Product size (bp)
ASNS-F	AGAGATTCTCTGGCGACCAAAAGA	203
ASNS-R	CTGGGTAATGGCGTTCAAAGACTT	
GAPDH-F	GTCAACGGATTTGGTCGTATT	223
GAPDH-R	GATCTCGCTCCTGGAAGATGG	
BMV-F	CCGAGGTGTCTGTTGAGGTA	195
BMV-R	CGTCTATGCGATGGTAGGTCT	

Primer sequences were designed using Oligo 5.0 Software.
F, designates a forward primer; R, designates a reverse primer

4. Gene structure. While QPCR is well suited for high-throughput analysis of gene expression, constantly changing the target mRNA sequence can be challenging. It is always useful to dedicate some extra time for checking up on the structure of the gene whose expression is being analyzed. For example, some genes have multiple promoters, which result in multiple tissue or developmental specific transcripts. Primer design must include a consideration of such issues. Primer design is a crucible step in QPCR experiments, because it will set the stage for the later analysis. Careful primer design will save time and money.

3.3.2. Synthesis of Primers

Roche recommends that primers be highly purified to remove extraneous salts left over from the synthesis process. We routinely use standard primers synthesized by Sigma-Genosys without extra purification and without problems. A working primer concentration of 0.5 μ*M* is our standard concentration for use in the LightCycler. Precautions should be taken against primer degra-

Table 3
Specifications for *ASNS* Primers Developed Using Oligo 5.0 Software

	Length (bp)	T_m (°C)	GC content (%)
Product	203	81.2	39.9
Forward primer	20	68.4	50.0
Reverse primer	20	68.9	50.0

Table 4
Titration of $MgCl_2$ to Optimize Primer Sets

	Final Concentration						
	3 m*M*	4 m*M*	5 m*M*	6 m*M*	7 m*M*	8 m*M*	9 m*M*
50 m*M* $MgCl_2$ (μL)	1.2	1.6	2	2.4	2.8	3.2	3.6
water (μL)	12.5	12.1	11.7	11.3	10.9	10.5	10.1

Using a 50 m*M* stock of $MgCl_2$, the final concentration of $MgCl_2$ in the 20 μL QPCR can be varied until an optimal concentration is achieved. The $MgCl_2$ titration described here was used in conjunction with the Master Mixture described in **Subheading 3.4.1.** by varying the amount of water and $MgCl_2$.

dation. Inappropriate storage of primers can result in primer degredation, effectively lowering the concentration of primer solutions. Low primer concentration will result in late amplification or nonamplificaion of target sequences, making quantification difficult or impossible. In our hands, newly synthesized primers will amplify effectively at a working concentration of 0.5 μ*M*. However, because 200 μ*M* primer stocks stored at –20°C in a frosty freezer will degrade after as little as one month, we now routinely use 10 μ*M* as our working concentration. To guard against primer degradation, aliquot concentrated primers (200 μ*M*) and store at –70°C. Thaw only enough aliquots for use in one or two experiments. For long-term storage, lyphilize aliquots and store at –70°C.

3.3.3. Optimization of Primer Sets

Each primer set must be optimized prior to use for quantification. In general, varying the $MgCl_2$ concentration and the annealing temperature will result in clean product formation devoid of primer dimers. Other optimization efforts may be required, however.

1. Use cDNA templates to optimize primer sets. Titrate $MgCl_2$ concentration between 3–7 m*M* (**Table 4**). In our experience, the most common optimal $MgCl_2$

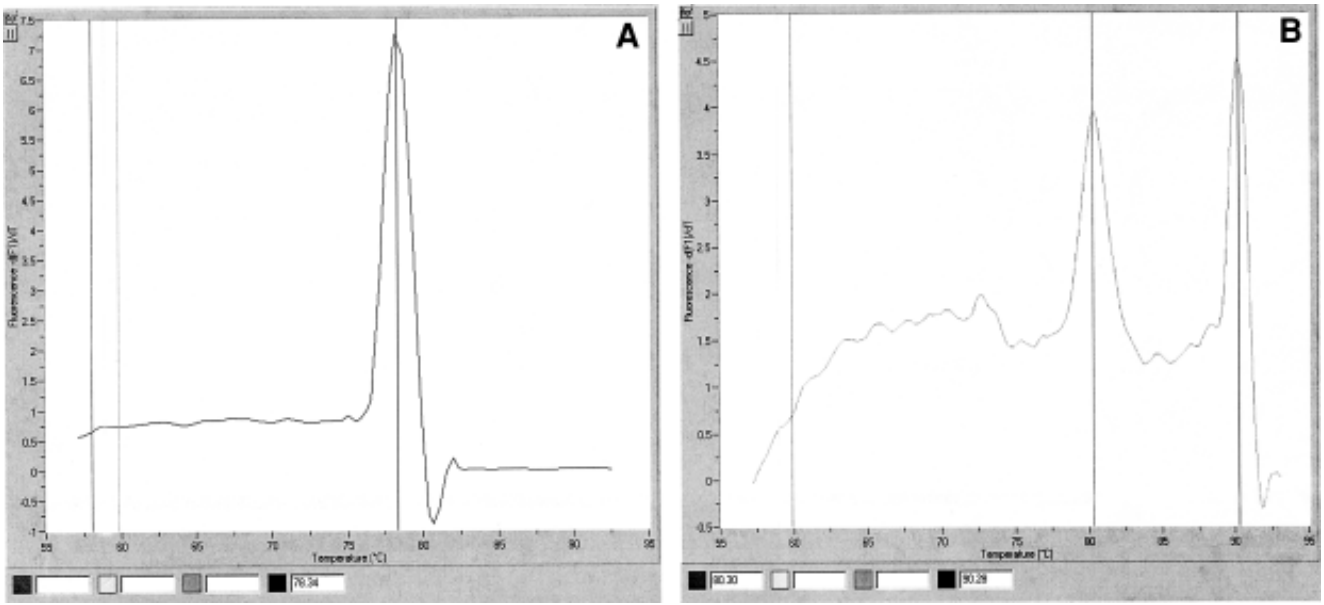

Fig. 1. Analysis of QPCR products by thermal denaturation producing a melting curve. (**A**) Melting curve for *ASNS* primer set with a clear sharp peak at 78.34°C, devoid of primer dimers. This indicates that the primer set has been properly optimized. A T_m of 81.2°C was predicted by the primer design software for the *ASNS* product (**Table 3**). (**B**) Melting curve of a primer set for another gene revealing the presence of primer dimers, with at least two separate products melting at 80.30°C and 90.28°C, respectively. Further optimization of this primer set would be required before it could be used for quantitation.

Table 5
Touchdown Program Used to Determine Highest Annealing Temperature Between 65°C and 59°C

	Step	Temperature (°C)	Time (s)
Cycles 1–10	denature	95	15
	anneal	*65*	20
	elongate	72	11
Cycles 11–20	denature	95	15
	anneal	*64*	20
	elongate	72	11
Cycles 21–30	denature	95	15
	anneal	*63*	20
	elongate	72	11
Cycles 31–40	denature	95	15
	anneal	*62*	20
	elongate	72	11
Cycles 41–50	denature	95	15
	anneal	*61*	20
	elongate	72	11
Cycles 51–60	denature	95	15
	anneal	*60*	20
	elongate	72	11
Cycles 61–70	denature	95	15
	anneal	*59*	20
	elongate	72	11

This program can be easily altered to query higher or lower ranges for optimal annealing temperatures. For the BMV and *GAPDH* primer set, temperatures between 58°C and 50°C were also investigated.

concentration is 4 m*M*, although some primer sets may require as much as 9 m*M* (**Table 4**). For all three primer sets, *ASNS*, *GAPDH*, and BMV, 4 m*M* was the optimal $MgCl_2$ concentration.

2. Ascertain the annealing temperature for each primer set using a touchdown program. During a touchdown program, the annealing temperature is progressively lowered by 1°C after the passage of 10 cycles (**Table 5**). At some point, the permissive temperature for the primer set will be reached, and amplification of the product will begin. This allows for the determination of the highest annealing temperature for the primer set and prevents the accumulation of nonspecific product and primer dimers. The annealing temperatures selected for the primer sets queried were: *ASNS*, 60°C; *GAPDH*, 55°C; and BMV, 54°C.

3. If primer dimers still persist, reducing annealing time may prove helpful. For example, with GC-rich target sequences, which we have found to be the most problematic to optimize, reducing annealing time to 0 s usually prevents dimer formation.
4. Using a "hot start" method may also cut down nonspecific product formation. Commercial anti-*Taq* DNA polymerase antibodies are available, which are easily added to almost any PCR. After incubation with the antibody, polymerase activity is prevented until the temperature rises above 70°C, thus ensuring a hot start and cutting down on the formation of primer dimers. We regularly use Platinum *Taq* DNA polymerase in our QPCR preparations, which contains thermolabile monoclonal antibodies to *Taq* DNA polymerase and ensures an automatic hot start.
5. After attempts to optimize primers, verify specific product formation by determining product T_m, using a melting curve (**Fig. 1A**). During a melting curve program, the temperature is increased in a linear fashion, while fluorescence is monitored constantly. At the product's T_m, fluorescence levels rapidly decrease as the product denatures. The determined T_m can be compared to the theoretical T_m calculated during primer design. Variation between theoretical and actual T_m can vary as much as +/– 3°C. Products with T_m more divergent than +/– 3°C are suspect and may be the result nonspecific product formation.
6. Check products by agarose gel electrophoresis to verify product length. Save products from optimized primer sets for the construction of standard curves (**Subheading 3.2.3.**). Product identity can also be verified by sequencing.

3.3.4. Construction of Standard Curves by Serial Dilution

Relative quantitation relies on the construction of an arbitrary standard curve for each primer set (**Fig. 2**). The points of the standard curve can be arbitrarily designated as 10^{-1}, 10^{-2}, 10^{-3}, etc. Once saved using the LightCycler software, standard curves can be imported and reused in analysis of subsequent QPCRs.

1. Save QPCR products generated during the optimization of each primer set.
2. Serially dilute products up to $1:1 \times 10^8$. In our hands, dilutions from $1:10^3$ through $1:10^8$ usually generate a robust standard curve.
3. Use the product dilutions as target sequences under the optimal conditions determined during primer optimization. At this step, it is vital to label the points of the standard curve and designate them as "standard curve" using the LightCycler program. Details about navigating the software are found in the LightCycler Operator's Manual ***(10)***.
4. Freeze the optimized PCR product and at least one standard from the standard curve for later use. In order for the electronically saved standard curve to be imported and used for later analysis, at least one of the points of the standard curve must be physically rerun in subsequent QPCRs, in which the specific standard curve is desired.

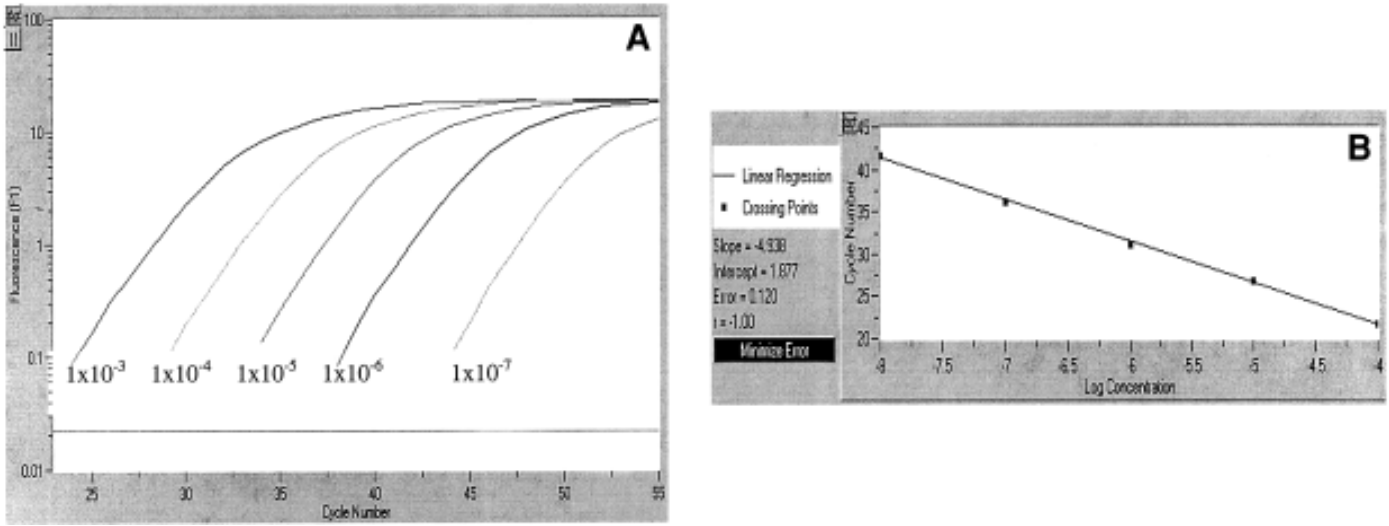

Fig. 2. Generation of a standard curve for *ASNS* primer set. The *ASNS* product produced during the optimization of the *ASNS* primer set was retained and serially diluted. Dilutions between 1×10^{-3} and 1×10^{-7} subsequently underwent QPCR amplification using the *ASNS* primers, generating a relative standard curve. The standard curve is relative (arbitrary), rather than absolute, because the exact copy number of the starting template is not known. Relative concentration of each point of the standard curve is related to the crossing point value (C_T), which corresponds to the cycle at which the product's logarithmic accumulation can be distinguished above background noise.

Table 6
Reaction Conditions Used for SYBR Green I reactions in the LightCycler

Step	Time	Temperature
Denaturation	15 s	94°C
Annealing	20 s	50–60°C
Extension	5 s/100 bp	72°C
Melting curve		up to 95°C

The annealing temperatures selected for the primer sets were: *ASNS*, 60°C; *GAPDH*, 55°C; and BMV, 54°C.

3.4. QPCR

Commercial kits for QPCR using SYBR Green I are available from several companies. In our experience, homemade SYBR Green I mixes work just as effectively. SYBR Green I must be carefully protected from light. Extended exposure will result in bleaching of the dye and a loss of fluorescence signal during QPCR.

3.4.1. Homemade Mixture

1. Dilute SYBR Green I in water to make 100X solution and store at –20°C as 10-μL aliquots.
2. SYBR Green I aliquots were thawed only once on the day of the reaction. Dilute the 100X dye to 10X concentration by adding 90 μL water.
3. Make a master mixture using the following recipe. Master mix for one reaction with 4 m*M* $MgCl_2$ final concentration: 12.5 μL PCR-grade water, 2.0 μL PCR buffer (-$MgCl_2$), 1.6 μL 50 m*M* $MgCl_2$, 0.4 μL 10 m*M* dNTP, 0.5 μL forward primer (0.5 μ*M*), 0.5 μL reverse primer (0.5 μ*M*), 0.5 μL 10X SYBR Green, 0.5 μL 100X BSA, 2.0 μL cDNA, and 0.5 μL Platinum *Taq* DNA polymerase.

3.4.2. Reaction Conditions

1. Analyze cDNA from H1299 Ad βgal, H1299 Ad WT p53, and H1299 Ad p53-D281G in triplicate for each primer set: *ASNS*, *GAPDH*, and BMV. Run separate reactions for each primer set (*see* **Table 6**).
2. Include one point from the already established relative standard curve corresponding to each primer set.
3. Include a negative control for each primer set.
4. Perform the QPCRs for 50 cycles under the optimal conditions found during primer optimization.

3.4.3. Verification of Product by Melting Curve Analysis and Agarose Gel Electrophoresis

1. After completion of each QPCR run, perform melting curve analysis to verify formation of only one specific product.
2. Recover the PCR product by inverting the capillaries in microcentrifuge tubes and pulsing in a microcentrifuge.
3. Analyze products using standard agarose gel electrophoresis techniques to verify correct product size.

3.5. Interpreting QPCR Data

After importing the electronically saved relative standard curve unique to each primer set, use the LightCycler software to determine the relationship of the samples relative to the standard curve. Repeat this procedure for each primer set. **Table 7** demonstrates interpretation of QPCR data to determine *ASNS* gene expression normalized to BMV.

Use the equation to the line obtained from the *ASNS* standard curve ($y = -4.938 \times +1.877$) (**Fig. 2B**) to quantitate the relative amounts of *ASNS* cDNA by linear extrapolation of the C_T values. The LightCycler software automatically performs this extrapolation after designation of a standard curve. Likewise, determine the relative amounts of BMV by linear extrapolation, using the equation to the line obtained from the BMV standard curve (curve not shown). Divide the average relative concentration of *ASNS* in each sample by the average relative concentration of BMV in the same sample, thus normalizing the amount of *ASNS*. The normalized amount of *ASNS*, ($ASNS_N$), is a unitless number that represents the relative amount of mRNA in the samples. In order to simplify interpretation of the results, the control sample H1299 βgal was considered to express a baseline level of *ASNS*, and its expression of *ASNS* was designated as "1". All other expression values are understood relative to this control sample. Divide each normalized value by the Ad βgal normalized value. Values generating decimals were expressed as negative numbers by dividing –1 by the decimal. Following this convention in **Table 7**, the relative expression of *ASNS* in Ad WT p53 is presented as –2.2 instead of 0.464. H1299 Ad p53 was found to express *ASNS* 2.2-fold less than H1299 Ad βgal, and H1299 Ad p53-D281G expressed *ASNS* 18.3-fold more than H1299 Ad βgal.

4. Notes

1. Choosing *GAPDH* as the housekeeping gene of reference. Suzuki et al. (2000) reviewed the pitfalls of relying on *GAPDH* as a housekeeping gene ***(11)***. *GAPDH* levels have been seen to vary in certain cellular conditions, e.g., heat shock, hypoxia, high levels of glucose or insulin, and exposure to UV light ***(11)***. Some studies have indicated that *GAPDH* regulation is dependent on the cell cycle ***(11)***. Elevated *GAPDH* transcript levels have been observed in breast carcinoma cell

Table 7
ASNA Gene Expression Normalized to BMV, Relative to H1299 Ad βgal

	ASNS		BMV			$ASNS_N$
H1299 Sample	C_T	Relative concentration	C_T	Relative concentration	Normalized to BMV	Relative to Ad βgal
Ad βgal	43.22	2.484E-09	30.95	9.109E-06		
	43.47	2.203E-09	29.98	1.401E-05	1.785E-04	1.0
	43.54	2.215E-09	29.74	1.554E-05		
average		*2.301E-09*		*1.289E-05*		
Ad WT p53	44.72	1.210E-09	29.9	1.452E-05		
	44.33	1.459E-09	29.64	1.628E-05	8.300E-05	–2.2
	44.82	1.154E-09	29.78	1.526E-05		
average		*1.274E-09*		*1.535E-05*		
Ad p53-D281G	37.94	3.090E-08	30.93	9.167E-06		
	37.70	3.469E-08	30.61	1.057E-05	3.269E-03	18.3
	37.79	3.328E-08	30.63	1.051E-05		
average		*3.296E-08*		*1.008E-05*		

Interpretation of QPCR data by normalization to BMV and expressed relative to the control sample, H1299 Ad βgal, allows relative quantitation of *ASNS* gene expression levels. *ASNS* was up-regulated in the presence of mutant p53-281G and down-regulated in the presence of WT p53.

Table 8
Normalization of ASNS Expression to GAPDH

	ASNS		*GAPDH*		$ASNS_N$	
H1299 Sample	C_T	Relative concentration	C_T	Relative concentration	Normalized to *GAPDH*	Relative to Ad βgal
Ad βgal	43.22	2.484E-09	36.63	2.931E-07		
	43.47	2.203E-09	37.28	2.130E-07	8.979E-03	1.0
	43.54	2.215E-09	36.85	2.626E-07		
average		*2.301E-09*		*2.562E-07*		
Ad WT p53	44.72	1.210E-09	35.72	4.593E-07		
	44.33	1.459E-09	36.16	3.698E-07	2.804E-03	–3.3
	44.82	1.154E-09	35.14	5.341E-07		
average		*1.274E-09*		*4.544E-07*		
Ad p53-D281G	37.94	3.090E-08	34.53	8.221E-07		
	37.70	3.469E-08	33.93	1.110E-06	3.333E-02	3.7
	37.79	3.328E-08	34.07	1.034E-06		
average		*3.296E-08*		*9.887E-07*		

Normalization of *ASNS* expression data relative to *GAPDH* did alter the relative expression levels of *ASNS*. However, the same trend was maintained, with *ASNS* down-regulated by WT 53 and up-regulated by mutant p53-D281G.

Table 9
Non-normalized *ASNS* Gene Expression

	ASNS		$ASNS_N$
H1299 Sample	C_T	Relative concentration	Relative to Ad βgal
Ad βgal	43.22	2.484E-09	
	43.47	2.203E-09	1.0
	43.54	2.215E-09	
average		*2.301E-09*	
Ad WT p53	44.72	1.210E-09	
	44.33	1.459E-09	–1.7
	44.82	1.154E-09	
average		*1.274E-09*	
Ad p53-D281G	37.94	3.090E-08	
	37.70	3.469E-08	14.3
	37.79	3.328E-08	
average		*3.296E-08*	

Analysis of the data relative to the H1299 Ad βgal control, but without normalization to a housekeeping gene or other standard, still maintained the trend of WT p53 down-regulating *ASNS*, and mutant p53-D281G up-regulating its expression.

lines, mouse cell lines, and hepatocellular carcinoma tissues ***(11)***. *GAPDH* has been implicated in a wide-range of activities, some of which are independent of its role in glycolysis, including endocytosis, translational control, nuclear tRNA export, DNA replication, DNA repair, and apoptosis ***(11)***. Evidence indicates that *GAPDH* is involved in apoptosis of non-neuronal and neuronal cells ***(12)***. Furthermore, *GAPDH* seems to be up-regulated by p53 during neuronal apoptosis ***(13)***. In light of this information, the choice of *GAPDH* as a housekeeping gene should be considered cautiously, especially in studies involving p53. Other housekeeping genes, such as *β-actin*, *18S* or *28S rRNA*, should also be considered carefully, because they have their own drawbacks ***(11)***. We reanalyzed our QPCR data by normalizing expression of *ASNS* to the expression of *GAPDH* (**Table 8**). The relative expression levels of *ASNS* did change compared to normalization with BMV (**Table 7**), although the overall pattern still persisted with *ASNS* down-regulated by wild-type p53 and up-regulated by mutant p53-D281G.

2. Non-normalized gene expression. An alternative to normalizing gene expression, to either a housekeeping gene or to an exogenously applied nucleic acid sequence such as BMV RNA, is to analyze data without normalization (**Table 9**). Assuming that RNA amounts used for cDNA synthesis were equal between all samples (i.e., H1299 Ad βgal, H1299 Ad WT p53, and H1299 Ad p53-D281G) and assuming that cDNA synthesis efficiency was equal in all samples, there is no

need to normalize *ASNS* expression. Our main concern using this method of analysis is the assumption that cDNA synthesis efficiency is equal. The presence of salts or other "minor" contaminants can easily disrupt the efficiency of the reverse transcriptase enzyme. Without a reference, such as a housekeeping gene, or a quality control standard, such as BMV RNA, there is no way to compensate for variations in cDNA synthesis efficiency and/or variation in total RNA concentration between samples. In order to have confidence in such data, it would be necessary to repeat the entire experiment (e.g., starting from adenoviral infection) a number of times.

3. Use of an exogenous quality control RNA: BMV. The use of BMV as a reference RNA to monitor cDNA efficiency rests on two points. First, the amount of RNA between samples, H1299 Ad βgal, H1299 Ad WT p53, and H1299 Ad p53-D281G, must be equal. The use of the ultrasensitive RiboGreen quantitation method to quantify total RNA prior to cDNA synthesis allows us to feel confident on this issue. Second, we must ensure that we are spiking the RNA samples with equal amounts of BMV prior to cDNA synthesis. This is a very serious point; pipeting error at this stage could very easily skew further data generated from subsequent QPCR analysis. To lessen the risk of pipeting error, we pipet BMV RNA in vol >6 μL. Additionally, multiple independent cDNA preparations (at least three) individually spiked with BMV RNA are used to repeat the analyses. Consistent results between preparations is our standard for successful quantitation of gene expression by QPCR.
4. Further QPCR applications. QPCR has the potential for a broad range of applications including mutation detection, absolute quantitation, and detection of transcriptional splice variants. More extensive coverage of theory, applications, and analysis of data is available ***(14)***.

Acknowledgments

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7

Transactivation and Transrepression Studies with p53

Mariano J. Scian, Rebecca Frum, Swati Palit Deb, and Sumitra Deb

Summary

The methods outlined in this chapter are designed to facilitate the study of the transactivation and transrepression properties of p53 (as well as p63 and p73). Once a gene of interest is identified, its presumptive promoter region can be cloned upstream of a luciferase gene in a plasmid. The most common reason for transfection experiments is to study gene expression patterns in the presence or absence of a particular gene product (e.g., p53). Three methods of transfection are outlined in this chapter: (*i*) cationic lipofection; (*ii*) calcium phosphate precipitation; and (*iii*) BES precipitation. The first method is ideal for the study of transactivation and transrepression properties of p53 (or other transcription factors). The last two are more suited for experiments where larger numbers of transfected cells are needed. Several examples of transfections and their respective results are provided.

Key Words

p53, promoter studies, transactivation, transrepression and transfection methods

1. Introduction

The *p53* gene is located on chromosome 17p and encodes an oligomeric 393-amino acid nuclear phosphoprotein that acts as a tumor suppressor. Wild-type (WT) p53 acts as a sequence-specific DNA-binding protein and transcriptional activator as well as a transcriptional repressor (reviewed in **refs. *1–5***). The consensus DNA-binding site contains two copies of the 10-bp motif 5'-PuPuPuCWWGPyPyPy-3', which can be separated from 0–13 bp ***(6)***.

Elevated levels of WT p53 can lead to apoptosis in certain cell types or induce cell cycle arrest in G_1 or G_2 ***(1–5)***. WT p53 levels increase in response to cellular stress situations, such as DNA damage. The elevated level of p53 induces expression of genes that are involved in various aspects of cellular growth regulation. These target genes of WT p53 are systematically being identified using various methods, such as serial analysis of gene expression, differ-

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ential display, subtractive hybridization, and gene array (or oligonucleotide array) analysis *(7,8)*. WT p53 has also been shown to repress transcription from a number of cellular genes ***(9–15).***

Two homologues of p53 have been identified in recent years: p63 and p73. Both p73 and p63 have structural and functional similarity with p53, although p63 and p73 are more similar to each other than to p53 *(16,17)*. Several isoforms have been identified for these family members; these vary in their N- or C-terminal regions. Splice variants have been demonstrated for p73, such as p73 α, -β, -γ, -δ, with p73-α and p73-β being the most prominent forms. Similar splice variants have been identified for p63 *(16,18–20)*. p73 has been shown to transactivate promoters with p53-binding sites *(21,22)*. Therefore, transactivation studies can also be conducted with p73 or p63 individually or in comparison to p53.

Once a gene of interest is identified, its presumptive promoter region can be cloned upstream of a luciferase gene in a plasmid. After cloning or identifying a p53 (p73 or p63) target gene, many researchers wish to analyze the characteristics of the particular promoter by reintroducing into cells. The most common reason for transfection is to study gene expression in the presence or absence of a particular gene product (e.g., p53). Therefore, selection of a cell line to be transfected is critical. When studying p53, it is important to select a cell line with the p53 genotype of choice. In our laboratory, to avoid any effects from endogenous p53, we routinely use Saos-2 cells, which do not express p53 *(23)*. Saos-2 osteosarcoma cells are easily transfectable by any of the methods outlined below. However, other cell lines may vary in their susceptibility to transfection, and it may be necessary to optimize the protocols outlined below to suit the cell line.

2. Materials

2.1. Cationic Lipofection Method

1. Transfection carrier vector (e.g., pCMV-Bam) *(24)* with p53 (WT or mutant) cDNA insert.
2. Luciferase reporter vector (e.g., pGL3 or pGL2 from Promega [Madison, WI, USA]) with desired promoter insert.
3. LIPOFECTAMINE 2000 (Invitrogen, Carlsbad, CA, USA).
4. Opti-MEM™ I media (Invitrogen).
5. 24-well plates.
6. A transfectable p53-null cell line (e.g., Saos-2) *(23)*.

2.2. Whole Cell Lysate Preparation

1. 5X Reporter lysis buffer (Promega).
2. Phosphate-buffered saline solution.

2.3. Calcium Phosphate Transfection Method

It is important to measure the amounts of each component and vol of each solution as accurately as possible.

1. 2X (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (HEPES)-buffered solution (HEBS): 250 m*M* NaCl, 10 m*M* KCl, 10 m*M* dextrose, 40 m*M* HEPES. Make 100 mL of the solution. First dissolve the components in 95 mL of water, then adjust the pH to 7.08 using one drop of 10 *N* NaOH and then with 0.1 *N* NaOH. When the desired pH is reached, complete the vol to 100 mL. Filter-sterilize using a 0.2-μm filter under sterile conditions and store the solution at 4°C.
2. 2 *M* $CaCl_2$ solution (100 mL). Filter-sterilize as described above and store the solution at –20°C.
3. 100X PO_4 (50 mL): 120 m*M* Na_2HPO_4. Filter-sterilize as described above and store the solution at 4°C.

2.4. β-Galactosidase Assay

1. 2X β-Galactosidase assay buffer: 200 m*M* sodium phosphate, pH 7.3, 2 m*M* $MgCl_2$, 100 m*M* β-mercaptoethanol, 1.33 g/mL O-nitrophenyl-b-β-galactopyranosidase (ONPG). This solution can be made in quantity, aliquoted, and stored at –20°C for future use.
2. 500 m*M* Na_2CO_3.

2.5. BES Transfection Method

As with the solutions used in the calcium phosphate precipitation method, it is important to measure the amounts and vol as accurately as possible.

1. 2X (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid])(BES) solution: 50 m*M* BES, 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4. Bring the vol to 100 mL using sterile water, adjusting the pH to 6.95 using 0.1 *N* NaOH. Filter-sterilize the solution with a 0.2-μm filter under sterile conditions; make aliquots of 1 mL and store at –20°C. It is important to not freeze-thaw the BES solution repeatedly, as this will decrease the effectiveness of the solution.
2. 2.5 *M* $CaCl_2$ solution. Bring the vol to 100 mL and make a few aliquots of 1 mL. Filter-sterilize with a 0.2-μm filter under sterile conditions, and store at –20°C.
3. 0.5 m*M* kynureate solution (optional). Filter-sterilize and store the solution at 4°C.

3. Methods

The methods outlined below describe three different protocols of transfection. The cationic lipofection method can primarily be used for the study of transactivation and transrepression properties, while the latter two are also used for studies where larger numbers of transfected cells are necessary (e.g., cell cycle profiling).

3.1. Cationic Lipofection Method

The following protocol is designed to transfect cells growing in 24-well plates. If larger wells or plates are to be transfected, adjust the vol to be used accordingly (*see* **Table 1**).

1. Twenty-four hours prior to transfection, plate cells in a manner so that they are 90–100% confluent the day of transfection. For example, for Saos-2 cells, approx 2.5×10^5 cells in a vol of 500 µL per well must be plated when using LIPOFECTAMINE 2000.
   Note: other cell lines may require different starting plating conditions. For reference, refer to the product sheet provided with the transfection reagent, the manufacturer's Web site, or technical support. If the cell line of choice is not listed, it will be up to the researchers to choose the starting cell amounts and optimization (*see* **Note 1**).
2. For convenience during transfection, dilute all the DNAs to be used so that the final concentration is 100 ng/µL. This dilution can be done using either sterile water or Opti-MEM I. Other DNA concentrations can also be used.
3. The day of transfection, prior to starting the transfection procedure, replace the media in the wells with freshly prepared complete media. Opti-MEM I or media without serum can be used instead. In such a case, remove the media without serum or Opti-MEM I 4–8 h after transfection and replace it with complete media.
4. Take out one 1.5-mL Eppendorf® tube per well to be transfected and label accordingly.
5. Dilute the DNA so that the final concentration does not exceed 1 µg/50 µL. In the examples below, all DNAs used are diluted to a concentration of 100 ng/µL. Since the total amount of DNA used is 1 µg, 40 µL of Opti-MEM I must be added to each Eppendorf tube to make up the vol to 50 µL.
6. In a 50-mL Falcon® tube, dilute the LIPOFECTAMINE 2000 by adding 2 µL of LIPOFECTAMINE 2000 into 48 µL of Opti-MEM I per well to be transfected. For example, when transfecting 24 wells, add 48 µL of LIPOFECTAMINE 2000 in 1152 µL of Opti-MEM I. Other cationic lipofection agents may require different dilutions. See the manufacturer's recommendations for such cases.
7. Vortex mix gently and incubate the LIPOFECTAMINE 2000 dilution for 3 min at room temperature.
8. At the end of the incubation period, add 50 µL of the diluted LIPOFECTAMINE 2000 solution to each of the DNA-containing Eppendorf tubes. The total vol per tube now should be 100 µL.
9. Spin down the tubes, vortex mix slowly, and incubate the mixture for 15 min to allow the LIPOFECTAMINE-DNA complexes to form. *Do not vortex mix the tubes after the incubation period is finished*. This will break apart the LIPOFECTAMINE-DNA complexes, decreasing the effectiveness of the transfection.
10. After incubation, add the contents of each Eppendorf tube drop-wise to the appropriate well. Mix the contents of the well by gently shaking or rocking the plate after each addition.

Table 1
Conversion Table

Type of plate	Relative surface area (compared to 24-well plate)	Plating vol	DNA dilution vol	LF2000 dilution vol
96-Well plate	0.2	100 µL	25 µL	25 µL
24-Well plate	1	500 µL	50 µL	50 µL
12-Well plate	2	1.0 mL	100 µL	100 µL
35-mm Plate	4	2.0 mL	200 µL	200 µL
6-well plate	5	2.5 mL	250 µL	250 µL
60-mm Plate	10	5.0 mL	500 µL	500 µL
100-mm Plate	28	14.0 mL	1.4 mL	1.4 mL

Vol are per well or plate to be transfected.

Note: the information on this table was adapted from the LIPOFECTAMINE 2000 data sheet. If using other cationic lipofection reagents, please refer to data sheet provided with the product.

11. Grow the cells for 24–48 h before harvesting, depending on the cell line transfected. It is recommended to check the cells 24 h after transfection, since different cell lines react differently to the cationic lipofection. If the cells have been noticeably affected by the transfection and appear to be dying, harvesting the cells after 24 h may be necessary (*see* **Notes 2–6**).

3.2. Preparation of Cell Lysates

Please note that the following protocol is for preparing cell lysates after transfection of cells growing in 24-well plates. When larger wells or plates are used, adjust the vol accordingly using the vol provided in **Table 1**.

1. Dilute the 5X Reporter lysis buffer to a 1X concentration using sterile water.
2. Aspirate the media from the wells.
3. Wash the cells with 300 µL of phosphate-buffered saline solution 2X.
4. Add 100 µL of 1X Reporter lysis buffer to each well and incubate for 5 min at room temperature.
5. Scrape the cells from the wells using a rubber policeman or similar device.
6. Transfer the lysis buffer and cells to a 1.5-mL Eppendorf tube. Keep the Eppendorf tubes on ice from this point on.
7. Pipet up and down to mix the contents of the tube and aid in the lysis of the cells. Freeze-thawing once or twice can be done to increase the protein extraction. Quick-freezing the cell lysates in a dry ice–ethanol bath and thawing them in room temperature water can accomplish this.
8. Once all wells have been harvested, spin the whole cell lysate at 8000–10,000 rcf for 2–5 min at 4°C.
9. Transfer the supernatant to a new tube and discard the pellet.
10. Determine the protein concentration of each cell lysate using Protein Assay Reagent (Bio-Rad, Hercules, CA, USA) or your method of choice. Normalize the amounts of protein in each extract and perform the luciferase assay.

3.3. Calcium Phosphate Precipitation Method

1. Prepare and test the solutions needed for this method prior to use. A "test" transfection using a β-galactosidase containing plasmid and performing the subsequent β-galactosidase assay (*see* **Subheading 3.4.**) should suffice (*see* **Notes 7–9**).
2. Twenty-four hours prior to transfection, plate the cells at a density of 6×10^6 cells/10-cm plate. The plating cell density may need to be changed depending on the cell line used.
3. The following day, prior to beginning the transfection procedures, allow the transfection solutions to reach room temperature.
4. Two hours before adding the precipitation solution to the plate, remove the media from the plate, and replace it with 9.0 mL of Dulbecco's modified Eagle medium (DMEM) containing the normal serum needed for the cell line.

5. For every plate to be transfected, take out and label appropriately two 1.5-mL Eppendorf tubes, one for the DNA and one for the HEBS/PO_4 solution.
6. In a 50-mL Falcon tube, mix 100 μL of the 100X PO_4 solution for every 9.9 mL of the HEBS solution prepared earlier to generate the working HEPES/PO_4 solution.
7. Add 500 μL of the working HEBS/PO_4 mixture to the first set of Eppendorf tubes.
8. To the second set of Eppendorf tubes, add the appropriate amount of DNA (normally up to 10 μg), and make up the vol to 437.5 μL with sterile water.
9. Immediately prior to the subsequent bubbling step, add 62.5 μL of the 2 *M* $CaCl_2$ solution to the DNA tubes.
10. Vortex mix gently, and spin down these tubes.
11. Using a pipet-aid, set up a "bubbling apparatus." Clamp the pipet-aid to a vertical stand, and tape the blowout button, so that when the tip of a 1-mL pipet is submerged into a liquid medium, it produces a slow steady stream of bubbles. This may take several attempts. The bubbling step is very important to the outcome of the transfection. We have found that a speed of 20 bubbles/10 s gives the best results.
12. Add the DNA solution drop-wise to the Eppendorf containing the HEBS/PO_4 solution while bubbling from the bottom of the Eppendorf tube using a 1-mL pipet. A fine precipitate will form. Use a new 1-mL sterile pipet for each addition.
13. Let the mixed solutions incubate for 30 min in an undisturbed space.
14. After incubating the DNA with the HEBS/PO_4, gently mix, and add the contents (1 mL per plate) of the tubes drop-wise to the appropriate plate. Place the plates at 37°C, being careful not to disturb the precipitate, as this will decrease the efficiency of the transfection.
15. The following day, remove the media, and wash the precipitate from the plates 2X using Hank's balanced salt solution (HBSS). Add fresh complete media to the plates and return the plates to the incubator.
16. Collect the cells 24 h later, following the appropriate or desired protocol.

3.4. β-Galactosidase Assay

1. Transfect the cells following the calcium phosphate precipitation method described, and allow the cells to grow for 48 h after transfection.
2. Harvest the cells and prepare whole cell lysate as previously described.
3. Measure the protein concentration of the whole cell lysate using Bio-Rad's protein assay reagent or your method of choice in order to normalize the amounts of cell lysate to be added.
4. Add appropriate cell lysate amount into a separate tube. The maximum vol of cell lysate to be added to the reaction mixture should not exceed 20 μL.
5. Add lysis buffer to the same tube to a final vol of 150 μL.
6. Add 150 μL of the 2X-assay buffer prepared earlier to the Eppendorf tube.
7. Incubate the mixture at 37°C for 10 min.
8. Stop the reaction by adding 1 mL of 0.5 *M* Na_2CO_3.
9. Measure the OD of the extract using a spectrophotometer with the wavelength set at 420 nm.

3.5. BES Transfection Method

1. Prepare and test the solutions needed for this method prior to use (*see* **Subheading 2.3.**) by transfecting and performing a β-galactosidase assay (*see* **Subheading 3.4.**).
2. Twenty-four hours prior to transfection, plate the cells at a density of 6×10^6 cells per plate. The cells should be 40–50% confluent on the day of transfection. It may be required to adjust this number depending on the cell line used.
3. Two hours before the transfection, remove the media from the plate, and replace it with 8.5 mL of complete media. Place the cells to be transfected at 37°C and a CO_2 percentage of 2–4% while preparing the reagents.
4. Thaw aliquots of the 2X BES as needed.
5. In a 1.5-mL Eppendorf tube, mix 1.35 mL of sterile water and 150 µL of 2.5 *M* $CaCl_2$ to make a 0.25 *M* $CaCl_2$ solution. More can be made if necessary.
6. Add 500 µL of the 0.25 *M* $CaCl_2$ solution to each aliquot of BES and mix well.
7. Add up to 10 µg of DNA to each of these tubes, mix well by pipeting up and down, and let it incubate for 20 min at room temperature.
8. Add the DNA cocktail to the cells in a drop-wise fashion. Return the cells to the 2–4% CO_2 incubator.
9. The cells should be rinsed 2X with HBSS approx 15–18 h after the transfection. Add complete cell culture media to the plates.
10. Check the quality of the precipitate under the microscope, as this is a good indicator of the efficiency of transfection. The precipitate should be moderately fine and evenly distributed. A coarse precipitate indicates a poor transfection; an extremely fine precipitate is also nonoptimal.
11. Optional: after the second wash, add media containing 0.5 m*M* kynureate. This appears to improve cell survival after transfection.
12. Return the cells to the appropriate CO_2 percentage for the cells in use. Harvest cells 24–48 h later (*see* **Notes 10** and **11**).

4. Notes

1. As with many products and methods, optimization may become necessary, particularly when using a different cell line. The amounts of promoter–luciferase plasmid needed may vary from cell line to cell line. In such cases, it may be necessary to vary the amount of luciferase plasmid transfected. For example, 200 ng of a promoter (p21)–luciferase construct transfected with 10 ng of mutant p53 plasmid will produce a 12-fold induction when transfected into Saos-2 cells. This may not necessarily be the case when transfecting the same amount of luciferase and plasmid using a different cell line (e.g., H1299).
2. Not all cell lines are readily transfected with LIPOFECTAMINE 2000. Some cell lines are more susceptible to the reagent than others. It is, therefore, important to check the transfected cells 24 h after transfection for any indication of incompat-

ibility. This is generally indicated by a large number of dead cells seen in the media ("floaters"), with a lesser number of attached cells compared to the previous day. In a case like this, it may be necessary to prepare the whole cell lysates after 24 h rather than 48 h after transfection.

3. The protocol given in **Subheading 3.1.** can be adjusted to accommodate transfections in large wells or plates. Use **Table 1** as a guide.
4. When studying the transactivation and transrepression properties of WT p53, the first step is to determine how the promoter will react to the presence of the WT or mutant protein. Different promoters have WT p53-binding sequences of different affinities and may not be transactivated or transrepressed with the same degree; therefore, a transfection gradient is needed. **Table 2** provides an example of a gradient used in a transactivation experiment using the p21 promoter. A similar setting can be used for a transrepression study using a promoter that is transcriptionally inhibited in the presence of WT p53.

   Figure 1 shows the results of a gradient study in which the p21 promoter, which is a normal target of WT p53, was used. Here, increasing the amounts of p53 did not increase the luciferase activity obtained, possibly due to squelching. This indicated that low amounts of p53 are adequate. If necessary, the amounts of p53 can be increased. The same type of arrangement can be used for a transrepression study and a similar setup can be used to study mutant p53 transactivation properties. Most tumors expressing mutant p53 retain and overexpress the mutant protein ***(5,25)***. Therefore, to mimic the elevated levels of mutant p53 in tumor cells, the amounts of tumor-derived p53 mutant used are larger (**Table 3** and **Fig. 2**).

   In this case, increasing the amount of mutant p53 used in the transfection increased the amount of luciferase activity observed, suggesting that this promoter is affected in a concentration-dependent manner. Other transfections can be done to determine the response of the promoter in the presence of amounts lower or higher than amounts used in this assay.
5. After determining the amount of WT p53 needed to obtain an optimum response from the desired promoter, it may be necessary to study whether tumor-derived mutants of p53 will affect the promoter under the same conditions. **Table 4** is an example of such a transfection.

   Figure 3 shows the results of the transfection described in the above table. Here, transfection with 50 ng of the mutant p53 did not produce a significant response from the p21 promoter, as compared to WT p53. Similar studies can be conducted comparing the ability of WT p53 and the various isoforms of p63 and p73 to transactivate or transrepress a given promoter. Similarly, the effect of WT or mutant p53 on promoter deletion mutants can also be carried out.
6. When studying promoters of genes affected by tumor-derived mutants of p53, it may be necessary to use a higher amount of the mutant protein to show significant transactivation. In this case, adjusting the amounts used in the transfection protocol may become necessary. For example, 10 ng of WT p53 DNA will induce

Table 2
Example Setup for a Transactivation Study with WT p53 to Determine Optimum Response

Well no.	1	2	3	4	5	6
Promoter amount (e.g., p21.Luc)	200 ng	200 ng	200 ng	200 ng	200 ng	200 ng
pCMV-Bam	10 ng	—	50 ng	—	250 ng	—
pCMV-Bam WT p53	—	10 ng	—	50 ng	—	250 ng
Filler DNA (e.g., pUC 19)	790 ng	790 ng	750 ng	750 ng	550 ng	550 ng

CMV, cytomegalovirus.

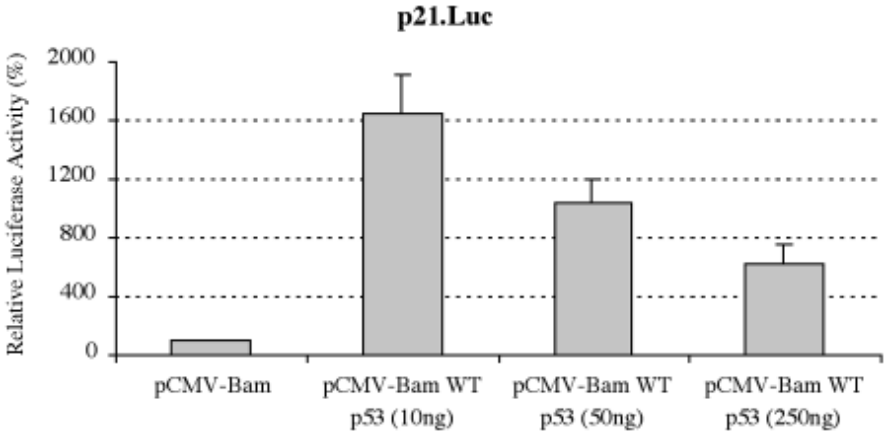

Fig. 1. Human osteosarcoma Saos-2 cells were transfected with the expression plasmid for WT p53 (pCMV Bam WT p53) or vector alone (pCMV-Bam) and p21.Luciferase (p21.Luc) using the LIPOFECTAMINE 2000 reagent as described in **Table 2**. The cells were harvested 48 h after transfection using Reporter lysis buffer, and luciferase activity was measured using a luminometer from Turner Designs (Sunnyvale, CA, USA). The vector reading was set to 100.

the p21 promoter approx 12- to 16-fold, whereas in the case of the epidermal growth factor receptor (EGFR) promoter, 800 ng of p53-D281G will produce approx a 10-fold induction.

7. Prior to doing any experimental transfections with the calcium phoshpate method, it is recommended to set up and carry out a set of transfections to generate a β-galactosidase standard curve. This can be done by transfecting various amounts of β-galactosidase plasmid (e.g., 1, 2, 5 μg, etc.), harvesting the cells after 48 h, and measuring the β-galactosidase activity. The number of starting cells and the bubbling rate can also be varied. This will generate a standard curve that can be used to approximate the efficiency of the transfection. The efficiency can only truly be known if the transfected cells are sorted, counted, and compared to the total number of cells.
8. The bubbling step is a critical step. The bubbling allows for the mixing of the DNA with the HEBS/PO_4 solution. As stated earlier, we have found that a bubbling rate of 20 bubbles/10 s gives the best results. Any deviation from this rate appears to decrease the transfection efficiency.
9. Here is an example of a transfection carried out using the calcium phosphate precipitation method.
   Figure 4 shows the results of the transfection described in **Table 5**. The β-galactosidase readings were as follows: attempt no. 1, 0.002 and 0.136 for the vector and β-galactosidase transfected cells, respectively; and attempt no. 2, 0.001 (vec-

Table 3
Example Setup for a Transactivation Study with Mutant p53 to Determine Optimum response

Well no.	1	2	3	4	5	6
Promoter (e.g., NFκB2.Luc)	200 ng	200 ng	200 ng	200 ng	200 ng	200 ng
pCMV-Bam	200 ng	—	400 ng	—	800 ng	—
pCMV-Bam p53-D281G	—	200 ng	—	400 ng	—	800 ng
Filler DNA (e.g., pUC 19)	600 ng	600 ng	400 ng	400 ng	—	—

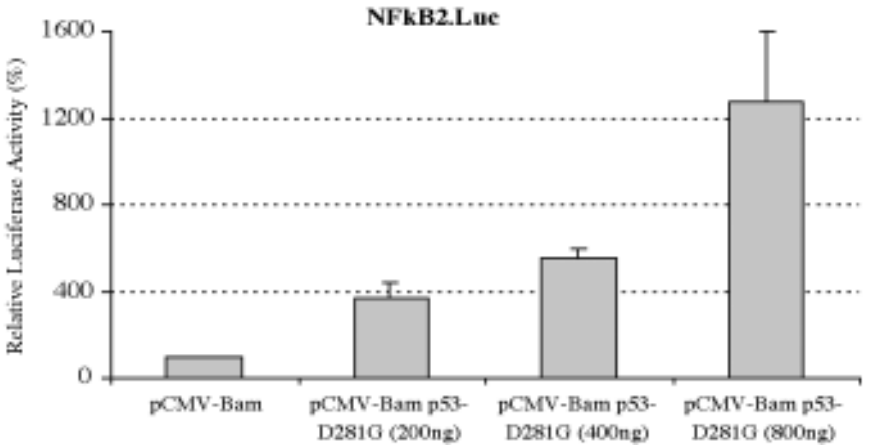

Fig. 2. Human osteosarcoma Saos-2 cells were transfected with pCMV-Bam p53-D281G or vector alone (pCMV-Bam and NfκB2.Luciferase (NFκB2.Luc), using the LIPOFECTAMINE 2000 reagent as described in **Table 3**. The cells were harvested 48 h after transfection, using Reporter lysis buffer, and luciferase activity was measured. The vector reading was set to 100.

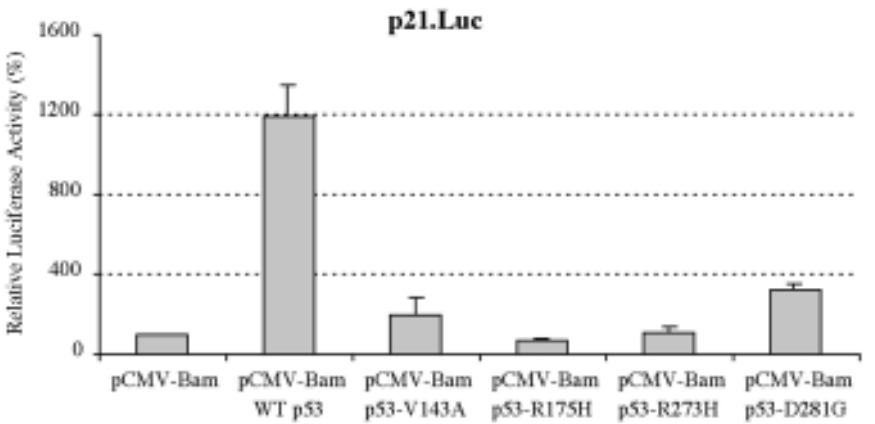

Fig. 3. Human Saos-2 cells were transfected with the expression plasmid for WT p53, p53-V143A, p53-R175H, p53-R273H, p53-D281G, or vector alone (pCMV-Bam) and p21.Luc using the LIPOFECTAMINE 2000 reagent as described in **Table 4**. The cells were harvested 48 h after transfection using Reporter lysis buffer, and luciferase activity was measured. The vector reading was set to 100.

Table 4
Example Setup for a Transactivation Study with WT p53

Well no.	1	2	3	4	5	6
Promoter (e.g., p21.Luc)	200 ng	200 ng	200 ng	200 ng	200 ng	200 ng
pCMV-Bam	50 ng	—	—	—	—	—
pCMV-Bam WT p53	—	50 ng	—	—	—	—
pCMV-Bam p53-V143A	—	—	50 ng	—	—	—
pCMV-Bam p53-R175H	—	—	—	50 ng	—	—
pCMV-Bam p53-R273H	—	—	—	—	50 ng	—
pCMV-Bam p53-D281G	—	—	—	—	—	50 ng
Filler DNA (e.g., pUC 19)	750 ng	750 ng	750 ng	750 ng	750 ng	750 ng

Table 5
Example of a Transfection Setup by the Calcium Phosphate Precipitation Method

Plate no.	1	2
Tube set no. 1		
HEBS/PO_4	500 μL	500 μL
Tube set no. 2		
2 *M* $CaCl_2$	62.5 μL	62.5 μL (added last)
Sterile water	to 437.5 μL	to 437.5 μL
DNA (pCMV Bam)	10 μg	—
DNA (pCMV β-galactosidase)	—	10 μg

Table 6
Example of a Transfection Setup Done Using the BES Method

Plate no.	1 (mock)	2
2X BES	500 μL	500 μL
0.25 *M* $CaCl_2$	500 μL	500 μL
DNA (pCMV Bam WT p53)	—	10 μg

tor) and 0.027 (β-galactosidase). Clearly, the first attempt resulted in a much better transfection. Reagents that produce low results such as this should be discarded and prepared again. Therefore, it is recommended to include a β-galactosidase transfection, which can be compared to the standard curve generated earlier, to get an idea of the efficacy of the transfection.

10. Unlike the calcium phosphate precipitation method, there is no bubbling step when using the BES method.
11. Here is an example of a transfection using the BES method (**Table 6**). Saos-2 cells were plated at equal densities (3×10^6) 24 h prior to transfection. Cells were then transfected with 10 μg of DNA using the BES method and allowed to grow for 24 h. The cells were then washed with cold phosphate-buffered saline (PBS), trypsinized, and collected in a 50-mL Falcon tube. Samples were then pelleted, resuspended in 1 mL of PBS containing 0.05 m*M* phenylmethylsulfonyl fluoride (PMSF), and analyzed by flow cytometry ***(26)*** after incubation of the cell pellet with the p53 antibody DO1 (Oncogene Research Products, Cambridge, MA, USA) and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Cells were then stained with propidium iodide. The transfected (T) cells were sorted and compared to untransfected (U) cells in the same plate. An untransfected plate was used as a mock (**Fig. 5**).

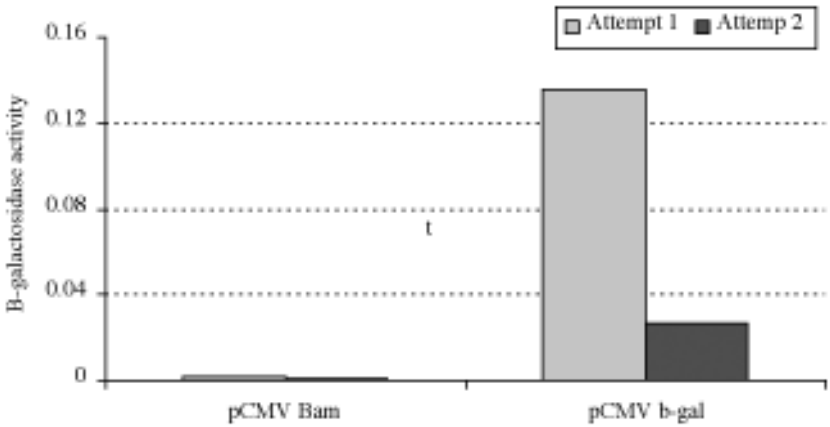

Fig. 4. Human Saos-2 cells were transfected with pCMV β-galactosidase or vector alone (pCMV-Bam), using the calcium phosphate precipitation method. The cells were harvested 48 h after transfection, using Reporter lysis buffer, and β-galactosidase activity was measured as described in **Table 6**. The readings were 0.002 and 0.001 for the vector transfected cells and 0.136 and 0.027 for the β-galactosidase transfected cells.

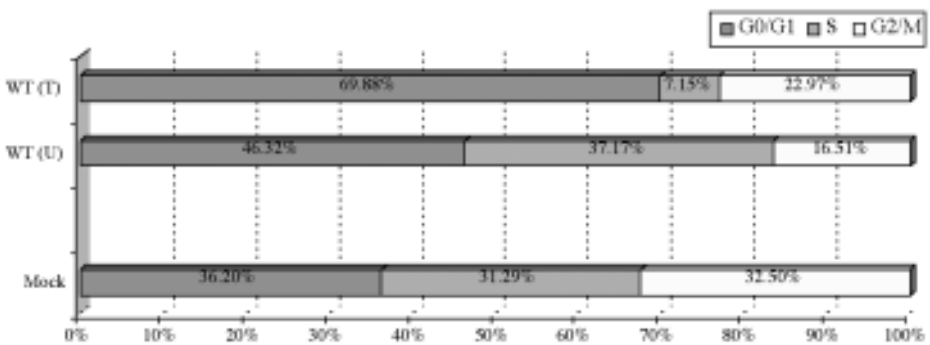

Fig. 5. Human Saos-2 cells were transfected with pCMV WT p53 or vector alone (pCMV-Bam), using the BES method. The cells were harvested 48 h after transfection, using Reporter lysis buffer, and incubated with a p53 specific antibody (DO 1) for 2 h at 4°C followed by an FITC-conjugated secondary antibody. Transfected cells were then sorted, and their DNA content was analyzed by flow cytometry. Presented is a graphical representation of the proportion of the transfected cells (T), compared to untransfected cells (U) from the same plate, found in each phase of the cell cycle. The mock represents an untransfected plate.

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8

Methods to Study p53-Repressed Promoters

Patrick Dumont, Anthony Della Pietra, and Maureen E. Murphy

Summary

There is substantial evidence in the literature that, in addition to functioning as an activator of transcription, the p53 tumor suppressor protein can also function as a sequence-specific transcriptional repressor of a separate set of genes. However, elucidation of the mechanism whereby p53 functions as a transcriptional repressor has been obscured by the use of artificial assays to measure this activity; these assays include transient transfection analyses, where both p53 and target promoters are overexpressed. This chapter describes alternative approaches for the definition of sequence elements that mediate transcriptional repression by p53. These include the McKay (immunobinding) assay, which measures the in vitro binding of large fragments of DNA, as well as chromatin immunoprecipitations (ChIPs), which measure in vivo binding. The use of such assays should better define the mechanism of transcriptional repression by p53 and should aid in the elucidation of the contribution of this activity to p53-dependent growth arrest and programmed cell death (apoptosis).

Key Words

p53, repression, transcription, immunobinding, chromatin immunoprecipitation

1. Introduction

The *p53* tumor suppressor gene continues to hold distinction as the most frequently mutated gene in human cancer; this gene is mutated in over 50% of human tumors of multiple histological types. Consequently, elucidation of the mechanisms whereby *p53* suppresses tumor formation has important consequences for understanding the development and treatment of many tumor types, including cancers of the brain, breast, bone, colon, liver, lymph, ovary, prostate, and stomach.

The p53 protein serves as an integral stress responder in the cell. It is normally inactive in cells, but becomes stabilized and activated as a sequence-

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specific transcription factor by multiple cellular stresses, including, but not limited to, DNA damage, hypoxia, and oncogene activation. The result of p53 activation is either growth arrest or apoptosis, depending upon cell type and environmental parameters, but it is currently accepted that it is p53's ability to induce apoptosis that underlies the strong selection for mutation of this gene during tumor development ***(1,2)***. Consequently, elucidation of the mechanism whereby p53 induces cell death has been a primary focus of studies on this tumor suppressor protein.

Although p53 is best characterized as an activator of gene transcription, compelling data from several laboratories indicate that other activities of p53 play an essential role in the ability of this protein to efficiently induce programmed cell death. For example, Caelles, Karin, and colleagues reported that, in an endocrine cell line containing wild-type p53, apoptosis could be induced that was p53-dependent, but this occurred in the absence of the induction of p53-response genes. Further, this cell death could not be inhibited with chemical inhibitors of new gene transcription, such as actinomycin D ***(3)***. Similarly, a study by Wagner, Hay, and colleagues ***(4)*** indicated that p53-dependent apoptosis, which was induced in the presence of inappropriately high levels of the c-myc proto-oncogene, could not be inhibited with cycloheximide, which inhibits *de novo* protein translation. Haupt, Oren, and others reported that synthetic mutants of p53, which are incapable of binding to DNA or activating transcription ***(5,6)***, could still induce apoptosis when overexpressed in human tumor cells. The take-home message from these studies was that p53 clearly had a transactivation-independent mechanism for apoptosis induction.

That p53 might also be a transcriptional repressor of gene expression was implied by early studies showing that this protein could repress the transcription of several viral and cellular promoters in transient transfection assays ***(7,8)***. However, attempts to determine the binding site specificity for p53 in these assays failed to reveal a promoter sequence required for repression, and the activity apparently mapped to basal promoter elements. Additionally, p53 was not found to occupy a discrete nucleic acid element in these "repressed" promoters, indicating an unusual lack of sequence specificity for this activity ***(8)***. The finding that inhibitors of apoptosis, such as bcl2, adenovirus E1B-19K, and the Wilms tumor suppressor gene *WT1* ***(9–11)*** could likewise inhibit transcriptional repression implicated this activity in this pathway, but also implied that such repression might be a side effect of cell death. Nonetheless, these early studies suggested that further study in this area was paramount.

The validity of this field of study was greatly increased when researchers began using subtractive hybridization and microarray analyses to reliably identify genes whose endogenous expression was decreased following physiological induction of wild-type p53. Genes identified in this manner include *cyclin*

B1, *cdc2*, *cdc25c*, *α-fetoprotein*, *Map4*, *MDR1*, *p14*^{ARF}, *presenilin 1*, *siah*, *stathmin*, *survivin*, *topoisomerase* IIa, and *wee*1, among others ***(12–23)***. For some of these studies, investigators used stable transfections of promoters linked to reporter genes in cell lines with inducible or endogenous p53 in order to circumvent problems of "squelching" of transcription associated with more artificial (transient) assays. Some of these studies also used nuclear run-on assays to show that decreased expression of these genes occurred at the level of transcriptional initiation ***(13)***. Such studies indicated for the first time that not only were endogenous genes transcriptionally repressed following p53 induction, but also that stably transfected promoters and truncation mutants thereof could recapitulate this effect reliably. These studies also indicated for the first time that sequence-specific binding of p53 to the promoters of these genes was required for transcriptional repression.

Identification of a p53 binding site in p53-repressed promoters required a reliable in vitro technique that was easily adaptable to large fragments of DNA and for which no *a priori* assumptions about the nature or sequence of the p53 binding site were made. Such a technique is the McKay, or immunobinding, assay, which relies on the in vitro association of p53 with candidate binding elements (up to 3 kb), followed by immunoprecipitation (IP) of bound DNA using p53 antisera ***(24)***. A variation of this technique was used to identify the first p53 binding sites in p53-activated genes ***(25)***. **Figure 1** depicts data on the p53-repressed Map4 promoter, which is sufficient to confer negative regulation by p53 in stably transfected cells (**Fig. 1A**) and interacts with p53 in a McKay assay (**Fig. 1B**). One disadvantage to immunobinding assays, like other in vitro promoter occupancy studies, such as electrophoretic mobility shift assays (EMSA) and DNase I protection (DNA footprinting), is that DNA elements can interact with a protein in vitro that are not necessarily able to, or accessible, in chromatin. Therefore, such in vitro binding assays are best supplemented using more in vivo approaches, such as chromatin immunoprecipitations (ChIPs). This latter assay uses formaldehyde cross-linking in order to determine whether a protein binds to a particular DNA fragment in vivo. **Figure 2** depicts results from ChIPs for the Map4 promoter. Protocols for both of these techniques will be presented here.

2. Materials

2.1. McKay Assay

1. McKay binding buffer: 10% glycerol, 5 m*M* ethylenediamine tetraacetic acid (EDTA), 20 m*M* Tris, pH 7.2, 100 m*M* NaCl, 0.1% Nonidet®P-40 (NP40). Supplement with protease inhibitors: 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL aprotinin, 1 μg/mL pepstatin, 1 μg/mL leupeptin.

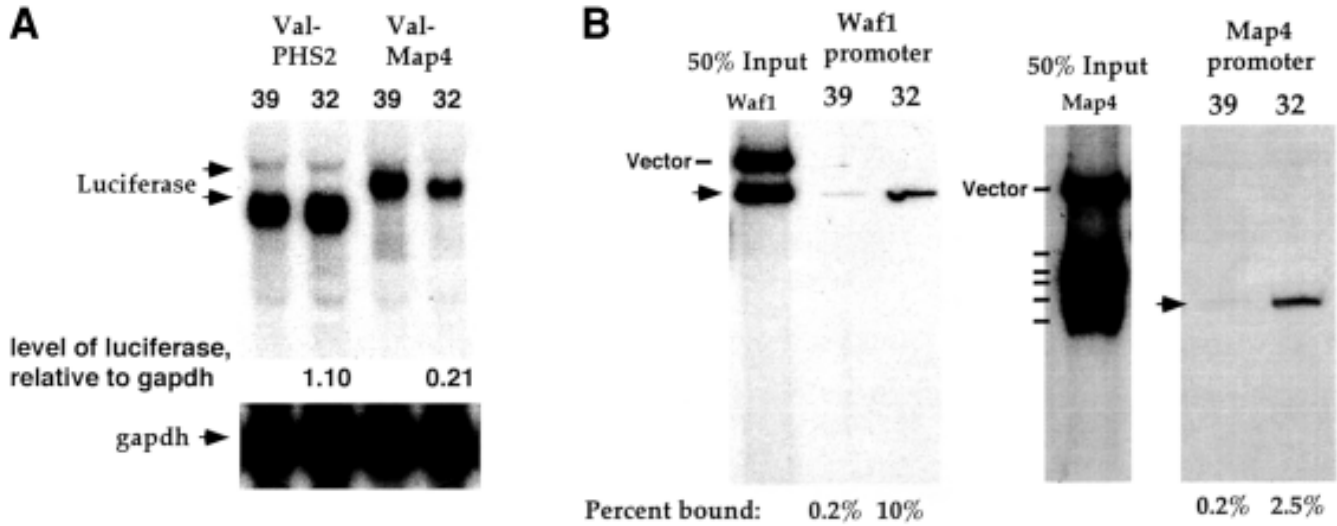

Fig. 1. (**A**) The Map4 promoter is sufficient to confer negative regulation by p53 in stably transfected cells containing inducible (temperature-sensitive) p53. Northern analysis of luciferase mRNA levels in pooled stably transfected cells containing temperature-sensitive p53 (Val5 cells: p53 is mutant and inactive at 39°C, wild-type and active at 32°C). The luciferase gene is linked to either the PHS-2 (COX2) promoter (negative control) or the Map4 promoter. Ratios given are the average levels of luciferase RNA at 32°C compared to 39°C from three independent experiments, relative to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) loading control. (**B**) Wild-type (32°C), but not mutant (39°C) p53 binds to the Map4 promoter in a McKay assay. A specific fragment of the Map4 promoter is immunoprecipitated with p53 from whole-cell extract from Val5 cells grown at 32°C (wild-type p53), but not mutant p53 (39°C). The waf1 promoter is shown as a positive control, and the vector fragment (denoted) serve as internal negative controls.

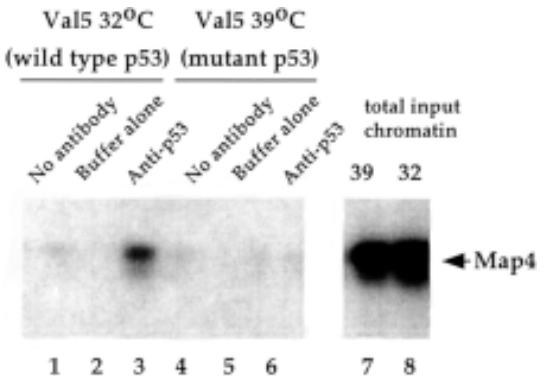

Fig. 2. Wild-type p53 binds to the Map4 promoter in vivo. ChIPs of the Map4 promoter in cells with wild-type p53 (32°C) or mutant p53 (39°C) indicate that only wild-type p53 binds to the Map4 promoter in vivo.

2. Protein A Sepharose® (Amersham Pharmacia Biotech, Piscataway, NJ, USA) (50% v/v): place 0.13 g dry beads in 1.5 mL TENN. Allow to swell for 30 min, with occasional vortex mixing. Pellet beads with a 30-s spin at 16,000*g* and then aspirate buffer. Wash 2× with 0.6 mL TENN and resuspend in 0.6 mL McKay binding buffer.
3. TENN: 50 m*M* Tris-HCl, pH 7.4, 5 m*M* EDTA, 0.5% NP40, 150 m*M* NaCl.
4. McKay washing buffer: 2% glycerol, 5 m*M* EDTA, 20 m*M* Tris-HCl, pH 7.2, 100 m*M* NaCl, 0.1% NP40.
5. McKay DNA loading dye: 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, pH 8.0, 20% glycerol, 0.05% bromophenol blue, and xylene cyanol.
6. poly(dI/dC): 1 μg/μL in McKay binding buffer, then sonicate at 30% power for 3 × 20 s to fragment.

2.2. Chromatin Immunoprecipitations

1. Cell lysis buffer: 5 m*M* 1,4-piperazinediethanesulfonic acid (PIPES), pH 8.0, 85 m*M* KCL, 0.5% NP40. Supplement with protease inhibitors (see below).
2. Nuclear lysis buffer: 50 m*M* Tris-HCl, pH 8.1, 10 m*M* EDTA, 1% sodium dodecyl sulfate (SDS). Supplement with protease inhibitors.
3. Immunoprecipitation (IP) dilution buffer: 0.01% SDS, 1.1% Triton® X-100, 1.2 m*M* EDTA, 16.7 m*M* Tris-HCl, pH 8.1, 167 m*M* NaCl. Supplement with protease inhibitors.
4. Dialysis buffer: 2 m*M* EDTA, 50 m*M* Tris-HCl, pH 8.0, 0.2 % Sarkosyl.
5. IP wash buffer: 100 m*M* Tris-HCl, pH 8.5, 500 m*M* LiCl, 1% NP40, 1% deoxycholic acid.
6. Elution buffer (make fresh): 50 m*M* $NaHCO_3$, 1% SDS.
7. 5X Proteinase K buffer: 50 m*M* Tris-HCl, pH 7.5, 25 m*M* EDTA, 1.25% SDS.

8. Protease inhibitors: 100 m*M* PMSF in ethanol, use at 1:100. Aprotinin (10 mg/mL) in 0.01 *M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 8.0, use at 1:1000. Leupeptin (10 mg/mL) in water, use at 1:1000.

3. Methods

3.1. Immunobinding (McKay) Assay

3.1.1. Preparation of End-Labeled DNA

1. Digest 10 μg of genomic DNA samples, cloned into a plasmid, with restriction enzymes that will generate small (100–2000 bp) fragments. Following digestion in a 50 μL reaction, heat-inactivate the restriction enzyme at 65°C for 15 min.
2. Add in a microfuge tube 30 μL of distilled water (dH_2O), 10 μL of 5X deoxycytidine triphosphate (dCTP) buffer, 1 μL (200 ng) of DNA, 5 μL of α^{32}P-dCTP, and 3 μL of Klenow polymerase (1 U/μL). Incubate 30 min at 37°C, and run on a G-50 column (Amersham Pharmacia Biotech). Count 1 μL. Store at –20°C until ready to use. The radiolabeled vector portion of the digest serves as a good internal negative control.

3.1.2. Preparation of Cell Lysate

1. Harvest one 10-cm plate, each of cells containing wild-type p53 in the induced and noninduced conditions (e.g., cells +/– a DNA-damaging agent of UV radiation). A reliable standard is MCF7 cells treated with 0.5 μg/mL adriamycin for 12–24 h). Harvest cells by scraping into 10 mL of phosphate-buffered saline (PBS). Spin cells at 1300*g* for 8 min.
2. Lyse the cell pellet in 1 mL of McKay binding buffer that has been supplemented with protease inhibitors. Incubate cell lysates on ice 20 min; then vortex mix 3 × 20 s, and spin at 14,000*g* for 10 min in a cold room. Collect supernatant.

3.1.3. Binding Reaction, Washes, Elution of Bound DNA

1. Keep samples on ice at all times. Measure the protein concentration of samples using a Bradford assay (Bio-Rad, Hercules, CA, USA). Incubate 100 μg of each extract with 5×10^5 counts per minute (cpm) of labeled DNA and bring up to 100 μL with binding buffer. Add 0.5 μg of p53 antisera (either 421, 1620, or DO-1 monoclonal antibodies; Calbiochem-Novabiochem, San Diego, CA, USA) to each sample, then add 12.5 μL poly(dI/dC). Rotate at 4°C for 1 h (*see* **Note 1**).
2. Add 30 μL of protein A Sepharose (50% v/v, equilibrated in binding buffer). Incubate at 4°C with rotation for 30 min.
3. Add 750 μL of McKay washing buffer, vortex mix for 60 s, and spin at 14,000*g* for 30 s. Being careful to avoid the protein A pellet, aspirate the supernatant. Repeat the wash 2×.
4. To the remaining protein A pellet after the last wash, add 150 μL of Tris-EDTA (TE), then 150 μL of phenol–chloroform, and 1 μL of yeast tRNA (20 μg/μL in dH_2O; Invitrogen, Carlsbad, CA, USA). Vortex mix for 1 min and spin for 3 min at 14,000*g*. Carefully pull off the top aqueous layer and remove to a fresh tube.

Add 15 μL of 10 *M* NH_4OAc and 300 μL of ethanol (ETOH). Mix well, incubate at –80°C 15 min, and spin 15 min at 14,000*g* in the cold. Wash pellet in 70% ETOH and let air-dry 10 min.

5. Resuspend pellet in 10 μL of dH_20 by pipeting, and add 10 μL of McKay dye. Load onto a 4% nondenaturing acrylamide gel and run in the cold room in 0.5X Tris-borate EDTA (TBE) at 200 V for 2 h. As controls, load 20,000 cpm of each probe in 10 μL of McKay buffer. Dry down gel and expose to film overnight.

3.2. Chromatin Immunoprecipitations (ChIPs)

3.2.1. Preparation of Cross-Linked Cell Lysate

1. Use 10^7 cells for induced and noninduced situations per immunoprecipitation; fix them right on the plate in media by adding 37% formaldehyde to 1%. Incubate at room temperature with shaking for 10 min.
2. Add glycine to 0.125 *M*, and rock for 5 min at room temperature. Wash cells 2× with PBS and scrape into 10 mL of PBS supplemented with protease inhibitors (*see* **Note 2**). Resuspend cell pellet in 7 mL lysis buffer plus protease inhibitors. Keep cells on ice for 10 min and perform 5 strokes with a Dounce B homogenizer on ice. Centrifuge at 3200*g* for 7 min to pellet nuclei.
3. Resuspend nuclei in 500 μL of nuclear lysis buffer supplemented with protease inhibitors. Vortex mix well and incubate on ice 10 min. Sonicate chromatin using a sonicator 10 × 10 s, 2-mm tip, 60% power, and keeping cells on ice between sonications. The average length of sonicated chromatin should be about 1000 bp, and this should be worked out for each different cell line and checked on an agarose gel. Microfuge sonicated chromatin at top speed for 15 min (*see* **Note 3**).

3.2.2. Immunoprecipitation of Fixed Chromatin

1. Transfer supernatant to a fresh tube and add 80 μL of protein A agarose pre-adsorbed with bovine serum albumin (BSA) and DNA (Upstate Biotechnology, Waltham, MA, USA). Preclear by rocking for 30 min at 4°C. Spin at top speed for 5 min, and transfer to a fresh tube. Add 100 μL into each IP tube. To each 100 μL, add 800 μL of IP dilution buffer, supplemented with protease inhibitors, along with 1 to 2 μg of antisera specific for p53, or normal rabbit sera as a negative control. To a control tube, add dialysis buffer instead of fixed chromatin along with each antibody. Incubate for 3–12 h at 4°C with rotation (*see* **Note 4**).
2. Add 60 μL of protein A agarose to each sample and incubate for 1 to 2 h at 4°C. Spin at 14000*g* in a microfuge for 3 min, and decant supernatant. Save the supernatant from the "normal rabbit sera" sample and label as "total input chromatin."
3. Wash pellets 2 × 1 mL dialysis buffer and 4 × 1 mL IP wash buffer, rotating gently for 3 min between washes. After each wash, spin at 14000*g* for 3 min, and aspirate supernatant carefully.
4. Add 150 μL IP elution buffer (freshly made), vortex mix 30 s, and rock for 15 min at room temperature. Spin at 14000*g* and transfer supernatant to fresh tube. Repeat with another 150 μL elution buffer. Combine supernatants and spin

at 14000*g* for 5 min to remove any traces of protein A agarose. Add 2 µL 5 mg/mL RNase A and bring to 0.2 *M* NaCl. Incubate at 67°C for 4–12 h; this step reverses cross-links to allow for the polymerase chain reaction (PCR). Add 1 µL of yeast tRNA (20 µg/µL) and 650 µL of 100% ETOH and precipitate at –20°C overnight. Remember to treat "total input chromatin" the same way.

8. Spin samples at 14000*g* for 20 min, decant ethanol, and air-dry for 10 min. Dissolve pellet in 100 µL of TE; add 25 µL of 5X proteinase K buffer and 1.5 µL of pro K (20 µg/µL in dH_2O). Incubate at 45°C for 1.5 h.
9. Add 175 µL of TE and extract once with 300 µL of phenol–chloroform and once with 300 µL of chloroform. Add 30 µL of 5 *M* NaCl, 20 µg of yeast tRNA, and 750 µL of ETOH to each sample, and precipitate at –20°C for 2–12 h.
10. Spin samples at 14000*g* for 20 min, and wash pellet in 70% ETOH. Resuspend in 100 µL of dH_2O; use 1 µL for PCR. For total input chromatin, use 1 µL of a 1:1000 dilution.

3.2.3. PCR

1. Add 5 µL of 10X PCR buffer to 5 µL of 10m*M* dNTPs (10 m*M* each; Amersham Pharmacia Biotech), 1 µL of 20 µ*M* forward primer, 1 µL of 20 µ*M* reverse primer, and dH_2O to 49 µL. Add 1 to 2 µL of *Taq* DNA polymerase. Mix well and add 1 µL of freshly diluted chromatin from above. PCR at 94 °C for 5 min, and 30–35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, followed by 72°C for 10 min and a 4°C soak.
2. Run 10 µL of each PCR on an agarose gel.

4. Notes

1. The McKay assay is a relatively straightforward assay with few technical difficulties; high background binding of the vector sequences can be eliminated by titrating to lower protein concentrations or lower antibody concentrations (0.1–0.5 µg).
2. ChIPs are much less straightforward, and often many parameters need to be worked out before this protocol is informative. First, cross-linking times may need to be worked out, as over-crosslinking can destroy antigen availability. Similarly, while some protocols eliminate the glycine incubation, this eliminates unreacted formaldehyde, which can interfere with later antibody binding.
3. Some researchers find that sonication of chromatin to very small pieces (100–300 nucleotides) enhances reproducibility and quantitation. Also, some protocols incorporate a cesium chloride gradient for the purification of fixed chromatin ***(26)***, which can enhance signal by eliminating insoluble contaminants that might impede IP.
4. It is important to use multiple different antibodies for the IP, as they can be variably sensitive to the denaturation of proteins with formaldehyde. Typically, polyclonal antisera works better than monoclonal. For p53, rabbit polyclonal antisera (p53 FL393; Santa Cruz Biotechnology, Santa Cruz, CA, USA) works well, but monoclonal antibody DO-1 (Ab-6; Oncogene Science) also works well.

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9

Interaction of p53 with Cellular Proteins

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Summary

Cellular proteins that interact with p53 play a major role in both positive and negative regulation of this tumor suppressor and can fine-tune its response to specific cellular stresses. As a consequence, p53 biology will not be complete until these interacting proteins are fully characterized. This chapter outlines two methods for identifying and characterizing p53-binding proteins: (*i*) glutathione-S-transferase (GST) protein–protein interaction assay; and (*ii*) co-immunoprecipitation (co-IP) assay. These two methods are ideal for any laboratory to perform; the assays are short in duration, do not require specialized expertise to establish in the laboratory, give reliable and reproducible data, and are cost-efficient, because few reagents are needed. This chapter gives a basic description of these two techniques and provides tips that are not found in other protocol manuals on how to achieve the best results.

Key Words

GST pull-down assay, co-immunoprecipitation, protein–protein interaction

1. Introduction

The p53 tumor suppressor protein cannot function alone. Instead, it needs to interact with multiple cellular proteins that serve to both enhance and repress p53 function ***(1,2)*** (*see* **Table 1**). Unlike transcriptional or translational regulation, these protein–protein interactions help provide a mechanism for instant regulation of the p53 molecule by either affecting its transactivation activity and/or protein stability, as discussed below.

Many protein–protein interactions play critical roles in p53 transactivation. For example, p53's amino (N)-terminal transactivation domain directly contacts basal transcription factors, such as TBP ***(3,4)*** and TAFII31 ***(5,6)***, as well as transcriptional co-activators such as p300/CBP ***(7,8)***. Thus, the transcrip-

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Table 1
p53-Interacting Proteins

General transcription factors
TFIID components: hTAFII31 ***(5,6)***
TBP ***(5,6)***
TFIIH components: XPB ***(22–24)***
XPD
p62

Protein kinases
Casein kinase 2 ***(25)***
HIPK2 ***(10,11)***
JNK1 ***(26)***

Redox sensitive proteins
HIF-1α ***(27)***
Ref-1 ***(9)***

Miscellaneous p53 activators
14-3-3σ ***(28)***
53BP1 ***(29)***
53BP2 ***(29)***
HMG-1 ***(30)***

Viral proteins
AdE1B55 kD ***(31,32)***
EBV ENBA-5 ***(33)***
HBV X protein ***(34,35)***
HPV E6 ***(36,37)***
SV40 Tag ***(38,39)***

Protein acetylases/deacetylases/ deacetylase adaptors
HDAC1 ***(40,41)***
p300/CBP ***(7,8)***
PCAF ***(42)***
Sin3a ***(43)***
Sir2α ***(44,45)***

Ubiquitination and de-ubiquitination
E6-Ap ***(46)***
HAUSP ***(47)***
MDM2 ***(48)***

Replication and repair proteins
RP-A ***(49–51)***
TFIIH components: XPB ***(22–24)***
XPD
p62

p53 family members
p63 ***(52)***
p73 ***(52)***

MDM2 family member
MDMX ***(53)***

tional machinery is recruited to promoters to which p53 is bound. Other proteins can further stimulate p53's transcriptional activity in response to certain cell stress stimuli. The redox-sensitive protein Ref-1 binds to p53 and activates its DNA binding and transactivation function in response to redox stress ***(9)***. Several kinases also form stable interactions with p53, resulting in phosphorylation and activation. For example, the homeodomain interacting protein kinase-2 (HIPK2) has been recently shown to bind and phosphorylate p53 at Ser46 after high doses of ultraviolet (UV) light, resulting in induction of p53-dependent apoptosis ***(10,11)***.

Protein stability of p53 is also tightly regulated by protein–protein interactions. Most importantly, the oncoprotein MDM2 binds to the N-terminal domain of p53 ***(12)*** and negatively regulates its stability. MDM2 is a RING-finger containing E3-ubiquitin ligase that ubiquinates lysine residues on p53's carboxy (C) terminus, thus targeting p53 to the proteosome for degradation ***(13,14)***. The functional link between these proteins is clearly shown by knock-out mouse models, in which MDM2 homozygous null mice are embryonic lethal, though double-knock-out MDM2-p53 mice survive ***(15)***. This striking finding indicates that without MDM2 to keep the p53 protein in check, the organism cannot survive p53's growth suppressive effects. Importantly, the MDM2-p53 interaction is also regulated by cell stress stimuli. For example, UV and γ irradiation can activate kinases that phosphorylate p53 on serine residues within the MDM2-binding domain, thereby preventing MDM2 from binding ***(16–21)***.

As suggested by the above examples, p53 protein–protein interactions are often regulated by cellular stress signals. Thus, it is important, when studying a particular p53-interacting protein, to keep in mind that the interaction may be very stress-specific and that optimization of binding conditions is required. This will include testing several cellular stress agents and performing time courses to determine when maximal binding occurs posttreatment.

In this chapter, we describe two commonly used methods for studying p53 protein–protein interactions: (*i*) glutathione-S-transferase (GST) pull-down assays to study interactions in vitro and co-immunoprecipitations (Co-IPs) reactions to study interactions in cells. Briefly, the GST pull-down assay uses recombinant p53 fused to the GST protein, which can then bind to glutathione coupled to agarose beads. Mixing GST-p53 with a recombinant protein of interest to test for binding or with cell lysates to pull out novel p53-interacting proteins is then done. On the other hand, co-IPs use p53-specific antibodies coupled to protein A or G Sepharose to pull-down p53 from cell lysates. If a known protein is being tested for p53 binding activity, then the co-IPs can be visualized by running on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis. Alternatively, to identify novel p53-interacting proteins, the SDS-PAGE gel can be stained with silver solution for example, followed by cutting out the novel protein bands and subjecting them to protein sequencing.

2. Materials

2.1. GST Protein–Protein Association Assay

1. LB broth (Fisher Scientific, Pittsburgh, PA, USA; cat. no. BP1426-2).
2. Isopropyl-β-D-thiogalactoside (IPTG): (Fisher Scientific; cat. no. BP1620-10). Store in aliquots at –20°C.

3. Phosphate-buffered saline (PBS) buffer 20X: 160 g NaCl, 4 g KCl, 23 g $Na_2HPO_2{\cdot}7H_2O$, 4 g KH_2PO_4. Store at room temperature.
4. PBS 1X: Prepare from 20X stock using water and store at 4°C.
5. Glutathione–agarose beads (Sigma, St. Louis, MO, USA; cat. no. G-4510). Swell the lyophilized powder with deionized water at room temperature for at least 30 min (1 g swells to approx 14 mL gel). Wash and store beads in 1X PBS.
6. Protease inhibitors: 1 µg/mL aprotinin, 1 µg/mL leupeptin, 4 µ*M* pepstatin A, 0.2 m*M* phenylmethylsufonyl-fluoride (PMSF) (add fresh just before use).
7. Dithiothreitol (DTT): make 1 *M* stock in water and store in aliquots at –20°C. Add to buffers just before use to make a final concentration of 1 m*M*.
8. GST-lysis buffer: 1X PBS, 0.1% Nonidet® P-40 (NP40), 10% glycerol. Store at 4°C. Add 1 m*M* DTT and protease inhibitors just before use.
9. GST-lysis buffer plus 500 m*M* NaCl: 1X PBS, 0.1% NP40, 10% glycerol, 500 m*M* NaCl. Store at 4°C. Add 1 m*M* DTT and protease inhibitors just before use.
10. SDS protein sample buffer 4X: 200 m*M* Tris-HCl, pH 6.8, 400 m*M* DTT, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol.

2.2. Immunoprecipitation of Cellular Proteins

1. PBS buffer 1X: *see* **Subheading 2.1.**, **item 4** above.
2. Lysis buffer: 50 m*M* Tris-HCl, pH 8.0, 5 m*M* ethylenediamine tetraacetic acid (EDTA), 150 m*M* NaCl, 0.5% NP40. Store at 4°C. Add 1 m*M* DTT and protease inhibitors just before use.
3. SNNTE buffer: 50 m*M* Tris-HCl, pH 7.4, 5 m*M* EDTA, 5% sucrose, 1% NP40, 0.5 *M* NaCl. Store at 4°C. Add 1 m*M* DTT and protease inhibitors just before use.
4. RIPA buffer: 50 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 1% Triton® X-100, 0.1% SDS, 1% (w/v) Na Deoxycholate. Store at 4°C. Add 1 m*M* DTT and protease inhibitors just before use.
5. Protein G Sepharose® (Amersham Pharmacia Biotech, Piscataway, NJ, USA; cat. no. 17-0618-01). Store at 4°C.
6. Anti-MDM2 (Ab-3, monoclonal, clone 4B11) (Calbiochem-Novabiochem, San Diego, CA, USA; cat. no. OP46).
7. Anti-MDM2 (C-18, rabbit polyclonal) (Santa Cruz Biotechnology, Santa Cruz, CA, USA; cat. no. sc-812).
8. Anti-p53 (Ab-1, monoclonal, clone PAb 421) (Calbiochem-Novabiochem; cat. no. OP03).
9. Anti-p53 (Ab-2, monoclonal, clone PAb 1801) (Calbiochem-Novabiochem; cat. no. OP09).
10. Anti-p53 (Ab-4, monoclonal, clone PAb 246) (Calbiochem-Novabiochem; cat. no. OP32).
11. Anti-p53 (FL-393, rabbit polyclonal) (Santa Cruz Biotechnology; cat. no. sc-6243).

3. Methods

3.1. GST Protein–Protein Association Assay

The following protocol is adapted from **refs. *54*** and ***55***, with some modifications (**Fig. 1**).

3.1.1. Purify GST-Fusion Proteins

1. Inoculate 5-mL starter cultures from single colonies of GST-fusion protein and GST-0 (control GST empty vector) constructs. Grow overnight with shaking at 37°C.
2. Add starter cultures to 500 mL of LB broth and incubate with shaking at 37°C until OD_{600} is between 0.6–0.8. Then add 500 µL 0.4 *M* IPTG to the cultures (final is 0.4 m*M*), and incubate for an additional 2–6 h.
3. Centrifuge cultures for 15 min at 5000*g*.
4. Resuspend cell pellets in 25 mL GST-lysis buffer.
5. Lyse cells by passage 2× through a French Press (*see* **Note 1**). Centrifuge lysates for 20 min at 30,000*g* to pellet insoluble fraction.
6. Incubate supernatents with 1 mL glutathione–agarose beads 50% slurry (500 µL bead bed vol) for 5 min at room temperature.
7. Centrifuge for 1 min at 1000*g* in swinging bucket rotor to pellet agarose beads.
8. Wash beads 3× with the following buffers: (*i*) 25 mL GST-lysis buffer; (*ii*) 25 mL GST-lysis plus 500 m*M* NaCl; and (*iii*) 25 mL GST-lysis buffer. During each wash, incubate with rotation at room temperature for 5 min, followed by centrifugation for 1 min at 1000*g*.
9. Check protein expression and purity on SDS-PAGE with Coomassie® brilliant blue staining. Compare the levels of GST-fusion and GST-0 proteins on the stained gel, such that equal amounts are used in the pull-down assay. In addition, these proteins can be run alongside bovine serum albumin (BSA) as a protein standard in order to determine the protein concentration of the GST-fusion protein. Titrate BSA in a range between 100 ng to 2 µg.

3.1.2. In Vitro GST-Fusion Protein Pull-Down Assay

This protocol calls for one protein to be immobilized on glutathione–agarose beads, for example GST-p53, and another protein, for example a candidate p53-binding protein, to be expressed in a soluble form. The soluble protein can be either in vitro translated (Transcription and translation [TNT]-coupled reticulocyte lysate system; Promega, Madison, WI, USA; cat. no. L4610) with ^{35}S methione (NEN® Life Science Products, Boston, MA, USA; cat no. NEG 709-A), purified by one of several established methods (6X histidine-tagged pro-

teins; Qiagen, Valencia, CA, USA; cat. no. 30210, or intein-fusion proteins; New England Biolabs, Beverly, MA, USA; cat. no. E6900S), or can be from cell nuclear extracts to identify novel p53-interacting proteins (*see* **Note 2**).

1. Combine GST-fusion protein (typically 1 μg) immobilized on glutathione–agarose beads with soluble protein (typically 5 μg or an excess of GST-fusion protein) in 30 μL final vol of GST-lysis buffer. As a control sample, add only the GST-fusion protein without adding the soluble protein.
2. Incubate 45 min at room temperature with mixing (*see* **Note 3**).
3. Following the incubation, wash 3× with GST-lysis buffer with light vortex mixing each time (*see* **Note 4**). Pellet agarose beads by centrifugation in microfuge at 5000*g* for 15 s.
4. Finally, remove supernatent and add 4X SDS loading buffer to a final of 1X concentration. Analyze protein interactions on SDS-PAGE and either stain with Coomassie brilliant blue or transfer to polyvinylidene difluoride (PVDF) membrane for Western blotting.

3.2. Immunoprecipitation

When dealing with an uncharacterized p53 binding protein, it is important to test several antibodies for immunoprecipitation (IP) because: (*i*) the antibody may interfere with the protein–protein interaction; and (*ii*) the p53 posttranslational modifications, such as acetylation and phophorylation, may negatively affect the antibody's ability to bind. *See* **Note 5** for information on p53 antibodies. For example, the PAb 421 epitope maps to amino acids 371–380, which is a region that is modified by acetylation, phosphorylation, and ubiquination in cells after various stress-inducing agents. Though this antibody works well for IP, it may only recognize a subset of p53 molecules in the cell that are unmodified in this region. PAb 246 recognizes p53 in a wild-type conformation. This antibody also works well for IP, though it may be possible that some p53-interacting proteins may affect p53 conformation, such that this antibody no longer recognizes it. The epitope for PAb 1801 is in the N terminal of p53, amino acids 46–55. Similar to PAb 421, this region is also posttranslationally modified after p53-activating agents. Refer to **Fig. 1** for the stepwise procedure.

3.2.1. Prepare Cell Lysate

1. Aspirate the medium from the cultured cell plate and wash the cells 2× with cold 1X PBS. Finally, add cold 1X PBS to each plate.
2. Scrape the cells from the plate using a plastic policeman. Transfer the suspensions to 15-mL tubes by pipet, followed by rewashing the plate with cold 1X PBS to both clean the policeman and to collect leftover cells in the plates. Collection tubes should be stuck deeply into the ice during the procedure.

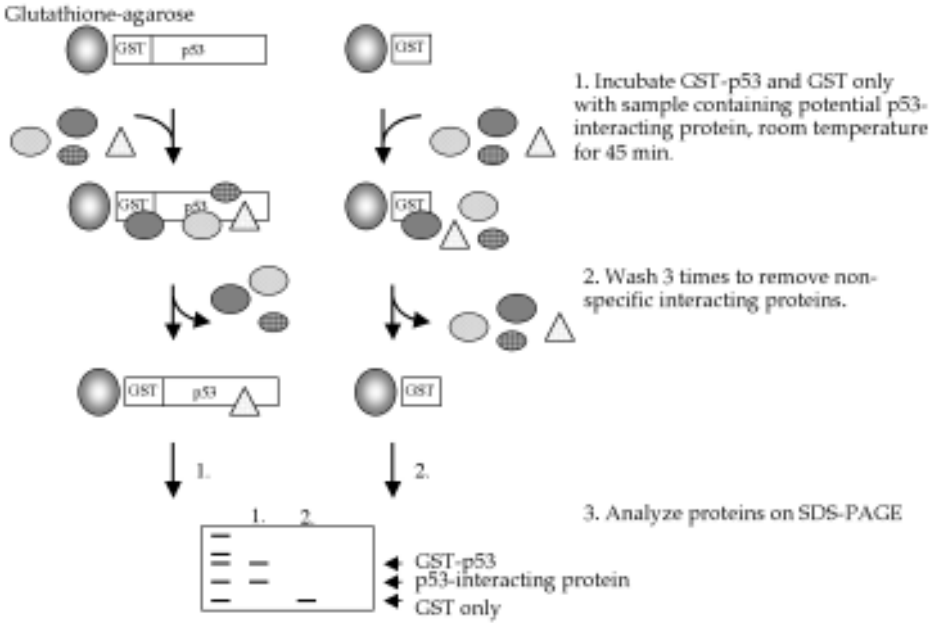

Fig. 1. Schematic diagram showing GST-pull-down assay. IP is performed in a similar manner if GST-beads are replaced with immunoaffinity beads. *See* text for the details on both assays.

3. Centrifuge the cells in a swinging bucket rotor for 3–5 min at 3000*g* and 4°C to pellet the cells. Aspirate the PBS, and then the cells are ready for IP or can be stored at –80°C for at least 1 mo.
4. Add 1.0 mL of cold lysis buffer per 10^7 cells. Resuspend the sample by vortex mixing and leave on ice for 20–30 min with occasional mixing. Transfer the lysate to a 1.5-mL tube and spin in a 4°C microfuge for 5–10 min at maximal speed. Transfer supernatant to a fresh tube and keep on ice.

3.2.2. Preclearing the Lysate

1. Add 30 µL of protein G Sepharose (50%) per 1.0 mL of lysate.
2. Rotate at 4°C for 30–60 min. Spin in a 4°C microfuge for 15 s at 5000*g*.
3. Carefully add supernatant to a new 1.5-mL tube or store at –80°C.

3.2.3. IP Reaction

1. Measure the protein concentration of the precleared cell lysate (*see* **Note 6**).
2. In a 1.5-mL tube, combine at least 300 µg cell lysate with 1 µg antibody of choice and 20 µL protein G Sepharose (50% slurry).
3. Add lysis buffer to bring vol to 500 µL

4. Rotate at 4°C for at least 2 h or overnight.
5. Centrifuge samples in room temperature microfuge for 15 s at 5000*g*.
6. Aspirate supernatent and wash beads with the following buffers (*see* **Note 4**) with centrifugation between each time: (*i*) 1 mL lysis buffer; (*ii*) 1 mL SNNTE buffer; (*iii*) 1 mL SNNTE buffer; and (*iv*) 1 mL lysis buffer.
7. Finally, aspirate supernatent and quickly spin the sample. Use a pipet to carefully remove excess supernatent, leaving approx 20 µL total vol in the tube. Add 4X SDS sample buffer to a final of 1X concentration. The samples are now ready to be boiled and loaded onto SDS-PAGE, followed by Western blotting analysis (*see* **Note 7**).

4. Notes

1. Bacterial cell lysis can be performed several ways, including French Press, sonication, and freeze-thaw, as described in *Current Protocols in Molecular Biology* ***(55)***. We use a Spectronic Unicon French Pressure System with a 40-K manual-fill cell (FA-031) at an internal pressure of 20,000 psi.
2. To identify novel p53-interacting proteins, one can use either whole cell lysates (*see* **Subheading 3.2.1.**) or nuclear extracts. Nuclear extract preparations have been described elsewhere ***(56,57)***. The lysate or extract should be incubated as described in **Subheading 3.1.2.** with beads containing either GST only or GST-p53 fusion protein. Additionally, an unrelated GST-fusion protein can also be used as a control. After running the samples on SDS-PAGE and after Coomassie brilliant blue or silver staining, proteins found only in the GST-p53 lane can be cut out of the gel and sent out for peptide sequencing by mass spectrometry, for example.
3. We perform mixing of GST-fusion proteins immobilized on glutathione–agarose in Eppendorf® tubes using a vortex mixer with a 3-in. rubber platform attachment. We place the Eppendorfs in a Fisherbrand Flat Rack held on by a rubber band while vortex mixing at low speed (Fisher Scientific; cat. no. 05-544-4) or alternatively use an attachable platform head that holds Eppendorf tubes (Fisher Scientific; cat. no. 12-812B).
4. Wash conditions will vary depending on the proteins tested, and so various wash buffers should be tested in order to maximize binding while minimizing background. Many protein–protein interactions are salt-dependent, while other interactions are hydrophobic in nature. Thus, using high ionic-strength washes may be good in some cases, while low ionic-strength washes will be good in others. For GST pull-down assays, the wash buffers we commonly use are: (*i*) GST-lysis buffer; (*ii*) GST-lysis plus 500 m*M* NaCl, to remove background proteins whose interactions are salt-sensitive; (*iii*) 1:3 diluted GST-lysis buffer in water, to remove background proteins whose interactions are hydrophobic; and (*iv*) RIPA buffer, which is a stringent wash good for removing background when using in vitro translated proteins with ^{35}S-methione label. For IPs, we use: (*i*) Lysis buffer, (*ii*) SNNTE (high salt); (*iii*) 1:3 diluted lysis buffer in water (low salt); and (*iv*) RIPA.

5. There are many antibodies to choose from when performing an IP of p53. Four are listed in **Subheading 2.**, including PAb 421, PAb 246, PAb 1801, and anti-p53 polyclonal. We typically use the first three for IPs, though there are others as listed in the Calbiochem-Novabiochem catalog.
6. To determine protein concentrations, we use the 5X protein assay dye from Bio-Rad (Hercules, CA, USA; cat. no. 500-0006) by diluting it in water to 1X concentration. We add 1 mL of 1X protein dye reagent to a 5-mL tube (Sarstedt, Newton, NC, USA; cat. no. 55.476), add 2 μL of cell lysate, vortex mix briefly, leave it at room temperature 5 min, transfer each solution into a 1-mL plastic cuvette, and then measure the color change by spectrophotometer set to wavelength 595 nm. A standard curve should be made using a purified protein of known concentration such as BSA. We have found that as an estimation of protein concentration, the following conversion works well:
protein concentration = (20 μL/? μL) × OD_{595} = [protein] μg/μL where ? equals the amount of lysate added to the protein assay reagent. For example, if 2 μL of cell lysate were added to the reagent, and the OD_{595} was 0.2, then:
protein concentration = (20 μL/2μL) × 0.2 = 2 μg/μL.
7. When the IP reactions are run on SDS-PAGE and analyzed by Western blotting, often the immunoglobulin (IgG) heavy chain signal is very prominent. Depending upon the molecular weight of your protein of interest, the IgG signal may overlap. This is especially true for p53, which migrates just above the IgG heavy chain. To avoid this problem, it is beneficial to perform the IP with a monoclonal antibody, e.g., PAb421, followed by Western blot analysis with a polyclonal antibody, e.g., anti-p53 p.c. The opposite is true as well, e.g., using a polyclonal antibody for IP followed by a monoclonal antibody for Western blotting.

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10

Interaction of p53 with the Adenovirus E1B-55 kDa Protein

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Summary

The E1B-55 kDa oncoprotein of adenovirus type 5 targets the tumor suppressor protein p53. This includes four distinct activities: (*i*) biochemical interaction of E1B-55 kDa with p53; *(ii)* inhibition of p53-induced transcription; (*iii*) relocalization of p53 from the nucleus to the cytoplasm; and (*iv*) in the simultaneous presence of E1B-55 kDa and the adenovirus E4-34 kDa (E4orf6) protein, extensive destabilization of p53. These activities can be observed experimentally, using co-immunoprecipitation of p53 with E1B-55 kDa, luciferase reporter assay of p53 activity, immunofluorescence to localize p53 and E1B-55 kDa, and immunoblot analysis of p53 levels. These experimental systems can be useful when analyzing novel interaction partners and modulators of p53, or in deciding whether adenovirus oncoproteins interact with novel growth regulatory proteins. Protocols describing the four methods are provided in this chapter.

Key Words

adenovirus, destabilization, immunoblot, immunofluorescence, immunoprecipitation, luciferase, oncoprotein, p53, relocalization, reporter assay

1. Introduction

Small DNA tumor viruses have evolved early gene products that mediate the transformation of primary cells in vitro and the induction of malignant tumors in animals. Adenovirus type 5 expresses three major transforming proteins from its E1 region, and each of them binds and inactivates cellular tumor suppressor gene products ***(1)***. In addition, the E4 region encodes proteins that can further contribute to malignant transformation ***(2)***.

The oncoprotein E1B-55 kDa of adenovirus type 5 specifically targets the p53 protein. In particular, the following phenomena can be observed as consequences of the interaction between the two proteins.

1. A specific complex is formed that contains E1B-55 kDa and p53 ***(3)*** (*see* **Notes 1–6**).

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2. The N-terminal transactivation domain of p53 is bound by E1B-55 kDa and rendered inactive ***(4)***. Therefore, p53-mediated transcription is compromized. Moreover, E1B-55 kDa contains a transcriptional repression domain that further reduces p53 activity when the complex of p53 and E1B-55 kDa binds to a p53-responsive promoter ***(5)*** (*see* **Notes 7–15**).
3. p53 is relocalized from the nucleus to the cytoplasm, where it co-localizes with E1B-55 kDa in discrete cluster-like structures ***(6,7)*** (*see* **Notes 16–23**).
4. When the E4-34 kDa protein of adenovirus type 5 (the product of E4 open reading frame 6) is present in addition to E1B-55 kDa, this strongly destabilizes p53 ***(8–11)*** through ubiquitination and proteasome-mediated degradation ***(12)***. The cooperation of E1B-55 kDa and E4orf6 is likely based on their ability to form a complex ***(13)*** that is predominantly localized in the nucleus ***(14)*** but capable of undergoing nucleocytoplasmic shuttling ***(15)***. The E1B-55 kDa and E4-34 kDa proteins are part of a larger complex that contains cullin family member Cul5, elongins B and C, and the RING-H2 finger protein Rbx1(ROC1) and mediates p53 ubiquitination **(12)** (*see* **Notes 24–30**).

These four phenomena can each be addressed experimentally, using the following methods:

1. Immunoprecipitation, to detect of a complex containing p53 and E1B-55 kDa (**Notes 1–6**).
2. Reporter assays, to detect the inhibitory effect of E1B-55 kDa on p53-mediated transcription (**Notes 7–15**).
3. Immunofluorescence, to detect the intracellular relocalization of p53 by E1B-55 kDa (**Notes 16–23**).
4. Immunoblot analysis, to detect the destabilization of p53 by E1B-55 kDa and E4-34 kDa (**Notes 24–30**).

The reader might use these methods for the following reasons:

1. The E1B-55 kDa protein represents a useful and well-studied example of a negative regulator for p53. Therefore, it can serve as a positive control when analyzing a different putative viral or cellular modulator of p53 activity. Such regulators include the oncoproteins from other DNA tumor viruses, simian virus 40 (SV40) T antigen and papillomavirus E6 proteins, as well as the cellular mdm2 protein (for review, *see* **ref. *16***).
2. Vice versa, E1B-55 kDa may target more cellular proteins in addition to p53. The techniques outlined below can be followed to investigate the existence of such additional interactions. For instance, it was found that, unlike p53, the p53-homologue p73 did not detectably interact with E1B-55 kDa by any of these assays ***(10,11,17–19)*** nor did the p63 proteins ***(11)***. Failure to interact with E1B-55 kDa was one of the first indications that p53-homologues have different functions than p53 itself. Since E1B-55 kDa has evolved to affect cell growth and death, any novel cellular target of it would represent a strong candidate for a cellular growth regulatory protein.

2. Materials

2.1. Co-Immunoprecipitation of p53 with Adenovirus-E1B-55 kDa (see Notes 1–6)

1. Lysis buffer: 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 5 m*M* ethylenediamine tetraacetic acid (EDTA), 0.05% bovine serum albumin, and 0.01% Nonidet® P-40. Store <1 wk at 4°C after adding bovine serum albumin and Nonidet P-40. Can be stored at least 1 yr without these components.
2. 6X Laemmli buffer: 350 m*M* Tris-HCl, pH 6.8, 30% (v/v) glycerol, 10% (w/v) sodium dodecyl sulfate (SDS), 9.3% (w/v) dithiotreitol (DTT), and 0.012% (w/v) bromophenol blue. Store in aliquots at –20°C (up to 1 yr), but heat to 37°C for 10 min after thawing to redissolve SDS and DTT.
3. Human embryonic kidney 293 cells (HEK293 cells) from ATCC (Manassas, VA, USA).
4. Dulbecco's modified Eagle media (DMEM) (Life Technologies [Rockville, MD, USA] or home-made brands).
5. The monoclonal mouse antibody 2A6 against Ad5 E1B-55 kDa ***(20)***, hybridoma supernatant kindly provided by A. J. Levine, Rockefeller University.
6. The plasmid pOS7-p53 to express p53 by transcription and translation in vitro was obtained from N. Horikoshi and T. Shenk, Princeton. The vector pOS7 contains a T7 promoter that is recognized by the corresponding phage RNA polymerase, followed by an internal ribosomal entry site from echomyocarditis virus and a multiple cloning site. The plasmid contains the ampicillin resistance gene (*bla*) and the the pBR322 origin of replication, yielding low-to-moderate copy numbers when grown in *Escherichia coli*.
7. TNT® T7-coupled reticulocyte lysate system (Promega, Madison, WI, USA; cat. no. L4610). L-[^{35}S]methionine (555 Mbq/mL, >37 TBq/mmol; Amersham Pharmacia Biotech, Piscataway, NJ, USA; cat. no. AG1094).
8. Protein A Sepharose® (supplied as dry powder; Sigma, St. Louis, MO, USA).

2.2. Reporter Assay to Measure the Inhibition of p53 Activity by E1B-55 kDa (see Notes 7–15)

1. H1299 cells (human adenocarcinoma of the lung; *p53* –/–; ATCC).
2. The reporter plasmid pGL2-BP100 was generated by cloning the p53-binding region of the *mdm2*-promoter, together with a minimal promoter comprising TATA box and initiator region, into the reporter plasmid pGL2basic (Promega), as described ***(21)***.
3. Expression plasmid pCGN-E1B-55 kDa ***(15)***. The vector plasmid pCGN, obtained from H. Zhu and R. Prywes, Columbia University, New York, is derived from the vector pCG ***(22)*** and contains the cytomegalovirus (CMV) major immediate early promoter, a herpes simplex virus (HSV) thymidine kinase 5' untranslated region (UTR), and the coding region of the hemagglutinine (HA)-tag immediately after the start codon. This is followed by a multiple cloning site, an intron from the rabbit β-globin gene, and a polyadenylation site. The plasmid

contains the ampicillin resistance gene (*bla*) and the colE1 origin of replication, yielding high copy numbers when grown in *E. coli*.
4. Expression plasmid pRcCMV-p53 ***(23)***; the vector plasmid pRcCMV came from Invitrogen (Carlsbad, CA, USA). The transfection reagent LIPOFECTAMINE 2000 was obtained from Invitrogen Life Technologies.
5. Reporter lysis buffer and luciferase substrate solution (Promega; cat. no. E1501).

2.3. Visualized Intracellular Relocalization of p53 by E1B-55 kDa (see Notes 16–23)

1. 4-Well chamber slides for cell culture, with removable gasket (Nalge Nunc International, Rochester, NY, USA; cat. no. 177437).
2. Phosphate-buffered saline (PBS): 236.9 m*M* NaCl, 2.7 m*M* KCl, 8.1 m*M* Na_2HPO_4, 1.1 m*M* $MgCl_2$, 1.5 m*M* KH_2PO_4, 1.2 m*M* $CaCl_2$. Store up to 1 yr at 4°C.
3. Fixing solution: 236.9 m*M* NaCl, 2.7 m*M* KCl, 8.1 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 4% w/v paraformaldehyde. Dissolve paraformaldehyde by briefly heating to approx 70°C under a fume hood, until the solution is no longer turbid, then cool to room temperature. Can be stored at least 1 yr in aliquots at –20°C.
4. Blocking solution: PBS supplemented with 10% (v/v) fetal bovine serum or newborn calf serum.
5. Monoclonal mouse antibody HA.11 against the hemagglutinine HA-tag, raw ascites fluid (Berkeley Antibody Company [BabCO], Richmond, CA, USA).
6. Polyclonal rabbit antibody FL-393 against p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).
7. Monoclonal mouse antibody 2A6 against Ad5 E1B-55 kDa ***(20)***, hybridoma supernatant kindly provided by A. J. Levine, Rockefeller University, New York.
8. Alexa 488 coupled anti-mouse immunoglobulin (IgG) and Alexa 594 coupled anti-rabbit IgG secondary antibodies (Molecular Probes, Eugene, OR, USA).
9. Mounting medium: Fluoprep (bioMerieux, Hazlewood, MO, USA) supplemented with 2.5% (w/v) 1,4-diazabicyclo [2.2.2]octane (DABCO; used as a stabilizer of fluorescence). Stir until completely dissolved, then let stand to remove air bubbles. Store in the original Fluoprep flask.

2.4. Destabilization of p53 by E1B-55 kDa and E4-34 kDa, Detected by Immunoblot (see Notes 24–30)

1. RIPA-buffer: 20 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 10 m*M* EDTA, 10 m*M* iodacetamide, 1% (v/v) Triton®-X-100, 1% (w/v) Desoxycholic acid, 0.1% (w/v) SDS. Store at 4°C, stable at least 1 yr.
2. Milk: PBS supplemented with 5% (w/v) milk powder and 0.05% (v/v) Tween® 20. Make fresh and use within 24 h.
3. Super Signal Ultra (Pierce Chemical, Rockford, IL, USA) chemiluminescent substrate for peroxidase.
4. Monoclonal mouse antibody Pab1801 against p53 (Calbiochem-Novabiochem, San Diego, CA, USA).

5. Expression plasmids as in 2B, in addition the plasmid pCMVE4orf6, obtained from T. Dobner and T. Shenk, Princeton. The vector pCMVneoBam ***(24)*** contains the CMV major immediate early promoter, followed by an intron from the rabbit β-globin gene, a *Bam*HI cloning site to accommodate the insert, and a polyadenylation signal. The plasmid contains the ampicillin resistance gene (*bla*) and the the pBR322 origin of replication, yielding moderate copy numbers when grown in *E. coli*.

3. Methods

3.1. Co-Immunoprecipitation of p53 with Adenovirus-E1B-55 kDa (see *Notes 1–6)*

All quantities are indicated for one sample. A typical experiment will include several samples, such as a positive control (wild-type p53 and HEK cells) and negative controls (replacing wild-type p53 with the mutant p53L22Q/W23S, which is unable to bind E1B-55 kDa ***[23]*** and/or replacing HEK293 cells with, e.g., HeLa cells, which do not contain E1B-55 kDa). The quantities should be scaled up accordingly.

1. Expand HEK293 cells (express E1B-55 kDa) in flasks or dishes; 2×10^6 cells/sample will be needed. Prepare cell lysate for one more sample than required in the experiment, since some loss will occur during preparation and centrifugation steps.
2. Prepare p53 by in vitro transcription and translation, using the expression plasmid pOS7-p53 and the TNT T7-coupled reticulocyte lysate system, adding ^{35}S-methionine, exactly as described in the TNT manual. Ten microliters of programmed reticulocyte lysate will be required per sample.
3. Wash cells once with PBS at room temperature.
4. Add 1 mL of PBS and harvest cells by scraping with a rubber policeman.
5. Transfer the cell suspension to a 1.5-mL Eppendorf® tube and spin for 5 min at 800*g* in a tabletop centrifuge (Eppendorf Scientific, Westbury, NY, USA). Remove supernatant.
6. Add 1 mL of PBS to the flask/dish to suspend the remaining cells. Transfer to the same Eppendorf tube, spin, and remove supernatant as in the previous step.
7. Resuspend cells in 100 µL lysis buffer by pipeting up and down 5×. Combine the lysate from 3–5 samples in one 1.5-mL tube.
8. Pass through an insulin syringe (1-mL syringe; 0.45 × 12 mm needle) back and forth 5×. This is possible without excessive loss, if at least three samples (300 µL total) are combined and processed together. Avoid foaming. Avoid spilling, which can easily occur, when the fluid is released from the syringe back into the tube.
9. Spin for 5 min at 14,000*g* in a tabletop centrifuge at 4°C. Transfer supernatant to a new 1.5-mL Eppendorf tube. Discard pellet. Avoid the transfer of any particular material, rather leave some 30 µL back in the first tube, and try not to touch the wall of the tube with the pipet tip.
10. Repeat the previous step to remove any residual insoluble material.

11. Re-aliquot HEK 293 cell lysate into samples of 100 μL (aliquots can be stored at –70°C at least 1 yr if desired; however, any precipitate that may have formed after thawing should be removed by centrifugation as in **step 9**). Add 10 μL of reticulocyte lysate containing radioactively labeled p53. Mix by gentle vortex mixing and incubate at 30°C for 30 min.
12. To each sample, add 660 μL lysis buffer and 100 μL DMEM.
13. Swell protein A Sepharose beads in PBS (can be stored at 4°C for 1 wk), then gently centrifuge (e.g., in a 15-mL conical tube, using a swing-out rotor at 1400*g* for 5 min) and remove supernatant. Wash 2× with lysis buffer. Resuspend in lysis buffer to obtain a suspension containing approx 30% packed vol of beads. Add 90 μL of this suspension to each sample. Use a pipet tip that has been "widened" by cutting off the thinnest part, and vortex mix the bead suspension between each pipeting step to ensure even distribution of the beads.
14. Incubate 20 min at 4°C with gentle agitation (slow-moving rotator) for preclearing.
15. Spin for 1 min at 14,000*g* in a tabletop centrifuge at 4°C. Transfer supernatant to a new 1.5-mL Eppendorf tube. Discard pellet.
16. Add antibody against E1B-55 kDa, 20 μL of a 2A6 hybridoma supernatant.
17. Briefly vortex mix, then incubate for 2 h at 4°C with gentle agitation (slow-moving rotator).
18. Add 30 μL of the beads suspension (10 μL packed vol of beads) to each sample, as described in **step 13**. It is essential that all samples receive the same amount of beads. This can be checked by briefly spinning the tubes and comparing the size of the pellets.
19. Briefly vortex mix, then incubate for 2 h at 4°C with gentle agitation (slow-moving rotator).
20. Spin for 1 min at 14,000*g* in a tabletop centrifuge at 4°C. Discard supernatant into the radioactive waste, but leave approx 50 μL in tube, and avoid removing any beads. If desired, keep 20 μL for analysis of supernatant.
21. Add 500 μL of lysis buffer. Vortex mix for 1 min. It is preferable to use a device for vortex mixing several tubes simultaneously, to ensure equal washing conditions for each sample. For example, use an automated Eppendorf mixer, if available. Spin for 1 min at 14,000*g* in a tabletop centrifuge at 4°C. Discard supernatant into the radioactive waste, but leave approx 50 μL in tube, and avoid removing any beads.
22. Repeat the previous washing step.
23. Repeat the previous washing step 2×, but use lysis buffer that was supplemented with 0.5% (instead of 0.05%) (w/v) bovine serum albumin. This will decrease background precipitation, conceivably by blocking nonspecific binding sites on the beads.
24. Repeat the previous washing step 2× using nonsupplemented lysis buffer.
25. Remove residual buffer using an insulin syringe (1 mL syringe; 0.45 × 12 mm). Avoid the removal of any beads by moving slowly and by pressing the needle with the open end against the wall of the tube.
26. Add 30 μL of 6X Laemmli buffer, mix by gentle vortex mixing and boil at 95°C

for 3 min. Vortex mix briefly, then spin for 1 min at 1400*g* in a tabletop centrifuge at room temperature.

27. Apply 10 µL of the samples to a 10% SDS polyacrylamide gel and run.
28. Fix gel for 15 min in 30% methanol/10% acetic acid, then place on 3 layers of Whatman paper (Whatman, Clifton, NJ, USA) and cover with Saran® wrap. Do not place Saran wrap underneath the gel. Dry under vacuum, and while heating (takes approx 3 h), expose to X-ray film or a BioImager™/PhosphorImager® plate (Molecular Dynamics, Sunnyvale, CA, USA).

3.2. Reporter Assay to Measure the Inhibition of p53 Activity by E1B-55 kDa (see Notes 7–15)

1. The day before transfection, seed four wells in a 6-well dish (one well has a diameter of 3.5 cm) with H1299 cells (10^5/well).
2. Assemble the following mixtures of plasmids:

   a. 100 ng pGL2BP100, 50 ng pRcCMV, 2.2 µg pCGN.
   b. 100 ng pGL2BP100, 50 ng pRcCMV, 2.2 µg pCGN-E1B-55 kDa.
   c. 100 ng pGL2BP100, 50 ng pRcCMV-p53, 2.2 µg pCGN.
   d. 100 ng pGL2BP100, 50 ng pRcCMV-p53, 2.2 µg pCGN-E1B-55 kDa.

3. Transfect each well with one of the mixtures, using LIPOFECTAMINE 2000 exactly as recommended by the manufacturer. Leave the cells in 2 mL/well of DMEM/10% fetal calf serum (FCS).
4. After 24 h, harvest by scraping off the attached cells into the culture media. Transfer 1 mL of the cell suspension to an Eppendorf tube. Spin at 800*g* for 2 min in a tabletop centrifuge. Gently remove supernatant. Transfer the rest of the cell suspension to the same tube and repeat centrifugation. Gently remove supernatant and briefly vortex mix the dry pellet. Add 100 µL of reporter lysis buffer and vortex mix briefly. Let stand for 15 min on ice. Vortex mix briefly and centrifuge at 14,000*g* for 1 min. Transfer supernatant to new tube (can be stored at 4°C for several hours or at –20°C indefinitely).
5. Quantitate luciferase activity in a luminometer. Expect little difference between (a) and (b), an approx 100-fold increase comparing (a) and (c), and a 5- to 10-fold decrease when comparing (c) and (d).

3.3. Visualized Intracellular Relocalization of p53 by E1B-55 kDa (see Notes 16–23)

1. The day before transfection, seed 5×10^4 H1299 cells in each well of a 4-well chamber slide.
2. Assemble the following mixtures of plasmids:

   a. 50 ng pRcCMV, 500 ng pCGN.
   b. 50 ng pRcCMV, 500 ng pCGN-E1B-55 kDa.
   c. 50 ng pRcCMV-p53, 500 ng pCGN.

d. 50 ng pRcCMV-p53, 500 ng pCGN-E1B-55 kDa.

3. Transfect each well with one of the mixtures, using LIPOFECTAMINE 2000 exactly as recommended by the manufacturer. Leave the cells in 0.5 mL/well of DMEM/10% FCS.
4. Incubate for 24 h.
   All steps of the following immunostaining protocol are performed at room temperature, except when overnight blocking (**step 7**) is desired for convenience. All washing steps are carried out with 0.5 mL of PBS.
5. Wash cells once, then add fixing solution for 15 min.
6. Wash 2×, then add PBS supplemented with 0.2% (v/v) Triton X-100 for 25 min.
7. Wash 3×, then add blocking solution for 15 min at room temperature or overnight at 4°C.
8. Dissolve first antibodies as follows in the same aliquot (0.5 mL) of blocking solution: rabbit anti-p53 1:300 (1.6 μL); mouse anti-HA-tag 1:1000 (0.5 μL). Mix and add 100 μL to each well. Incubate 1 h.
9. Wash 3× and dissolve the secondary antibodies, as follows, in the same aliquot (0.5 mL) of blocking solution: Alexa 488-coupled anti-mouse IgG 1:500 (1 μL) and Alexa 594-coupled anti-rabbit IgG 1:500 (1 μL). Mix and add 100 μL to each well. Incubate for 1 h in the dark.
10. Wash 3× and dissolve 4,6-diamidino-2-phenylindole (DAPI) from a 10 mg/mL H_2O stock solution 1:5000 in PBS (2 μg/mL final concentration). Add 1 mL to each well. Incubate for 10 min in the dark.
11. Wash 3× and from now on, work quickly and avoid drying the cell monolayer. Remove the gasket from the slide, and remove any jelly that might still stick to the slide, using a pair of tweezers. Remove residual PBS with a paper towel from the edges of the slide, without touching the monolayer. Put the slide face-up onto a paper towel. Add one small drop of mounting medium to each well. Gently apply a glass cover slip (24 × 50 mm) onto the four wells. Cover with another paper towel and stabilize the cover slip on the slide at the edges, using thumbs and fourth fingers of both hands. Use second fingers to gently press the cover slip onto the slide for approx 10 s. This will allow excess mounting medium to be released and soaked up by the towel. Avoid moving the cover slip sidewards, since this may damage the monolayer. Remove towels and allow to dry for 1 h at room temperature in the dark. Watch with an immunofluorescence microscope.
12. Expect a staining pattern in cytoplasmic clusters for E1B-55 kDa (HA-tagged; green) in (b) and (d), and no staining above background in (a) and (c).
13. Expect an even nuclear staining of p53 (red) in (c), no staining above background in (a) and (b), and a staining pattern in cytoplasmic clusters that co-localize with the green stain in (d).

3.4. Destabilization of p53 by E1B-55 kDa and E4-34 kDa, Detected by Immunoblot (see Notes 24–30)

1. The day before transfection, seed eight wells in 6-well dishes (one well has a diameter of 3.5 cm) with H1299 cells (10^5/well).

2. Assemble the following mixtures of plasmids:

   a. 100 ng pRcCMV, 500 ng pCGN, 1.7 μg pCMVneoBam.
   b. 100 ng pRcCMV, 500 ng pCGN, 1.7 μg pCMV-E4-34 kDa.
   c. 100 ng pRcCMV, 500 ng pCGN-E1B-55 kDa, 1.7 μg pCMVneoBam.
   d. 100 ng pRcCMV, 500 ng pCGN-E1B-55 kDa, 1.7 μg pCMV-E4-34 kDa.
   e. 100 ng pRcCMV-53, 500 ng pCGN, 1.7 μg pCMVneoBam.
   f. 100 ng pRcCMV-53, 500 ng pCGN, 1.7 μg pCMV-E4-34 kDa.
   g. 100 ng pRcCMV-53, 500 ng pCGN-E1B-55 kDa, 1.7 μg pCMVneoBam.
   h. 100 ng pRcCMV-53, 500 ng pCGN-E1B-55 kDa, 1.7 μg pCMV-E4-34 kDa.

3. Transfect each well with one of the mixtures, using LIPOFECTAMINE 2000 exactly as recommended by the manufacturer. Leave the cells in 2 mL/well of DMEM/10% FCS.
4. After 24 h, harvest by scraping off the attached cells into the culture media. Transfer 1 mL of the cell suspension to an Eppendorf tube. Spin at 3000 rpm for 2 min in a tabletop centrifuge. Gently remove supernatant. Transfer the rest of the cell suspension to the same tube and repeat centrifugation. Gently remove supernatant and briefly vortex mix the dry pellet. Add 200 μL of RIPA buffer and vortex mix briefly. Add 50 μL of 6X Laemmli buffer, vortex mix, and boil at 95°C for 3 min. Shake on an Eppendorf mixer at highest speed for 30 min (this helps to shear genomic DNA and reduce the viscosity of the solution). Spin at 14,000*g* for 10 min in a tabletop centrifuge. Transfer supernatant to new tube (can be stored at 4°C for several h or at –20°C indefinitely).
5. Apply 30 μL of each sample to the pockets of an SDS polyacrylamide gel. Run gel and transfer to a nitrocellulose membrane using standard methods.
6. Briefly rinse membrane in PBS. Incubate in milk for 30 min with gentle agitation.
7. Incubate with antibody Pab 1801 to p53, diluted 1:5000 in milk.
8. Wash briefly with PBS/0.05% Tween 20. Repeat 2× and wash with milk for 15 min (gentle agitation).
9. Repeat **step 8**.
10. Incubate with secondary antibody, peroxidase-coupled anti-mouse IgG, diluted 1:10000 in milk.
11. Wash briefly with PBS/0.05% Tween 20. Repeat 2× and wash with milk for 15 min (gentle agitation).
12. Repeat **step 11** twice. Finally, wash 3× with PBS/0.05% Tween 20.
13. Mix 500 μL of each component of Super Signal Ultra peroxidase substrate.
14. Place membrane into a translucent plastic seal bag, add substrate, and seal by heating. Distribute substrate, e.g., by rolling a pipet over the bag. Cut off one corner of the bag, and squeeze out excess substrate. Reseal bag. Quickly go to the dark room.
15. Expose X-ray films to the blot, varying the exposure times between 0.5 s and several hours.
16. Expect a signal at approx 50 kDa in lanes (e) to (g). The signal will be much weaker (approx 100-fold less) in (h), reflecting extensive destabilization of p53. No signal should be detected in (a) to (f).

4. Notes

1. Instead of pOS7-p53, it is possible to use the more widespread pRcCMV-p53 ***(23)*** for transcription and translation in vitro, since the latter contains a T7 phage promoter as well. However, yields will be approx 10-fold lower. Therefore, the use of an internal ribosomal entry site upstream of the p53 coding sequence is advisable. Commercially available vector systems with this feature include the pCITE vectors (Novagen, Madison, WI, USA).
2. Radioactive contamination of the gel should be avoided as much as possible. If applying input controls derived from the programmed reticulocyte lysate, or when analyzing supernatants, do not use the same gel chamber, but run a second gel, since any free ^{35}S-methionine can diffuse from the loading pockets to the running buffer. It will then evenly penetrate the gel, creating considerable background. In contrast, the immunoprecipitated samples do not contain detectable free ^{35}S-methionine, due to the washing steps. Therefore, when necessary, very long exposure times (up to 2 mo) can be chosen without obtaining a background signal. Several layers of Whatman paper help to avoid contamination with radioactivity that may stick to the dryer.
3. In many institutions, it will be preferable to use nonradioactive systems, and one might attempt to detect p53 by immunoblot analysis. However, two issues need to be kept in mind when establishing such a system. First, HEK293 cells contain high amounts of endogenous p53, which becomes stabilized by E1B-55 kDa. This p53 will be detected in Western blots, making it difficult to distinguish it from p53 (and mutants) that was created by transcription and translation in vitro. To circumvent this difficulty, one might attempt to use adenovirus-infected H1299 cells (*p53 –/–* and easy to infect with adenovirus) or *p53 –/–* cells, which were transfected with an E1B-55 kDa expression plasmid (cf. **Subheading 3.4.**). Secondly, in SDS polyacrylamide gels, p53 runs very close to the Ig heavy chain of antibodies used for immunoprecipitation. Binding of the secondary antibody used in the immunoblot to this heavy chain frequently leads to a strong signal that obscures the detection of p53. This can be avoided using an antibody to p53 that is directly coupled to peroxidase (e.g., the monoclonal antibody DO-1 coupled to peroxidase, available from Santa Cruz Biotechnology).
4. The only commercially available antibody to E1B-55 kDa (monoclonal antibody from rat, clone 9C10, e.g., from Oncogene Research Products [Cambridge, MA, USA]) works less than antibody 2A6 in our hands. When looking for commercially available antibodies, note that E1B-55 kDa is entirely different from the more widely studied E1B-19 kDa protein (a bcl-2-like inhibitor of apoptosis).
5. Unlike p53, the p53-homologues p73 and p63 do not bind detectably to E1B-55 kDa ***(10,11,17–19)***. We found that swapping only five amino acids between p53 (residues 24–28) and p73 (residues 20–24) abolishes the interaction between p53 and E1B-55 kDa and enables p73 to bind E1B-55 kDa ***(10)***.
6. The interaction between E1B-55 kDa of adenovirus type 5 and p53 is readily detectable by immunoprecipitation. In contrast, the large E1B gene product from adenovirus type 12 appears to interact only weakly with p53, making it difficult

to observe this interaction by immunoprecipitation ***(11,25)***. However, adenovirus type 12 E1B inhibits p53 activity in reporter assays (*see* **Subheading 3.2.**), it relocalizes p53 to the cytoplasm (*see* **Subheading 3.3.**) and it destabilizes p53 when co-expressed with an E4orf6 protein (*see* **Subheading 3.4.**).

7. It is important to keep the amounts of p53 expression plasmid as low as indicated. We found that even very low amounts of p53 (e.g., expressed from 10 ng of plasmid) can induce reporter expression as efficiently as microgram amounts of p53 expression plasmid ***(26)***. If p53 is expressed to much higher levels than required to "saturate" transcriptional activity, it becomes difficult to observe negative regulation by E1B-55 kDa or any other p53-antagonist.
8. For expression of E1B-55 kDa, it seems important to keep the 3' UTR of the mRNA in the expression plasmid. We have previously worked with constructs that contained only the coding region, and expression was very poor.
9. Plasmids that contain the E1B-55 kDa coding region, such as pCGN-E1B-55 kDa, appear to slow down the growth of commonly used *E. coli* strains, and they tend to undergo recombination. Special precautions are necessary to prevent this from happening. We routinely grow these plasmids in *E. coli* of the SURE strain (Stratagene, La Jolla, CA, USA), which lacks many enzymes involved in DNA recombination, and we always grow cultures from freshly transformed single colonies directly to the required amounts, avoiding the storage of transformed bacteria at 4°C or as frozen glycerol cultures.
10. It has been suggested that the E4-34 kDa (E4orf6) protein alone is also capable of inhibiting p53 activity ***(27)***. We and others did not observe this, but did find a stronger inhibitory effect of E1B-55 kDa and E4-34 kDa together, compared to E1B-55 kDa alone ***(10,19)***.
11. Most, if not all, p53-responsive reporter plasmids can be used in this assay.
12. An alternative system with good transfection efficiencies consists in the combination of Saos2 cells (human osteosarcoma, *p53* –/–) and the transfection reagent FuGene6 (Roche Molecular Biochemicals, Indianapolis, IN, USA). The advantage of this system is that the transfection has very little toxic effect on the cells. However, the overall transfection efficiency is not as good as in the H1299/LIPOFECTAMINE 2000 system. Further, inhibition of p53 activity by E1B-55 kDa is less prounced when using Saos2 cells and FuGene6, possibly because a lower proportion of transfected cells takes up several plasmids simultaneously.
13. The large E1B gene product of adenovirus type 12 works about as well as that of adenovirus type 5 in this assay.
14. Unlike p53, the p53-homologues p73 and p63 are not inhibited by E1B-55 kDa in this assay and in similar experiments ***(10,11,17–19)***. We found that swapping only five amino acids between p53 (residues 24–28) and p73 (residues 20–24) abolishes the inhibition of p53 by E1B-55 kDa and enables E1B-55 kDa to inhibit p73 activity ***(10)***.
15. It is important to keep the cells free of mycoplasms. Otherwise, the transfection efficiency will drop extensively. In our hands, mycoplasms were efficiently removed and did not reappear after adding tetracyclin (2 μg/mL) and

ciprofloxacin (10 µg/mL) to the culture medium. We routinely test our cells for the presence of mycoplasms, using immunofluorescence (Ridascreen; r-biopharm).

16. The paraformaldehyde solution is made in a buffer very similar to PBS, but lacking bivalent cations (Ca^{2+} and Mg^{2+}). It is possible to prepare the solution in regular PBS, but we found that the paraformaldehyde is then quite difficult to dissolve completely.
17. If in need to speed up drying of the mounting medium, it can be carried out at 37°C for 15 min.
18. If no epitope tag is available, E1B-55 kDa protein can be detected with a monoclonal antibody (clone 246 ***[20]***). We use a hybridoma culture supernatant, diluted 1:20 in blocking solution. The only commercially available antibody (monoclonal antibody from rat, clone 9C10, e.g., from Oncogene Research Products) works less well in our hands, but does detect E1B-55 kDa when present in large amounts. When looking for commercially available antibodies, note that E1B-55 kDa is entirely different from the more widely studied E1B-19 kDa protein (a bcl-2-like inhibitor of apoptosis).
19. When using an anti-E1B-antibody, the protocol can be used to detect E1B-55 kDa and p53 in adenovirus-transformed cells, such as HEK 293 cells. However, these cells come off the dish quite easily, and care must be taken to wash them gently. Also, they must be free of mycoplasms (*see* **Note 15**) to show an acceptable morphology after staining.
20. If the amount of available antibody is limiting, the protocol can be modified to use less antibody solution. In this case, we spot as much antibody solution as affordable (we have used down to 10 µL/well) on parafilm and placed the slide upside down onto it. However, this will result in somewhat uneven staining intensities within the wells.
21. This assay system can also be used to detect the relocalization of E1B-55 kDa by the E4-34 kDa protein ***(14)***. Heterokaryon assays, employing the same staining technique, reveal nucleocytoplasmic shuttling of the complex ***(15)***. In the presence of E1B-55 kDa and E4-34 kDa, co-expressed p53 is usually not detectable due to its rapid degradation (*see* **Subheading 3.4.**).
22. In contrast to E1B-55 kDa form adenovirus type 5, the large E1B gene product of adenovirus type 12 was found mainly in the nuclei of cells that were transformed by this virus ***(7)***. However, when strongly expressed by transient transfection, this E1B protein was also found in cytoplasmic clusters, together with p53 ***(11)***.
23. Unlike p53, the p53-homologues p73 and p63 are not relocalized by E1B-55 kDa in this assay and similar experiments ***(10,11)***. We found that swapping only five amino acids between p53 (residues 24–28) and p73 (residues 20–24) abolishes the relocalization of p53 by E1B-55 kDa and leads to the co-localization of E1B-55 kDa and p73 in intranuclear track-like structures ***(10)***.
24. The best antibodies for detection of human p53 in our immunoblots were the mouse monoclonals Pab1801 und DO-1. The popular antibody Pab421, which works very well in immunoprecipitations and electrophoretic mobility shift analysis, yields only a weak signal in immunoblots.

25. The signal may be so strong that <1 s of exposure to the X-ray film is sufficient. This can result in exhausting the substrate and bleaching of the signal. Therefore, it is important to take the first exposures quickly after adding the chemiluminescent substrate. If bleaching still occurs, reduce the amount of secondary antibody (we sometimes use as little as a 1:100000 dilution).
26. Detection of p53 can be performed simultaneously with reporter assays. In this case, lyse the cells from one well in 100 µL of reporter lysis buffer (as described **Subheading 3.2.**, **step 4**), remove a portion (e.g., 20%) for luciferase assay, add 100 µL of RIPA buffer to the remainder (including the pellet with the nuclei), vortex mix, add 50 µL of 6X Laemmli buffer, and boil. Proceed as in **Subheading 3.4.**, **step 4**.
27. It is possible to detect E1B-55 kDa and E4-34 kDa in these immunoblots as well. However, they tend to appear in multiple bands of higher molecular weight than expected. Some, but not all, of these bands may be due to SUMOylation ***(28)***.
28. Since the destabilization of p53 by the adenovirus oncoproteins is mediated by the ubiquitin–proteasome pathway, p53 should be restabilized by the addition of a proteasome inhibitor ***(29)***. However, the addition of the proteasome inhibitor MG132 only moderately increased the levels of p53 in the presence of E1B-55 kDa and E4-34 kDa (our unpublished observations). We do not know why p53 degradation seemed to be inhibited incompletely under these circumstances.
29. The homologous proteins from adenovirus type 12 mediate p53 degradation to a similar extent as those from adenovirus type 5 ***(11)***, and even combinations of oncoproteins across virus types remain capable to destabilizing p53 (our unpublished observations).
30. p53 mutants that do not interact with E1B-55 kDa are not degraded in this assay ***(10,12)***. However, interaction with E1B-55 kDa is not sufficient for degradation. For instance, a p73 mutant, which carries amino acids 24–28 from p53, efficiently binds E1B-55 kDa but remains stable in the presence of E1B-55 kDa and E4-34 kDa ***(10)***. The C-terminal portion of p53 is at least partially required for destabilization ***(10)***.

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11

In Vivo Footprinting and DNA Affinity Chromatography for Analysis of p53 DNA Binding Ability

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Summary

p53 is a sequence-specific DNA binding protein. The p53 consensus is two copies of 5'-RRRC(A/T)(T/A)GYYY-3'. The interaction of p53 with specific DNA binding sites (DBS) has been analyzed extensively using electrophoretic mobility shift analysis (EMSA). These studies do not address the interaction of p53 with nuclear chromatin or the stability of p53-DBS interactions. In vivo footprinting examines the dynamic interactions of p53 protein in nuclear chromatin. p53 DBS affinity chromatography compares the stability of p53 from different cellular extracts with different DBS. Isogenic strains expressing high p53 levels, and deleted for p53, are required for controlled experiments using both methods. Different systems can be used to generate sufficient p53 protein (including DNA damage), and this results in the analysis of different forms of p53. A comparison of different cellular sources of high levels of p53 (in the presence and absence of DNA damage) vs different p53 DBS is required to appreciate the complexity of the regulation. Methods for comparing p53 from three different cellular sources with different DBS are presented here. The p53 research community needs to expand this analysis to complete the picture of how p53 differentially regulates transcription of target genes in nuclear chromatin.

Key Words

p53, DNA, binding, affinity, in vivo, footprinting, chromatography, chromatin

1. Introduction

p53 is a tumor suppressor protein implicated in many human cancers ***(1)***. Key to the ability of p53 to control cell growth is its sequence-specific DNA binding ability ***(2,3)***. The regulation of the p53-DNA interaction is very complex ***(4,5)***. This interaction is modulated by many different factors ***(6,7)***. There

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is a great divergence among the natural p53 response elements, with a consensus that emerges as a repeat of 5'-RRRC(A/T)(T/A)GYYY-3' ***(8)***. So far, 300 potential sites have been identified in the human genome ***(9)***. The p53 protein is susceptible to posttranslational modifications, such as phosphorylation and acetylation at multiple sites, and these are believed to modulate p53 DNA binding ability as well as activity ***(6,10)***. p53 interacts with many cellular proteins, and these interactions can also regulate the activity of p53 ***(4,7)***. Different combinations of the p53 interactions mentioned above give rise to p53-DNA interactions, which may vary in their affinity and stability of DNA binding.

DNA binding techniques measure different aspects of a given protein's ability to interact with a particular site. The source of the protein being used for the study also directs the investigated question. The common gel shift assay, also known as electrophoretic mobility shift analysis (EMSA), has been carried out extensively with purified p53, and more references exist than are sited here ***(2,11–15)***. This assay has been used to demonstrate the DNA sites that p53 can interact with and their relative affinities. The phenomenon of "caging," in the case of EMSA, forces association of p53 and DNA, and thus, complexes are essentially trapped upon entering the gel. In general, this assay has not assessed the instability of p53-DNA complexes. Less frequently, we and others have used in vitro footprinting to analyze p53-DNA interactions ***(2,14,16–19)***. Using this technique, an appreciation for the fast on–off rate that exists for p53-DNA interactions, when using purified protein, was obtained. The rate of p53 dissociation from DNA is very fast and can be observed when comparing the ability to detect a footprint vs the ability to see the protein interact with DNA using the EMSA ***(14)***. In addition, p53 protein expressed in bacteria demonstrates more instability than p53 expressed in insect cells, most likely due to posttranslational modification systems present in eukaryotic cells ***(14,20)***. In order to examine how p53 regulates transcription of genes in eukaryotic cells using the endogenous DNA binding sites (DBS), we have carried out in vivo footprinting and compared the observations to experiments using p53-DBS affinity chromatography (DNA affinity chromatography with p53 binding sites).

In vivo footprinting using ligation-mediated polymerase chain reaction (LM-PCR) is a standard technique that can demonstrate clear protection of certain DBS by their cognate proteins in the nucleus on chromatin ***(21–23)***. There is no other technique that demonstrates the real time interaction of transcription factors with chromatin, as does this technique. However, only a limited number of studies have been published that present p53 footprints on nuclear chromatin ***(24–27)***. The three DBS that have been examined in this way are in the genes *mdm2*, *p21*, and *gadd45*. The published papers examining the interaction of p53 with nuclear chromatin discuss the transient nature of the p53-DNA interaction in vivo. p53 is a transcription factor that controls the regulation of

many genes ***(28)***, but demonstrating its interaction with DBS in vivo has presented complications. This is most likely due to the fact that we expect p53 to behave like other transcription factors that show clear protection of sites in vivo, and it does not. The p53 protein differentially coordinates transcription to allow for growth arrest or apoptosis and is a central controller for these phenomena ***(7)***. The mechanisms by which it does this are not yet understood. Clear protection of both the p53 DBS in the *mdm2* gene and the *gadd45* gene have been demonstrated ***(25–27)***. However, one report documented little to no protection of these same sites with either different treatment or assay conditions ***(24)***. The DNA binding activity of high levels of p53, produced in response to DNA damage, is potentially different from high levels of p53 in cells that have not undergone DNA damage because of differential phosphorylation of p53. In addition, DNA damage may affect chromatin configuration through events such as the acetylation of histones. Only one report has been able to document the protection of the *mdm2* site in nuclear chromatin, and this was using high levels of p53 in the absence of DNA damage ***(25)*** (in the report where no protection of mdm2 was seen, DNA damage was used to increase the level of p53). The *mdm2* gene is paradoxical due to the p53-mdm2 feedback loop, whereby the MDM2 protein disables p53 activity. The differing ways in which high levels of p53 were achieved may account for the one source of p53 protein showing protection of the *mdm2* site on chromatin, while the other did not. It has also been seen that the in vitro protection of the gadd45 site is substantially different from the in vivo footprint and that DNA damage does not change how p53 interacts with this site in nuclear chromatin ***(26,27)***. The protocol for in vivo footprinting has the following major steps: (*i*) isolation of DNase-treated nuclei from experimental cells; (*ii*) isolation of nuclei from the negative control cells; (*iii*) annealing and extension of the gene-specific oligonucleotide no. 1; and (*iv*) visualization of the Dnase-mediated cleavage sites by LM-PCR.

p53 DNA binding activity can be modulated in several ways. Its interaction with other proteins, including members of the general transcription machinery ***(29)***, can alter its activity. Posttranslational modification of p53 can have an effect on DNA binding as well as its ability to transactivate ***(6)***. Variations in the consensus p53 DNA binding site are another factor that influence the binding of p53 to its response element ***(13)***. Different cellular conditions can result in variable combinations of the above-mentioned modulations and may thus lead to different types of p53-DNA interactions with regard to the amount of p53 bound to the DNA element, the affinity of binding, and the type of p53 molecules bound to the DNA sites. The key to understanding p53 function is being able to dissect the subtle differences that may result in multiple types of interactions with DNA. DNA affinity chromatography has traditionally been

used to purify specific proteins ***(30)***. Purification of p53 from cellular extracts using DNA affinity chromatography with p53 DBS has not been reported; however, a p53 family member has recently been isolated using this method ***(31)***. We have used p53 DBS affinity chromatography to analyze the stability of the p53 interaction with specific sequences, as well as demonstrating some of the other proteins that are part of an associated complex ***(32)***. The amount of p53 present in an extract to be studied does not directly correlate with the amount of p53 protein bound to different p53 DBS columns (although EMSA data would suggest that p53 binds with high affinity). It appears that each p53 DBS selects for a subpopulation of p53 molecules with specific DNA binding characteristics (p53 "bindomers"), whose cellular concentration may not be as high in each given experimental condition (Molina, paper in preparation). We have noted that for the super consensus sequence (SCS), increased purification can be achieved if the cells have been exposed to DNA damage. We suggest that if the stability of a p53-DBS interaction increases, by virtue of particular posttranslational modifications due to DNA damage, then the enrichment for p53 in eluted fractions is greater. Because p53-DBS affinity chromatography can enrich for specific p53 molecules and other associated proteins, it facilitates biochemical analysis of these potential bindomers and their complexes. Using the same cell lines and DBS sequences for in vivo footprinting p53 in nuclear chromatin as for enrichment of p53 from DBS affinity chromatography will allow a picture to emerge that demonstrates what proteins interact with the p53 DBS under different conditions. The p53-DBS affinity chromatography procedure can be divided in four steps: (*i*) gel filtration of the nuclear extract intended to eliminate the nucleases present in the crude preparation; (*ii*) preparation of the p53-DBS affinity column, which includes labeling and concatemerizing the specific deoxyoligonucleotide, ligating the concatemerized DNA to a solid matrix, and finally, packing and equilibrating the column; (*iii*) p53-DBS affinity chromatography, which involves passing the partially fractionated nuclear extract through the column, washing the column to remove the remnant proteins nonspecifically bound to the DNA, and eluting the p53-DBS-bound protein; and (*iv*) detection of the eluted proteins by EMSA and/or Western blot.

2. Materials

2.1. Cell Lines and Extracts

A limiting factor for the biochemical analysis of p53 protein is its low cellular concentration under nonstressed conditions. To achieve a detectably strong signal, especially for in vivo footprinting assays, it is advisable to utilize a cellular system that overexpresses p53. In addition, investigators should have at their disposal isogenic strains null for p53 to be used as negative controls.

Two sets of cell lines that fulfill these conditions are the TR9-7 cell line ***(33)***, which expresses wild-type p53 and its parental line 041, as well as murine 3-4 cells ***(25,34)***, which express the temperature-sensitive (ts) mutant $p53^{Val135}$ and its parental line 10-1 ***(35)***. Another system that meets these requirements is the insect cell line *Spodoptera frugiperda* 21 (Sf21). These cells are easily infected with a baculovirus expressing the human *p53* gene and are able to overexpress p53 protein without entering apoptosis (*see* **Note 1**).

2.1.1. Cell Lines

1. TR9-7 is an isogenic human fibroblast cell line derived from MDAH041. It contains a tetracycline-regulated wild-type *p53* gene (generously provided by M. Agarwal ***[33]***) .
2. MDAH041 is a human fibroblast cell line lacking functional p53 protein due to frameshift mutation on one p53 allele at codon 184 and loss of the normal p53 allele ***(33)***.
3. 3-4 ***(25,34)*** is an isogenic mouse fibroblast cell line stably expressing p53 and is derived from 10-1 cells ***(35)*** by transfection with a plasmid (pLTRp53cGval135) expressing the ts mutant $p53^{Val135}$ ***(36)***.
4. 10-1 is a mouse fibroblast cell line that is null for p53 due to a deletion in the *p53* gene ***(35)***.
5. Sf21 cells are an insect cell line infected with a recombinant baculovirus expressing wild-type human *p53* ***(37)***.

2.1.2. Reagents

1. Dulbecco's modified Eagle medium (DMEM) supplemented with 5 mL of penicillin (5000 U)/steptomycin (5000 µg/mL) liquid (Life Technologies, Rockville, MD, USA) per 500 mL of medium. TR9-7 cells are selected by adding G418 (600 µg/mL) and hygromycin (50 µg/mL) to the medium. This medium also contains tetracycline at 2 µg/mL to keep p53 expression repressed when appropriate. 3-4 cells are also kept in selective medium with 1.0 mg/mL G418 (Gemini BioProducts, Calabasas, CA, USA).
2. 10% Fetal bovine serum (FBS), heat-inactivated (Life Technologies).
3. 500 mL TC-100 medium, pH 6.2, (Life Technologies) supplemented with 5 mL penicillin (5000 U)/streptomycin (5000 µg/mL) liquid, 30 µL 2.5 mg/mL amphotericin B, and 50 mL heat-inactivated FBS.

2.1.3. Solutions

1. Lysis stock buffer: 20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, 10 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* ethylenediaminie tetraacetic acid (EDTA), 0.1% Triton® X-100, 20% glycerol, 1 m*M* dithiothreitol (DTT), 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 50 µg/mL aprotinin, and 50 µ*M* leupeptin.

2. Cowie lysis buffer: 50 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 1% Nonidet® (NP40), 0.1% aprotinin, 1 m*M* DTT, 1% inhibitor cocktail, 0.35 m*M* PMSF.
3. Inhibitor cocktail: mix 10 µL each of the following reagents in a final vol of 1 mL in distilled water (dH_2O): 30 µg/µL leupeptin, 10 m*M* benzamide, and 1 µg/µL bacitracin.

2.2. In Vivo Footprint

In 1989, a PCR footprinting procedure was developed that enabled in vivo protein-DNA interactions to be viewed at specific regions of chromatin ***(21)***. The technique involves ligation-mediated PCR, and it permits amplification of an entire sequence ladder of DNA. The protocol that we used was kindly provided by Clifton E. McPherson ***(23)***. There are variations to the protocol. The procedure provided here uses isolated nuclei treated with DNase I. This DNA is prepared from nuclei extracted from either the 3-4 (murine) or TR9-7 (human) cells described in **Subheading 2.1.** Negative controls for each are the 10-1 cells or 041 cells, respectively, that have been treated with DNase I. After isolating the DNA, a gene-specific oligonucleotide no. 1 is annealed to denatured DNA and extended with Sequenase® DNA Polymerase. A linker oligonucleotide is then ligated to the end created by the extension (this allows for the ladder). PCR is then carried out using a second gene- specific oligonucleotide no. 2 and an oligonucleotide complementary to one strand of the linker. The last step is to carry out linear PCR on these templates using a radiolabeled gene-specific oligonucleotide no. 3. The products are subsequently viewed on a sequencing gel. Oligonucleotide no. 3 is also used for Sanger dideoxy sequencing, so that the sequence of the ladder is known.

2.2.1. Gene-Specific Primer Sets for Analysis of Specific Sites

1. Murine *mdm2*:
   no. 1: 5'-TCGAGGTAGAAATACCAACC-3'
   no. 2: 5'-CGAAGCTGGAATCTGTGAGG-3'
   no. 3: 5'-GGAATCTGTGAGGTGCTTGCAGCA-3'
2. Human *mdm2*:
   no. 1: 5'-ACAGCACCATCAGTAGGTAC-3'
   no. 2: 5'-AAGCTACAAGCAAGTCGGTG-3'
   no. 3: 5'-AAGTCGGTGCTTACCTGGATCAGCAG-3'
3. Human *gadd45*:
   no. 1: 5'-CCCTGAAAACATAACTTCCC-3
   no. 2: 5'-GAAGCTGACTCCTTAATGAGGG-3'
   no. 3: 5'-TGACTCCTTAATGAGGGGTGAGCCAG-3'

2.2.2. Linker **(21)**

no. 1: LMP-T1: 5'-GAATTCAGATC-3'
no. 2: LMP-B1: 3'-CTTAAGTCTAGAGGGCCCAGTGGCG-5'

2.2.3. Reagents

1. DNase I (Worthington Biochemical, Lakewood, NJ, USA).
2. Sequenase DNA polymerase, version 2.0 (13 U/μL; USB, Cleveland, OH, USA)
3. γ^{32}P-ATP (150 μCi/μL).
4. T4 Polynucleotide kinase (New England Biolabs, Beverly, MA, USA)
5. *Taq* DNA polymerase (6 U/μL) (Roche Molecular Biochemicals, Indianapolis, IN, USA).

2.2.4. Solutions

1. RSB: 10 m*M* Tris-HCl, pH 7.4, 10 m*M* NaCl, 3 m*M* $MgCl_2$, pH 7.4, 0.5% NP40, 1 m*M* PMSF or absent (depending on the step).
2. DNase I stop: 2 *M* NH_4OAc, 100 m*M* EDTA, 0.2% sodium dodecyl sulfate (SDS).
3. Sequenase buffer 1 (store at –20°C): 125 m*M* Tris-HCl, pH 7.5, 400 m*M* NaCl, 25 m*M* $MgCl_2$.
4. Sequenase buffer 2 (store at –20°C): 40 m*M* Tris-HCl, pH 7.5, 5 m*M* $MgCl_2$, 20 m*M* DTT, 0.1 m*M* dNTPs.
5. Ligase buffer 1 (store at –20°C): 80 m*M* Tris-HCl, pH 7.5, 180 μg/mL bovine serum albumin (BSA) 30 m*M* $MgCl_2$.
6. Ligase buffer 2 (store at –20°C): 12 m*M* ATP, 70 m*M* DTT.
7. Oligonucleotide no.1 mixture: 0.3 pmol/μL in dH_2O.
8. Oligonucleotide no.2 mixture: 5.0 pmol/μL in dH_2O.
9. Oligonucleotide no.3 mixture: 25 pmol/μL in dH_2O.
10. Linker oligonucleotide solution: 20 pmol/μL each of LMP-T1 and LMP-B1 in a solution of 250 m*M* Tris-HCl, pH 7.5, 5 m*M* $MgCl_2$. Heat at 95°C for 5 min, cool at room temperature for 20 min. Aliquot and store at –20°C. Thaw aliquots at room temperature immediately before use. This may be refrozen.
11. 10X *Taq* buffer: 650 m*M* Tris-HCl, pH 8.8, 100 m*M* β-mercaptoethanol, 165 m*M* $(NH_4)_2SO_4$.
12. 5X Oligonucleotide kinase buffer: 250 m*M* Tris-HCl, pH 9.5, 50 m*M* $MgCl_2$, 25% glycerol.

2.3. p53 DBS Affinity Chromatography

2.3.1. p53 Binding Site Oligonucleotides

1. MDM2-P2:
   Top:
   5'-GATCCCTGGTCAAGTTGGGACACGTCCGGCGTCGGCTGTCGGAGGAGCTAAGTCCTGACATGTCTCCG-3'
   Bottom:
   5'-GATCCGGAGACATGTCAGGACTTACCTCCTCCGACAGCCGACGCCGGACGTGTCCCAACTTGACCAGG-3'
2. SCS:
   Top:
   5'-TCGAGCCGGGCATGTCCGGGCATGTCCGGGCATGTC-3'

Bottom:
5'-TCGAGACATGCCCGGACATGCCCGGACATGCCCGGC-3'

3. RGC:
   Top:
   5'-TCGAGTTGCCTGGACTTGCCTGGCCTTGCCTTTTC-3'
   Bottom:
   5'-TCGAGAAAAGGCAAGGCCAGGCAAGTCCAGGCAAC-3'
4. mtRGC:
   Top:
   5'- TCGAGTTTAATGGACTTTAATGGCCTTTAATTTTC-3'
   Bottom:
   5'-TCGAGAAAATTAAAGGCCATTAAAGTCCATTAAAC-3'

2.3.2. Reagents

1. Sephacryl™ S-300 matrix (Amersham Pharmacia Biotech, Piscataway, NJ, USA).
2. Glass packing reservoir (2 × 25 cm).
3. 10 U/μL T4 polynucleotide kinase (New England Biolabs).
4. 1 U/μL T4 DNA ligase (Life Technologies).
5. γ^{32}P-ATP (10 μCi/μL) (NEN® Life Science Products, Boston, MA, USA).
6. 4B Sepharose®, CNBr-activated (Amersham Pharmacia Biotech).
7. Glycogen 20 mg/mL (Roche Molecular Biochemicals).
8. 1 *M* Ethanolamine-HCl, pH 8.0.
9. Herring sperm DNA (sonicated).
10. p53 DBS affinity column (1 mL of beads).
11. Amicon® Centricon 10 or 30 (Millipore, Bedford, MA, USA).

2.3.3. Solutions

1. TM+ buffer, comprising 50 m*M* Tris-HCl, pH 7.9, 1 m*M* EDTA, pH 8.0, 12.5 m*M* $MgCl_2$, 10 m*M* KCl, 0.1% NP40, 20% glycerol, and 1 m*M* DTT.
2. 10X T4 polynucleotide kinase buffer. 700 m*M* Tris-HCl, pH 7.6, 100 m*M* $MgCl_2$, 5 m*M* DTT.
3. 5X T4 DNA ligase buffer: 250 m*M* Tris-HCl, pH 7.6, 50 m*M* $MgCl_2$, 5 m*M* ATP, 5 m*M* DTT, polyethylene glycol 800, 25% (w/v).
4. TBE buffer, pH 7.6: 10.8 g Tris base, 5.5 g boric acid, 20 mL 0.5 *M* EDTA, pH 8.0.
5. 1 *M* and 10 m*M* Phosphate buffer, pH 8.0 (*see* **Note 12**).
6. Column storage buffer: 10 m*M* Tris-HCl, pH 7.6, 1 m*M* EDTA, pH 8.0, 0.3 *M* NaCl, 0.04% sodium azide (added prior to use).
7. Buffer Z: 25 m*M* HEPES, pH 7.8, 12.5 m*M* $MgCl_2$, 1 m*M* DTT, 0.1% NP40 (v/v), 20% glycerol (v/v).
8. Column regeneration buffer: 10 m*M* Tris-HCl, pH 7.8, 1 m*M* EDTA, 2.5 *M* NaCl, and 1% NP40. Store at room temperature. May separate into phases. Mix by swirling just before use.
9. Column storage buffer: 10 m*M* Tris-HCl, pH 7.6, 1 m*M* EDTA, 0.3 *M* NaCl, 0.04% sodium azide (w/v) (*see* **Note 15**).

3. Methods

3.1. Cells and Cell Extracts

3.1.1. Cell Growth

1. Grow TR9-7, MDAH041, 3-4, and 10-1 cells on 150-mm plates at 37°C in DMEM until 70% confluent. Grow Sf21 cells in suspension at 27°C in TC-100 medium, supplemented with 10% heat-inactivated FBS. Seed the cells onto 150-mm plates at a concentration of 2 to 3 × 10^7 cells/plate and allow them to attach.
2. At an appropriate time prior to harvesting, express p53.

3.1.2. Expression of p53

Make a curve of p53 expression vs time. It is recommended that initial experiments with all cell lines include a time course for p53 expression. These curves are essential, because different DBS will most likely show p53 interaction kinetics, which are variable.

3.1.2.1. TR9-7 Cells

1. Replace the growth medium with DMEM (plus 10% fetal calf serum [FCS]) without tetracycline. This system is designed to be repressive for p53 expression in the presence of tetracycline so that growing cells are not privy to the cell cycle effects of p53. We suggest culturing two sets of TR9-7 cells: one set with DMEM supplemented with tetracycline as a control (no p53) and another set with tetracycline removed 24 h (or at an appropriate time) prior to harvesting to allow for p53 expression.
2. If the effects of DNA damage are also being examined, then damage should be imparted at least 18 h after tetracycline removal when p53 is expressed. We have imparted damage using 0.1 m*M* camptothecin 20 h after tetracycline removal with good results.

3.1.2.2. 3-4 Cells

1. The cells must be shifted to 32°C to express p53. We perform the temperature shift 4 h prior to harvesting, but once again, a time course for p53 expression is strongly recommended.

3.1.2.3. SF21 Cells

1. Infect cells with a recombinant baculovirus expressing the human p53 gene at a multiplicity of infection (MOI) of 10:1.
2. Incubate cells at 27°C.
3. Harvest 48 h later by scraping the plates.

3.1.3. Cells Extracts

3.1.3.1. TR9-7 and 3-4 Cells

1. To harvest TR9-7, 3-4 cells, and their negative controls, wash the cells 2× with 1X phosphate-bufferd saline (PBS) at 4°C .
2. Harvest by scraping the plates with 1 mL of cytoplasmic lysis buffer per 150-mm plate (prepared by combining 8.8 mL of lysis stock buffer with 6.2 mL of dH_2O).
3. Centrifuge the cell suspension at 2300 rpm (600*g*) for 10 min at 4°C to pellet nuclei.
4. Remove the supernatant and resuspend the pellet in 1 mL of nuclear extraction buffer (prepared by combining 8.8 mL of lysis stock buffer, 1.47 mL of 5 *M* NaCl, and 4.7 mL of dH_2O).
5. Rock for 1 h at 4°C.
6. Centrifuge the nuclear extract at 14,000 rpm (23,425*g*) for 10 min at 4°C to further pellet debris and recover the supernatant.
7. Determine protein concentration via Bradford Microasssay (Bio-Rad, Hercules, CA, USA), aliquot the extracts and store at –80°C.

3.1.3.2. SF21 Cells

1. Centrifuge the suspension at 2000 rpm (478*g*) for 15 min.
2. Resuspend the pellet in 1.6 mL of Cowie lysis buffer per 150-mm plate and allow to lyse on ice for 30 min.
3. Centrifuge the cell extract at 2000 rpm (478*g*) for 15 min at 4°C to pellet debris and recover the supernatant.
4. Centrifuge the extract at 20,000 rpm (47,807*g*) for 30 min at 4°C to pellet debris.
5. Determine protein concentration via Bradford Microassay, aliquot, and store at –80°C.

3.2. In Vivo Footprinting of p53 DNA Response Elements

3.2.1. Extraction of Genomic DNA from DNase I Treated Nuclei after the Expression of p53

Ligation-mediated PCR is the method used to visualize "in vivo" footprints generated after DNase I treatment of cell nuclei. The protocol described here is for use with cells grown in culture, so purists would prefer to refer to it as intranuclear footprinting, due to the lack of an entire organism (*see* **Notes 1** and **2**).

1. Grow on 150-mm plates. Use at least 3 plates for each cell line under each set of conditions. Cells should be at no more than 70% confluent before expression of p53. Conditions and curves for activating p53 in the human TR9-7 and the murine 3-4 line are described in **Subheading 3.1.** in the section for cells.
2. Wash plates 2X with ice-cold PBS.
3. Scrape cells and put into a tube. Add 1X PBS if needed to get all cells off the plate.

4. Spin down at low speed at 4°C (2500 rpm [746*g*] in SS34 rotor) for 10 min.
5. Resuspend in 2 mL of RSB with PMSF to extract nuclei (*see* **Note 2**).
6. Homogenize with 20 strokes using pestle B, and check cells for trypan blue exclusion (continue to homogenize until all nuclei have been extracted).
7. Spin down at 4000 rpm (1912*g*) at 4°C for 10 min.
8. Wash nuclei in 2 mL of RSB without PMSF.
9. Resuspend in RSB without PMSF, so cell count is 2.0×10^6 cells/250 µL.
10. Treat with DNase I for 10 min at 32°C using 250 µL of nuclei in RSB with $CaCl_2$ added to a final concentration of 0.1 m*M*. It is important to perform a curve of DNase I to titrate the digestion. Use DNase I, 2932 U/mg. The curve should include at least 0.05, 0.1, and 0.5 µg of DNase I.
11. Add 250 µL of DNase I stop buffer, and then add proteinase K to a final concentration of 400 µg/mL. Incubate overnight at 37°C.
12. Extract once with phenol pH 8.0 and 3× with chloroform:isoamyl 24:1.
13. Ethanol precipitate using sodium acetate at a final concentration of 0.25 *M* before the addition of 2 vol of ice-cold 100% ethanol.
14. Resuspend the pellet in TE to a concentration of 1 µg/µL. Store at 4°C, and use samples as soon as possible (*see* **Notes 2–5**).

3.2.2. LM-PCR Protocol to View DNase I Mediated Cleavage Sites

1. Begin with 1 µg of genomic DNA in 1X Sequenase buffer 1 (25 m*M* Tris-HCl, pH 7.5, 80 m*M* NaCl, 0.5 m*M* $MgCl_2$) at a final vol of 15 µL for each sample.
2. Add 1 µL of 0.3 pmol/µL oligonucleotide no. 1. Denature at 95°C for 5 min; anneal at 50°C for 30 min, then chill on ice. Spin down to remove condensation prior to moving on to the next step.
3. Add 9 µL of Sequenase buffer 2 (40 m*M* Tris-HCl, pH 7.5, 5 m*M* $MgCl_2$, 20 m*M* DTT, 0.1 m*M* dNTPs) and 0.5 µL of Sequenase DNA polymerase, version 2.0 (13 U/µL). Mix gently.
4. Incubate at 37°C for 10 min, followed by heat inactivation at 68°C for 10 min. Then place on ice. Spin down to remove condensation prior to moving on to the next step.
5. With the tubes on ice, ligate by adding 20 µL of each ligase buffer 1 and ligase buffer 2, 5 µL of linker solution, and 3 µL of T_4 DNA ligase (1 Weiss U/µL; Life Technologies) to the mixture. Mix gently. Incubate at 20°C overnight, and then at 68°C to inactivate the enzyme.
6. Place tubes on ice, add 8 µL of 3 *M* NaOAc, and ethanol precipitate with 2 vol of ice-cold 100% ethanol. Resuspend the pellet in 14.5 µL of dH_2O. Transfer this DNA solution to a 500-µL PCR tube.
7. Add: 16 µL of 2.5 m*M* dNTP, 2 µL 5 pmol/µL oligonucleotide no. 2, 2 µL 5 pmol/µL LMP-T1 oligonucleotide, 1 µL 4 mg/mL BSA, 4 µL of 10X *Taq* buffer, and 0.5 µL of *Taq* DNA polymerase (5 U/µL).
8. In a thermal cycler, perform 20 cycles as follows: 1 min at 94°C to denature, 2 min at 65°C to anneal, and 3 min at 74°C to extend. Store samples at –20°C.

9. To visualize the footprints, 15 μL of the PCR from above was transferred to a fresh 500-μL PCR tube, and add 1.5 μL of a ^{32}P-radiolabeled oligonucleotide no. 3 (1 pmol/μL, as described below in **step 14**), 0.5 μL of 10X *Taq* buffer, 3 μL of 2.5 m*M* dNTP, and 0.5 μL of Taq DNA polymerase (*see* **Note 6**).
10. In a thermal cycler, perform 7 cycles as follows: 1 min at 94°C to denature, 2 min at 68°C to anneal, and 3 min at 74°C to extend. When cycling is complete, add 24 μL of TE, 1 μL of 5 mg/mL tRNA, and 5 μL of 3 *M* NaOAc.
11. Add 50 μL of $CHCl_3$, vortex mix, and centrifuge to separate phases. Add 2 vol of 100% ice-cold ethanol and precipitate.
12. Resuspend the pellet in 12 μL of sequencing loading buffer and resolve the DNA by electrophoresis using a 6% denaturing polyacrylamide gel. Run one-third of your sample to determine how to normalize samples for a second gel.
13. Sequence the DNA using ^{32}P-oligonucleotide no. 3 and Sequenase DNA polymerase, version 2.0.
14. Radiolabeling of oligonucleotide no. 3: use 1–3 μL of oligonucleotide no.3 (25 pmol/μL), 5 μL of 5X oligonucleotide kinase buffer, 1.25 μL of 100 m*M* DTT, 1–3 μL of γ^{32}P-ATP at 150 μC/μL, use 1 μL/25 pmol of oligonucleotide. Add dH_2O to 25 μL, and then 1 μL of T4 polynucleotide kinase. Place at 37°C for 30–60 min, 68°C for 10 min, and use a Sephadex® spin column (Amersham Pharmacia Biotech) to remove free nucleotides. Adjust vol to 1 pmol/μL and count 10 μL of a 1:100 dilution in a scintillation counter. Store at –20°C (*see* **Note 6**).

3.3. p53-DBS Affinity Chromatography

This procedure is divided into three parts: (*i*) gel filtration of the nuclear extract; (*ii*) p53-DBS affinity chromatography of the partially fractionated nuclear extract; and (*iii*) detection of the p53 present in the p53-DBS affinity elution fractions. To protect the integrity of the DNA affinity column, it is advisable to rid the nuclear extract of nucleases. This can be accomplished by subjecting the extract to gel filtration prior to loading it onto the DNA affinity column. Sephacryl S-300 is an appropriate matrix for this purpose. After partial fractionation of the nuclear extract, the fractions from the gel filtration column corresponding to the column bed vol can be pooled and concentrated for further use on the p53-DBS affinity column.

The partially fractionated and pooled nuclear extract is next loaded onto the p53-DBS affinity column. It is important to load as much p53 as possible, because only a fraction of the p53 present in the extract will be competent for binding to a particular p53 DBS. As much as 10 μg of p53 has been used for this purpose. The nuclear extract is passed through the affinity column at gravity flow. Because of the fast on–off rate of p53 on its DBS, it is advisable to pass the sample several times through the column (we recommend 10 passes). The column flow-through should be kept for further use as a control to evaluate the amount of p53 bound to the column. The p53 DBS affinity column is then washed, and the protein(s) eluted in 1-mL aliquots with increasing concentrations of KCl to sequentially elute p53 species with increased binding affinity.

Finally, the eluted p53-DBS affinity fractions can be analyzed using Western blot EMSA and to detect p53 and p53 DNA binding activity, as well as to look for the presence of other cellular proteins co-eluted with p53.

3.3.1. Gel Filtration of Cellular Extract

3.3.1.1. Packing of a 25-cm-Long Column Performed at 4°C

1. Mount the packing reservoir on a stand.
2. Bring 37 mL of Sephacryl S-300 resin to 50 mL with TM+ buffer.
3. Using a Buchner funnel, wash the resin 2× with 50 mL of TM+ buffer. Allow the resin to equilibrate to 4°C.
4. Mix the resin well, and in one operation, pour it down the wall of the packing reservoir with the help of a glass rod.
   Note: a very thin suspension will lead to poor packing of the column. Very gently, fill the reservoir with TM+ buffer. Assemble buffer reservoir, and connect to pump.
5. Open outlet, and run the pump at 12 μL/s for 2 h or until constant height. At this point, change the speed to 20 μL/s and run the pump for 1 h.
6. Equilibrate the column by running 50 mL (2-bed vol) of TM+ buffer at a speed of 6 μL/s. Check for even packing of the column by illuminating it from the back.

3.3.1.2. Gel Filtration Performed at 4°C

1. Equilibrate the column by running 50 mL (2-bed vol) of fresh TM+ buffer at a speed of 100 μL/15s.
2. Drain buffer from above the bed and apply sample.
   Note: the sample vol should not be more than 4% of the bed vol (1 mL).
3. Run the column until the sample enters the matrix, stop the pump, and fill the packing reservoir to the top with TM+ buffer.
4. Run the pump at 100 μL/15 s, and collect 25 fractions (1 mL each).
5. Discard the 7 first fractions (7 mL), corresponding to 6.5 mL of void vol plus 0.5 mL of tubing vol.
6. Pool the remaining 18 fractions. Concentrate the pooled fractions with Centricon, and keep at –80°C for further use (*see* **Notes 7–9**).
7. Sanitate the Sephacryl column by running the following solutions at a speed of 100 μL/15 s: 0.3 *M* NaOH, 30 min of contact time; 50 mL (2-bed vol) TM+ buffer, and 75 mL (3-bed vol) 20% ethanol.
8. Store the Sephacryl S300 column in 20% ethanol at 4°C.

3.3.2. Preparation of the p53 DBS Affinity Column

3.3.2.1. DNA Phosphorylation

1. To hybridize single-stranded oligonucleotides, prepare a reaction mixture as follows: mix 65 μg of top deoxyoligonucleotide and 65 μg of bottom deoxyoligonucletide in TE buffer for a total vol of 65 μL . Add 12 μL of 10X T4 polynucleotide kinase buffer.
2. Hybridize oligonucleotides by heating for 2 min at 88°C, 10 min at 65°C, 10 min at 37°C, and 5 min at room temperature.

3. Add 30 μL of 10 m*M* ATP, pH 7.0, 0.5 μL of γ^{32}P-ATP (10 μCi/μL), 10 μL of T4 polynucleotide kinase (10 U/μL), and 2.5 μL of dH_2O (total vol equals 120 μL). **Note:** from this point on, check radioactivity with a hand-held counter at each step to track DNA.
4. Incubate at 37°C for 2 h.
5. Add 100 μL of 5 *M* NH_4OAc and 30 μL of dH_2O to adjust to 2 *M* NH_4OAc and 250 μL final vol.
6. Heat at 65°C for 15 min to inactivate the enzyme. Cool down at room temperature for 10 min.
7. Ethanol precipitate the DNA with 3 vol (750 μL) of ice-cold ethanol and 1 μL of glycogen.
8. Resuspend the pellet in 225 μL of TE, pH 7.6.
9. Extract the DNA once with 250 μL of 1:1 (v/v) phenol:chloroform and once with 250 μL of 24:1 (v/v) chloroform:isoamyl.
10. Adjust NaOAc concentration to 0.3 *M* with 25 μL of 3 *M* NaOAc, pH 5.2, and ethanol precipitate 1× with 3 vol (750 μL) of ice-cold ethanol.
11. Resuspend the pellet in 65 μL of dH_2O, and keep at –20°C until ligation.

3.3.2.2. Ligation

1. Prepare a reaction mixture containing the 5'-phosphorylated double-stranded oligonucleotide from **step 11** (**Subheading 3.3.2.1.**) and 40 μL of 5X T4 DNA ligase buffer. Vortex mix 1 min to assure that the DNA pellet is resuspended. Add 53.3 μL of 10 m*M* ATP, pH 7.0, 30 μL of T4 DNA ligase (1 U/μL), and 11.7 μL of dH_2O for a total vol of 200 μL.
   Note: save 0.5 μL of this reaction mixture prior to ligation for use as a control when checking the progression of the ligation procedure by agarose gel electrophoresis.
2. Incubate at 15°C overnight. Monitor the ligation by agarose gel electrophoresis until you have enriched for 10-mers of oligonucleotides: 680 bp for mdm2, 350 bp for RGC, and 250 bp for SCS.
3. Once the ligation is complete, extract the DNA once with phenol, pH 8.0, 200 μL and once with 24:1 (v/v) chloroform:isoamyl alcohol, 200 μL.
4. Adjust salt concentration to 2 *M* NH_4OAc. Precipitate the DNA with an equal vol of 2-propanol and glycogen, 1 μL as carrier. Resuspend pellet in 225 μL of TE.
5. Precipitate the DNA 1X using a final concentration of 0.3 *M* sodium acetate and 3 vol of ice-cold ethanol.
6. Wash the pellet 2× with 800 μL of ice-cold 75% ethanol.
7. Dry the pellet for 5 min under vacuum. Resuspend in 10 μL of dH_2O, and store at –20°C until further use.

3.3.2.3. Preparing the Sepharose Matrix: 1 mL of Beads

1. Add 0.6 g of activated Sepharose 4B and 3 mL of 1 m*M* HCl. Mix by inversion for 1 min.

2. Transfer to a Buchner funnel and perform the following washes for 15 min each: 100 mL 1 m*M* HCl, 20 mL dH_2O, 20 mL 10 m*M* phosphate buffer, pH 8.0.

3.3.2.4. Coupling

Note: from this point on, it is necessary to track the binding of ligated oligonucleotides to the matrix by monitoring the radioactivity in the washes.

1. Transfer the washed Sepharose matrix to an Eppendorf® tube and add 0.4 mL of 10 m*M* phosphate buffer (pH 8.0) to make a thick slurry.
2. Add 10 μL DNA from **Subheading 3.3.2.2.**, **step 7**, and incubate on a rotating wheel at room temperature for 12 h or overnight.
3. In a fume hood, transfer the beads to a Buchner funnel, and wash 2× with 20 mL dH_2O, and 1× with 20 mL 1 *M* ethanolamine-HCl, pH 8.0.
4. To inactivate the CNBr, transfer the beads to a 15-mL falcon tube, and add enough 1 *M* ethanolamine-HCl, pH 8.0, to create a smooth slurry. Incubate 4–6 h on a rotating wheel at room temperature.
5. Transfer to a Buchner funnel, and perform the following washes: 2× with 20 mL 1 *M* phosphate buffer, pH 8.0, 20 mL 1 *M* KCl, and 20 mL column storage buffer.
6. Put the beads coupled to DNA in a plastic column, add 5 mL of column storage buffer, and store them at 4°C.
7. Before discarding, treat CNBr-contaminated waste and instruments by soaking them in a 10–20 mg/mL solution of sodium hydroxide and glycine. Let stand overnight in a fume hood and then discard.

3.3.3. Affinity Chromatography of Cellular Extract

Normalize the experiments for a given amount of p53 contained in the partially fractionated cellular extract (*see* **Notes 8–15**).

1. Assemble the p53-DBS affinity column on a stand and let it equilibrate at 4°C.
2. Equilibrate the beads by running 10 mL of Buffer Z (0.1 *M* KCl) through the column at gravity flow (*see* **Note 11**).
3. To the partially fractionated cellular extract, add herring sperm DNA (competitor) at 5 ng competitor per 1 μg of total protein. Incubate for 15 min at room temperature. Centrifuge at 13,000 rpm (20,198*g*) for 10 min to pellet the debris. Recover the supernatant and discard the pellet. Put the extract at 4°C (*see* **Note 15**).
4. Drain the column buffer from the bottom without allowing the beads to dry.
5. Load the cellular extract onto the column and pass it 10× at gravity flow. Collect the flow-through for further analysis.
6. Wash the column 4× with 2 mL Buffer Z (0.1 M KCl). Make sure to rinse the walls of the plastic reservoir. Collect the washes in 1-mL or 2-mL fractions for further analysis.
7. Elute the affinity column by running 1-mL fractions of Buffer Z, with KCl concentrations ranging from 0.2–1.0 *M*. Collect each fraction independently in Eppendorf tubes.

8. Concentrate each fraction to 100 μL, using Centricon 10 or 30 (*see* **Note 7**). Aliquot, and keep at –80°C for further use.

3.3.3.1. Column Regeneration

Work at room temperature.

1. Close the column outlet and add 5 mL of regeneration buffer to the beads.
2. Stir the beads in the regeneration buffer with a siliconized glass rod.
3. Open the outlet, and allow buffer to run through.
4. Repeat **steps 1–3**.
5. Run 5 mL of storage buffer through the column at gravity flow. Repeat 2×.
6. Keep the column in column storage buffer and at 4°C.

3.3.4. Analysis of the p53 DBS Affinity Fractions

1. Concentrate each p53 DBS elution fraction independently, using Centricon 10 or 30 (*see* **Notes 7** and **8**).
2. Use the p53 DBS elution fractions for Western blot, EMSA, or both, according to the objectives.

4. Notes

1. The p53 temperature-sensitive line 3-4, as well as the tetracycline-regulated cell line TR9-7, have been used to successfully observe p53-dependent changes in DNase I protection. If any other cell line is used, we suggest that you use one of these as a positive control. We have begun to work with the cell line p53 A2 that expresses very high levels of p53 controlled by the removal of the drug doxycycline (a generous gift from Bert Vogelstein) ***(38)***. It appears to be a very promising cell line that has an isogenic partner, null for p53, and should be considered for future experiments. Sometimes overexpressing lines stop expressing the protein of interest. We have had some trouble with the TR9-7 line and thawed older freeze downs when this occurred.
2. It is critical to determine that the nuclear DNA can be extracted from the cells without being significantly nicked in the absence of DNase I addition. This can be analyzed to some extent on an agarose gel, but the best method is to carry out LM-PCR using the primer sets on the extracted genomic DNA prior to beginning the key experiments. It is then critical to empirically determine the amount of DNase I required to obtain an even distribution of cutting sites on the region of interest. There must be enough cleavage to resolve a given area; however, too much cleavage will decrease the region that you are able to resolve.
3. There is a critical window during which p53 DNase I protection can be observed, and it is possible that this window will vary for the different p53 response elements. Therefore, a time curve for mRNA expression from the gene of interest should be analyzed to determine an early p53 target gene expression time point, which can be compared to one after 24 h of p53 expression. We have seen that, after 4 h of p53 expression, a clear footprint on the mdm2 p53 RE can be observed in 3-4 cells at the

mdm2 site ***(25)***. After DNA damage, shorter time points may be required. Protection of the gadd45 site has been observed after 4 h in ML-1 cells ***(26)***.

4. As we mentioned before, the regulation of the p53-DNA association is complex. Analysis with purified proteins does not always reflex this complexity, so we recommend using cellular extracts for comparisons as well.
5. The specific activity of the radioactivity used is very high. You will be using a preparation that is rather crude for this technique.
6. Use at least 10 μg of p53 contained in the cell extract, which corresponds to about 50 maxiplates (150 mm) of 3–4 cells at 70% confluency. This equals approx to 7–10 mg of total protein.
7. The choice of the Centricon cut-off number depends on your goal. If you want to isolate p53 protein only, it is better to choose the higher cut-off number, Centricon 30. This will allow the concentration to proceed faster. If you want to analyze associated proteins, it is advisable to use the small cut-off number, Centricon 10. This choice will allow you to retain most of the proteins present in each fraction, but it will retard the filtration procedure.
8. Concentrate the partially fractionated cellular extract to a convenient vol, and achieve a p53 concentration of about 10 μg of p53/mL of extract.
9. Once the Sephacryl S 300 column has been packed at 4°C, do not transfer to any other temperature, because it can de-equilibrate and incorporate air bubbles into the column bed.
10. Before loading the sample onto the p53-DBS affinity column, save at least 100 μL of sample for comparative analysis. Also, keep the washes and flow-though for the same purpose.
11. To prepare Buffer Z of different salt concentrations (0.2–1.0 *M*) for the affinity column elution, mix the corresponding amounts of Buffer Z (no salt) and Buffer Z (1.0 *M* KCl).
12. Prepare 1 *M* phosphate buffer by preparing a 1 *M* KH_2PO_4 solution and adjusting its pH to 8.0 with a 1 *M* K_2HPO_4 solution.
13. Do not concentrate the crude nuclear extract beyond 70 mg/mL. It will be too viscous and difficult to run through the Sephacryl S 300 column.
14. The p53-DBS affinity column should be operated at gravity flow. Applying other forces might deform the matrix and hinder the binding of p53 to the DNA.
15. All the buffers containing DTT should be prepared fresh for each use to assure its effectiveness.
16. All values are based on a Sorvall SS 34 rotor except for **step 3** of **Subheading 3.3.3.**, which is for a micro-centrifuge.

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12

Signaling to p53

The Use of Phospho-Specific Antibodies to Probe for In Vivo Kinase Activation

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Summary

Phospho-specific antibody technology has been recently adopted to study p53 phosphorylation both in vivo and in vitro. We have developed and carefully characterized p53 phospho-specific reagents directed to major amino- and carboxy-terminal regulatory sites. The specificities of both polyclonal and monoclonal reagents targeting the same phospho-epitope are discussed. We have defined the major chemical binding determinants for specific monoclonal reagents by determining the relative contribution of charge and sequence to epitope recognition. Remarkably, we have found that the utility of these reagents in different assay systems is not universal and depends both on epitope conformation and affinity. This is reflected in the striking differences in their ability to detect endogenous p53 and recombinant protein. Therefore, we conclude that this novel class of reagents is not generally applicable, but that the utility of each reagent must be determined empirically.

Key Words

antibody, phosphate, phospho-specific, polyclonal, monoclonal, kinase, signaling, noninvasive, ELISA, immunoblotting, bandshift

1. Introduction

1.1. p53 Regulation by Phosphorylation

p53 function as a tumor suppressor is linked to its function as a sequence-specific DNA-binding protein and stress-activated transcription factor that controls the expression of a large panel of gene products implicated in normal growth control, cell-cycle arrest, and apoptosis ***(1)***. The central core domain of p53 con-

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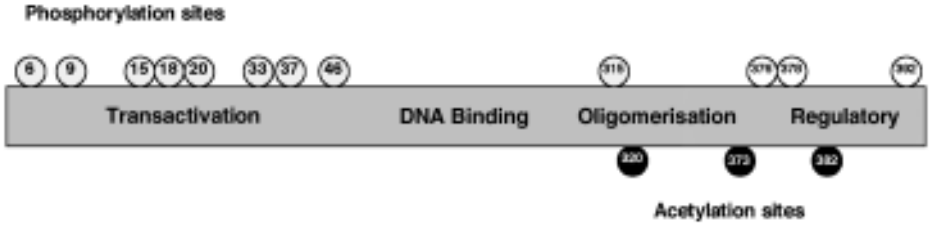


Fig. 1. Sites of posttranslational modification on human p53. Phosphorylation sites and acetylation sites mapped on p53 using conventional radiolabeling methods, phospho-specific antibodies, or mass spectrometry.

tains the sequence-specific DNA-binding domain and an oligomerization domain that is required to assemble p53 into its tetrameric structure (**Fig. 1**). Regulatory domains at the amino and carboxy termini of p53 modulate protein–protein interactions and DNA–protein interactions that affect the rate of p53-dependent transcription (**Fig. 1**) ***(2)***. The C terminus of p53 contains a negative regulatory domain ***(3)*** whose phosphorylation at Ser^{392} by CK2 ***(4)*** or Ser^{315} by cyclin-dependent kinases ***(5,6)***, as well as acetylation at multiple lysine residues by p300 and PCAF ***(7,8)***, stimulates the DNA-binding function of p53. The N-terminal regulatory domain of p53 contains the highly conserved *BOX-1* sequence (amino acids 13–27) that directs the binding of p53 to proteins, including the positive effector p300 ***(9)*** and the inhibitor MDM2 ***(10)***, the balance of which regulates the tumor suppressor activity of p53. Phosphorylation of *BOX-1* domain sites, including Ser^{15}, Thr^{18}, and Ser^{20}, can either reduce MDM2 binding ***(11)*** or stabilize the p53-p300 transcription complex ***(12)***, resulting in a net activation of p53 function. Thus, the concerted activation of the sequence-specific DNA binding function of p53 by posttranslational modification of its C-terminal domain and the kinase-dependent stabilization of the p53-p300 complex provides a working model to explain the basic mechanism of how p53-dependent gene expression can be activated. Understanding the regulation of the enzymes that target these sites of covalent modification on p53 is clearly necessary for dissecting the upstream regulation of p53 activity, and a variety of methods have been used to identify and measure changes in p53 phosphorylation.

1.2. Methods for Measuring Protein Phosphorylation

Commonly utilized methods for studying protein phosphorylation include phosphopeptide mapping and protein mobility shift by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Identification of site-specific protein phosphorylation involves the metabolic labeling of cultured cells with radioactive precursors such as ^{32}P-orthophosphate. The protein of interest is immunoprecipitated, digested with proteolytic enzymes, and the

resulting peptide fragments are resolved by two-dimensional electrophoresis. Peptide migration is compared to control phosphopeptides to identify phosphorylation sites. However, radionuclide exposure can activate stress response pathways, resulting in growth arrest and changes in the basal p53-phosphorylation state ***(13,14)***, thereby rendering conventional radiolabeling approaches unsuitable for studying kinase pathways that affect the physiological regulation of stress-response proteins. Mass spectrometry-coupled identification of phosphorylation sites complements the use of radiolabeling methods and has the important advantage of being noninvasive, in that cells are not labeled with a damaging precursor prior to cell harvesting. Several p53 protein phosphorylation and acetylation sites have been mapped using this method, and although certain known phosphorylation sites predominate ***(15)***, other known sites are not detected using this method, possibly due to low yields of phosphopeptide following sample processing.

More recently, phospho-specific antibodies have been developed and used as noninvasive probes for studying site-specific protein phosphorylation in vivo. A clear advantage of this technology is that it permits the relatively inexpensive development of reagents to both known and putative phosphorylation sites, with minimal sample processing resulting in improved signal retention. The combination of specificity and sensitivity of phospho-specific antibody technology has caused it to be widely adopted for studying phosphorylation of a range of cellular proteins, including cytoskeletal proteins, components of signaling pathways (including receptor kinases and transcription factors), neuronal proteins, and cell cycle regulatory proteins.

Initial studies revealed that phosphorylated proteins are not good antigens, as they are rapidly dephosphorylated by endogenous phosphatases in the bloodstream of laboratory animals. Furthermore, phosphorylation sites are not always within immunodominant regions of the protein. Therefore, the preferred immunogens are synthetic 10–12 amino acid peptides, consisting of the sequence surrounding the phosphorylation site to be studied. Initially, these peptides had to be phosphorylated following synthesis, thereby restricting the utility of this technique to studying the regulation of sites phosphorylated by known kinases. However, improvements in chemical synthesis technology now enable phosphate to be added during synthesis using an arylthio group that protects the peptide from dephosphorylation in vivo. Both polyclonal and monoclonal antibodies (MAbs) can be generated using phosphopeptides, and there are advantages to each approach. High titer polyclonal antibodies can be relatively easily and cheaply produced, but require affinity purification to remove nonspecific antibodies from antisera. However, although more costly and technically demanding to generate, MAbs have the advantage of being intrinsically more reproducible and additionally can be produced at high titre in ascites fluid.

The epitope of a phospho-specific antibody generally comprises two recognition determinants: (*i*) a specific amino acid sequence; and (*ii*) phosphate at a specific site, within this region. However, there is a useful class of phospho-specific reagents which detect phospho-tyrosine(Tyr), phospho-threonine (Thr), and phospho-serine(Ser) that do not have a specific epitope sequence context. These are widely used to detect phosphorylation of specific proteins following immunoprecipitation. However, despite their clear potential, several factors have to be considered before using these novel reagents. First, careful characterization is required to prevent the selection of reagents that cross-detect phosphate at different sites within the same sequence. Further, for reagents targeting consensus amino acid sequences phosphorylated by abundant kinases, there is a possibility of cross-reactivity with other cellular proteins in some assays.

1.3. Development of Immunochemical Methods for Studying p53 Phosphorylation

Polyclonal reagents targeting several p53 phospho-epitopes have now been generated, including many sites within N- and C-terminal regulatory domains. More recently, this technology has been adapted to include acetylated epitopes such as Lys^{320} and Lys^{382} (*see* **Fig. 1**). However, although several research groups have now generated anti-p53 phospho-specific and acetyl-specific antibodies targeting key residues, the binding specificities of all of the reagents have not all been published (*see* **Note 1**).

We generated p53 site-specific antibodies to study the regulation of p53 function by posttranslational modification at major sites that regulate either the DNA-binding activity or the p300-binding activity of p53. Reagents designed to specifically target both N-terminal (Ser^{15}, Thr^{18}, and Ser^{20}) and C-terminal (Ser^{315} and Ser^{392}) sites have been developed and used to assess signaling changes to p53. A comprehensive study using these reagents has shown that phosphorylation patterns on p53 protein are damage-regulated ***(4,6,16,17)*** and vary strikingly in normal human fibroblasts, depending on the type of damage or signal used to perturb cells ***(18)***. Increases in site-specific phosphorylation of p53 in normal human cells implicate kinase signaling in the regulation of p53 function. However, an observed decrease in the level of basal phosphorylation at constitutive sites indicates that phosphatases as well as kinases may also play a role in modulating specific protein–protein or protein–DNA interactions of p53 ***(18)***.

This review will first describe immunochemical assays used to characterize and measure the specificity or promiscuity of phospho-specific antibodies to the N-terminal *BOX-1* domain of p53 and will highlight the difficulties in acquiring reliable phospho-specific reagents. Significant differences in specificity between similar phospho-specific MAbs may be understood by deter-

mining their respective chemical mechanisms of epitope binding, and we describe methods for determining key binding residues within a phospho-epitope. The second part contains more sophisticated assays that measure: (*i*) the specificity of phospho-specific MAbs to the C-terminal regulatory domain of p53 using phage–peptide display; and (*ii*) the stoichiometry of p53 phosphorylation using native (i.e., non-denaturing) gel electophoresis. We discuss the utility of well-characterized monoclonal phospho-specific antibodies in studying the regulation of p53 site-specific phosphorylation in cell lines or in clinical material in order to ascertain whether kinase pathways target p53 in physiological situations. Lastly, the utility of a monoclonal phospho-specific antibody in the development of cell-free site-specific kinase assay–phosphatase assay opens the door to develop high-throughout inhibitor screening from crude lysates that preserves complex regulatory conditions not always optimized using highly pure kinases.

2. Materials

All reagents were supplied by Sigma (St. Louis, MO, USA), unless otherwise stated.

1. Keyhole Limpet Hemocyanin (KLH) (Sigma; cat. no. H2133) is prepared at 10 mg/mL in H_2O and stored at –20°C.
2. Phosphopeptides are synthesized by standard methods ***(19)*** and contain a C-terminal amino acid with a free primary amine to facilitate glutaraldehyde cross-linking to carrier protein prior to immunization. These are dissolved at 4 mg/mL in H_2O and stored at –20°C.
3. Glutaraldehyde is stored as a 70% solution at –20°C.
4. Protein G beads (suspension in 50% ethanol) for immunoglobulin (IgG) purification are from Amersham Pharmacia Biotech (Piscataway, NJ, USA).
5. Antibody elution buffer: 0.1 *M* glycine, pH 2.5.
6. Phosphate-buffered saline (PBS) buffer: 140 m*M* NaCl, 2.6 m*M* KCl, 10 m*M* Na_2HPO_4, 1.7 m*M* KH_2PO_4.
7. NaF is prepared as a 1 *M* stock solution and stored at room temperature. The working concentration is 50 m*M* (*see* **Note 2**).
8. Reacti-gel (6X) is obtained from Pierce Chemical (Rockford, IL, USA; cat. no. 20259).
9. Colorless enzyme-linked immunosorbent assay (ELISA) plates (Falcon®) are from Becton Dickinson (Franklin Lakes, NJ, USA; cat no. 3912) and opaque ELISA plates are Microlite 2 (DYNEX Technologies, Chantilly, VA, USA).
10. Streptavidin is stored as a 10 mg/mL stock solution at –20°C.
11. Biotinylated phosphopeptides are dissolved at 5 mg/mL in dimethyl sulfoxide (DMSO) and stored at –20°C.
12. ELISA coating buffer: 0.1 *M* sodium borate (B_4O_7), pH 9.0.
13. PBS-T: PBS containing 0.1% (v/v) Tween®-20.

14. 3BPBST: PBS containing 3% (w/v) bovine serum albumin (BSA).
15. 5MPBST: PBS containing 5% (w/v) fat-free milk powder.
16. Fat-free milk powder used is Marvel.
17. 1 *M* β-glycerophosphate (βGP) is stored at 4°C. The working concentration is 10 m*M* (*see* **Note 2**).
18. Okadaic acid (OA) (O-7760; Sigma; cat. no. 0-7760, sodium salt) is stored at –20°C as a 120 μ*M* stock solution in ethanol. The working concentration is 12 n*M* (*see* **Note 2**).
19. Phospho-p53 (Ser^{20}) and phospho-p53 (Ser^{15}) polyclonal antibodies are from New England Biolabs (Beverly, MA, USA).
20. Horseradish peroxidase (HRP)-conjugated secondary antibodies to mouse or α-rabbit IgG are supplied by Dako (Glostrup, Denmark).
21. Electrochemiluminescence (ECL)™-chemiluminescence solutions and Hyperfilm™ are obtained from Amersham Pharmacia Biotech.
22. TMB Liquid Substrate system for developing the ELISA is from Sigma (cat. no. T-8540).
23. The ELISA plate reader used is a Model 4000 (DYNEX Technologies), and the fluorimeter is a Flouroscan Ascent FL (Labsystems Affinity Sensors, Franklin, MA, USA).
24. Two sensor SA chips (Biacore, Piscataway, NJ, USA).
25. Biacore 2000 upgrade (Biacore).
26. Priming buffer: 1 *M* NaCl, 40 m*M* NaOH.
27. HBS buffer: 10 m*M* 4-(2-hydroxyethyl)-1-pipetrazineethanesulfonic acid (HEPES), pH 7.4, 150 m*M* NaCl, 3.4 m*M* ethylenediamine tetraacetic acid (EDTA), 0.05% Biacore Surfactant P20.
28. Stripping buffer: 10 m*M* glycine, pH 2.0.
29. Phage-peptide display kits (12-mer and cyclic 7-mer libraries) were obtained from New England Biolabs.
30. p53 polygrip oligomers: 5381 5'-TTT TTT TTT TAG ACA TGC CTA GAC ATG CCT-3'; 5382 5'-TTT TTT TTT TAG GCA TGT CTA GGC ATG TCT-3'. Stock oligomer concentration is 2 mg/mL in Tris-EDTA (TE) buffer.
31. Chroma-spin-10 columns were supplied by Clontech Laboratories (Palo Alto, CA, USA).
32. TE: 20 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA.
33. Cyclin-dependent kinase (CDK) buffer: 10% glycerol, 25 m*M* HEPES-KOH, pH 7.5, 50 m*M* KCl, 0.5 mg/mL BSA, 5 m*M* $MgCl_2$, 1 m*M* dithiothreitol (DTT).
34. DB buffer: 25 m*M* HEPES, pH 7.6, 15% glycerol, 50 m*M* KCl, 0.02% Triton® X-100, 1 m*M* $MgCl_2$, 1 mg/mL BSA. Add 5 m*M* DTT and 1 m*M* benzamidine directly before use.
35. Nondenaturing gel contains 4% polyacrylamide (29:1), 1X Tris-borate (TB) buffer, 0.1% Triton X-100, 0.1% ammonium persulfate, 0.1% N,N, N',N'-tetramethylethylene diamine (TEMED).
36. TB buffer: 45 m*M* Tris base and 44 m*M* H_3BO_3.
37. Nondenaturing gel tank buffer: 0.625X TB and 0.1% Triton X-100.
38. Tissue culture plates are from Nalge Nunc International (Rochester, NY, USA).

39. Urea lysis buffer: 7.0 *M* urea, 0.1 *M* DTT, 0.05% Triton X-100, 25 m*M* NaCl, 50 m*M* NaF, 0.02 *M* HEPES (pH 7.6).
40. Bradford assay reagent is from Bio-Rad (Hercules, CA, USA).
41. Laemmli buffer: 2% SDS, 25 m*M* Tris-HCl, pH 6.8, 10% glycerol, 0.02% bromophenol blue.
42. SDS-polyacrylamide gels (10%) are made as indicated ***(20)***.
43. SDS-PAGE buffer: 25 m*M* Tris, 190 m*M* glycine, 0.1% SDS.
44. Blotting buffer: 150 m*M* glycine, 20 m*M* Tris, 0.1% SDS (w/v) and 20% methanol, pH 8.3.
45. Nitrocellulose membrane (HyBond® C) is from Amersham Pharmacia Biotech.
46. Avidin and biotin solutions are from Vector Laboratories (Burlingame, CA, USA).
47. The ABC Elite biotin-streptavidin immunoperoxidase kit is supplied by Vector Laboratories.
48. Diaminobenzidine (DAB) in 0.03% hydrogen peroxide is from Dako.
49. DPX mountant is from Diachem International Ltd.
50. 50X Low salt buffer (LSB): 0.5 *M* HEPES, pH 7.4, 1.25 *M* KCl, 0.5 *M* NaCl, 55 m*M* $MgCl_2$, 5 m*M* EDTA. This is stored at 4°C.
51. Low salt extract (LSE) buffer: 1X LSB, pH 7.2, 0.1 *M* DTT, 1X PIM plus NaF. This is made fresh before use. PIM is 10X protease inhibitor mixture (PIM) (10 m*M* pefabloc, 0.1 mg/mL leupeptin, 5 μg/mL aprotinin, 10 μg/mL pepstatin, 50 μg/mL trypsin inhibitor, 5 m*M* benzamidine, 8 m*M* ethyene glycol bis(β-aminoethyl ether) N,N,N',N',-tetraacetic acid (EGTA), 5 m*M* EDTA, and 250 m*M* NaF.
52. High salt extract (HSE) buffer: 5 *M* NaCl, 10 m*M* $MgCl_2$, 10 m*M* DTT. This is stored at –20°C.
53. Salt wash buffer (SWB): 1X LSB, 0.5 *M* NaCl, 10 m*M* $MgCl_2$, 1 m*M* DTT. This is stored at –20°C.
54. LM-HK buffer: 50 m*M* HEPES, pH 7.5, 50 m*M* KCl. This is stored at –20°C.
55. LM-10XMED: 10 m*M* $MgCl_2$, 0.2 m*M* EDTA, 1 m*M* DTT. This is stored at –20°C.
56. 10 m*M* ATP, pH 7.5, is stored at –70°C.
57. 400 ng/μL tetrameric bacterially expressed p53 is stored at –70°C.
58. HiTrap MonoQ (5/5) column is obtained from Amersham Pharmacia Biotech.
59. Buffer A: 15% glycerol, 25 m*M* HEPES pH 8.0, 0.02% Triton X-100, 5 m*M* DTT, 1 m*M* benzamidine, 50 m*M* NaF.
60. 0.22 μ*M* Amicon® Filters are from Millipore (Bedford, MA, USA).

3. Methods

3.1. Immunization with Phosphopeptides and Antibody Generation

For the generation of p53 BOX-1 phosphospecific polyclonal sera, synthetic phosphopeptides based on the p53 *BOX-1* sequence (^{13}PLSQETFSDLWK LLP^{27}), with single phosphates at Thr^{18} or Ser^{20}, were C-terminally coupled to the hapten KLH and used to immunize rabbits using standard techniques ***(21)***. Panels of hybridomas were generated against similar p53 *BOX-1* phospho-

Table 1
N Terminally Biotinylated Synthetic Peptides

BOX-1 peptide	Sequence
Nonphos.	Biotin - SGSG - ^{10}VEPPLSQETFSDLWKLL^{26}
Ser^{15}-P	Biotin - SGSG - ^{13}PLS(PO$_4$)QETFSDLWKLLP^{27}
Thr^{18}-P	Biotin - SGSG - ^{13}PLSQET(PO$_4$)FSDLWKLLP^{27}
Ser^{20}-P	Biotin - SGSG - ^{13}PLSQETFS(PO$_4$)DLWKLLP^{27}

peptide antigens containing single phosphates at Ser^{15}, Thr^{18}, or Ser^{20}, using conventional technology ***(21,22)***.

1. Incubate 0.5 mL of peptide and 0.5 mL of KLH with glutaraldehyde at a final concentration of 0.1%, and cross-link at 37°C for 1 h.
2. Neutralize the glutaraldehyde by adding 0.1 mL of 1.5 *M* Tris-HCl, pH 8.0.
3. Inject the peptide–KLH conjugate antigen into mice (50 µg) for MAb generation or into rabbits (500 µg) for polyclonal antibody generation as described ***(22)*** (*see* **Note 3**).
4. Purify the MAbs from hybridoma serum supernatant or from ascites tumors using protein G beads (manufacturer's protocol), and dialyze the purified IgG against PBS to stabilize the MAbs for storage at –20°C.
5. Purify the IgG in the rabbit serum (25 mL) by applying the serum (containing 50 m*M* NaF phosphatase inhibitor) to a phosphopeptide column equilibrated in PBS (0.1 mg of peptide coupled to 0.1 mL of Reacti-Gel (6X) resin, according to manufacturer's suggestions). Wash the column with PBS, elute the IgG bound to the phosphopeptide using 0.5 mL of antibody elution buffer, and dialyze the purified IgG against PBS to stabilize the affinity purified polyclonal antibody for storage at –20°C.

3.2. Use of ELISA to Characterize p53 BOX-1 Phospho-Specific Antibodies

Streptavidin-capture peptide–ELISA was used to determine the specificity of the p53 BOX-1 phospho-specific polyclonal sera and monoclonal antibodies for a panel of N terminally biotinylated synthetic peptides (**Table 1**).

1. Coat microtiter wells with 5 µg/mL streptavidin in 50 µL H_2O, and incubate at 37°C overnight.
2. Block nonspecific binding sites on streptavidin using 200 µL/well 3BPBST.
3. Add 25 ng/well biotinylated p53 peptides in 50 µL 5MPBST/βGP/OA, and incubate for 1 h (*see* **Note 4**).
4. For phospho-Ser^{15} MAbs, KLH-coupled p53 peptides were directly coated onto microtiter wells at 2 µg/mL in ELISA coating buffer and incubated at 4°C overnight.

5. Wash wells with 3 × 200 μL PBS-T, then block nonspecific binding to the peptide by adding 200 μL/well 5MPBST/βGP/OA and incubate for 1 h.
6. Dilute primary antibodies in 5MPBST/βGP and add 50 μL/well and incubate for 1 h.
7. Antibody binding was detected by incubating with 1/1000 dilution either anti-mouse IgG-HRP (for MAbs) or anti-rabbit IgG-HRP (for polyclonal serum) in 5MPBST/βGP, which permits luminometric detection using 50 μL/well ECL. For colorimetric detection, develop using 50 μL/well TMB complete substrate. The color reaction is stopped by the addition of 50 μL/well 0.5 *M* H_2SO_4, and read at $O.D._{450\ nm}$ using a microplate reader.

All incubations following the coating step are for 1 h at room temperature in a humidified container, and wells are washed between steps with 3 × 200 μL PBST.

3.2.1. Characterization of p53 BOX-1 Phospho-Specific Polyclonal Sera: An Example of the IgG Complexity in Polyclonal Serum and the Relative Importance of Developing MAbs to Phosphorylation Sites

Binding of IgG from crude αphospho-Thr^{18} antiserum to nonphosphorylated peptide and also to peptides with phosphate substitutions at either positions Thr^{18} or Ser^{20} was examined (**Fig. 2**; dark bars). Removal of phospho-insensitive antibodies from crude serum by absorption with nonphosphorylated *BOX-1* peptide resulted in a reduced affinity of the polyclonal serum for the nonphosphorylated sequence, but maintained binding to both phosphopeptides (**Fig. 2**; white bars). Purification of anti-phospho-Ser^{20} antisera by absorption with nonphosphorylated peptide was also insufficient to achieve complete phospho-specificity (data not shown). Thus, the purified anti-phospho-Thr^{18} IgG (**Fig. 2**) did not display strict phospho-specific epitope binding, and if this IgG were used to study the level of "Thr^{18} phosphorylation" of p53, then misleading results would be obtained. These data indicate that the immune response to phosphopeptide antigens involves the generation of a mixture of B cell clones with either phospho-insensitive epitopes or with phosphate-dominant recognition determinants, as well as those with specific reactivity towards the challenging antigen (*see* **Note 1**). Due to these difficulties in working with polyclonal mixtures and further unreliability in acquiring reproducible preparations in different rabbits, we sought to generate phospho-specific MAbs to produce a permanent resource.

3.2.2. Examples of Specificity of p53 BOX-1 Phospho-Specific MAbs

MAbs have an obvious advantage over polyclonal antisera for phospho-specific reagent generation, as single clones can be selected that have the required specificity. Selected clones were characterized for their specificity for site-

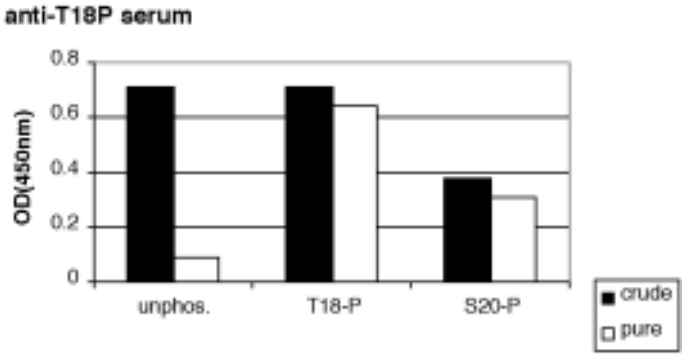


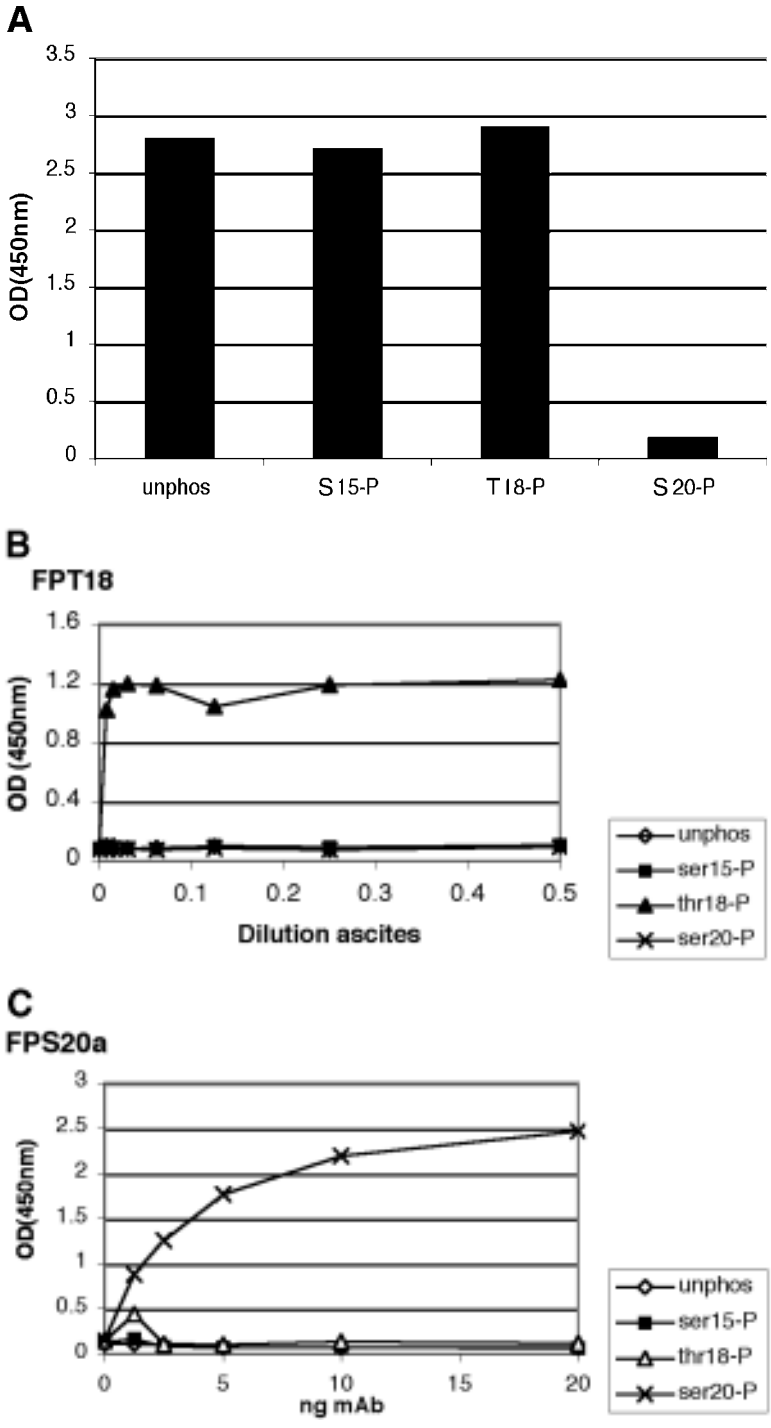
Fig. 2. Characterization of polyclonal anti-sera raised against p53 *BOX-1* phosphopeptides. Specificities of crude (shaded bars) and purified (white bars) α-phospho-Thr[18] polyclonal anti-sera for synthetic p53 *BOX-1* N terminally biotinylated peptides (unphos, nonphosphorylated; T18-P, phospho-Thr[18]; and S20-P, phospho-Ser[20]) determined by peptide–ELISA.

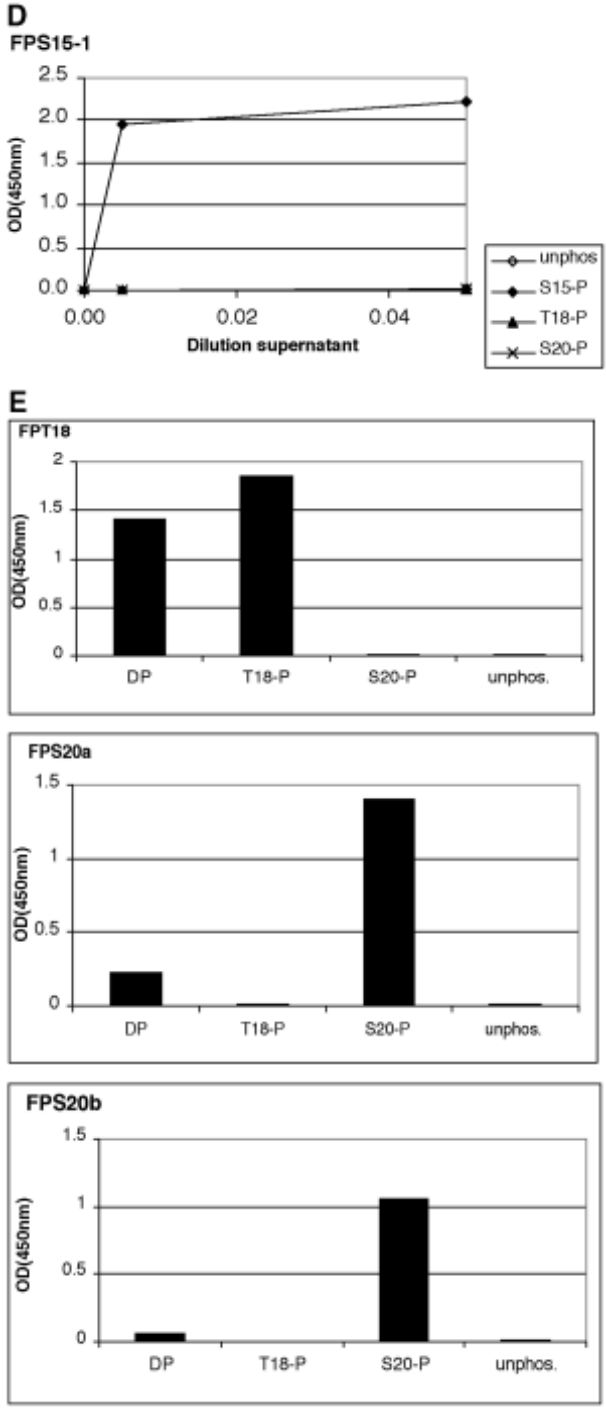
specific phosphorylation using a panel of p53-derived BOX-1 N-terminally biotinylated peptides (**Table 1**). The phospho-Ser[20]-sensitive MAb DO-1 was included as a control (**Fig. 3A**).

3.2.2.1. Differential Specificity of Phospho-Thr[18] MAbs

Four clones generated were selected by dot-blot from a panel of hybridomas generated against the phospho-Thr[18] peptide, and the specific binding of secreted antibodies to synthetic *BOX-1* peptides was assessed using the peptide-ELISA (*see* **Table 2**). Two of the clones (FPT18 3.1 and FPT18 6D7) were insensitive to phosphate substitution (data not shown) and bound equally well to all four BOX-1 peptides. A third clone had a similar specificity to MAb DO-1, binding efficiently to all peptides except phospho-Ser[20]. However, the fourth clone (FPT18) was specific for phosphate at Thr[18] (**Fig. 3B**). This antibody has been most useful for detecting Thr[18] phosphorylation of p53 in clinical samples ***(11)*** and for in vitro kinase assays (data not shown).

Fig. 3. Characterization of p53 *BOX-1* phospho-specific MAbs. The specificities of the indicated MAbs to the indicated phosphopeptides were determined by ELISA: (**A**) DO-1; (**B**) FPT18; (**C**) FPS20a; and (**D**) FPS15-1. (**E**) Phospho-specific MAbs were examined for tolerance to adjacent phosphorylation. The effect of dual-site Thr[18]/Ser[20] phosphate on the binding of MAbs FPT18 (top), FPS20a (middle), and FPS20b (bottom) to synthetic p53 BOX-1 N terminally biotinylated peptides (DP, diphospho-Thr[18]/Ser[20]; T18-P, phospho-Thr[18]; and S20-P, phospho-Ser[20]; unphos, nonphosphorylated) was determined using peptide-ELISA.


A
3.5
3
2.5
2
1.5
1
0.5
0
OD(450nm)
unphos
S15-P
T18-P
S20-P
B
FPT18
1.6
1.2
0.8
0.4
0
OD (450nm)
0
0.1
0.2
0.3
0.4
0.5
Dilution ascites
unphos
ser15-P
thr18-P
ser20-P
C
FPS20a
3
2.5
2
1.5
1
0.5
0
OD(450nm)
0
5
10
15
20
ng mAb
unphos
ser15-P
thr18-P
ser20-P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FPS15-1
OD(450nm)
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2.0
1.5
1.0
0.5
0.0
0.00
0.02
0.04
Dilution supernatant
unphos
S15-P
T18-P
S20-P
E
FPT18
OD(450nm)
2
1.5
1
0.5
0
DP
T18-P
S20-P
unphos.
FPS20a
OD(450nm)
1.5
1
0.5
0
DP
T18-P
S20-P
unphos.
FPS20b
OD(450nm)
1.5
1
0.5
0
DP
T18-P
S20-P
unphos.

Table 2
Specific Binding of MAbs ot BOX-1 Peptides

MAb	Nonphos	Ser^{15}-P	Thr^{18}-P	Ser^{20}-P	Specificity
FPT18	—	—	***	—	T18-P specific
PT18 3.1	***	***	***	***	Nonspecific
PT18 C12	***	***	***	—	DO1-like
PT18 6D7	***	***	***	***	Nonspecific

***, strong binding to the peptide.
—, no binding.

3.2.2.2. Specificity of Phospho-Ser^{20} MAbs

The specific binding of two clones (FPS20a and FPS20b), selected from a panel of hybridomas generated towards the phospho-Ser^{20}, to synthetic *BOX-1* peptides was assessed. Both clones had identical specificities for the phospho-Ser^{20} peptide and did not bind to phospho-Ser^{15}, phospho-Thr^{18}, or unphosphorylated peptides (one clone shown in **Fig. 3C**). These IgGs have a very low affinity for Ser^{20}-phosphorylated p53 protein and have not been useful for immunoblotting p53 from clinical material or cell lines. However, these IgG are useful for in vitro kinase assays in a cell-free system (*see* **Subheading 3.9.**). For immunoblotting, DO-1 epitope masking as a probe for Ser^{20} phosphorylation has been developed ***(16)***.

3.2.2.3. Specificity of Phospho-Ser^{15} MAbs

Following the primary screening of hybridomas generated towards the phospho-Ser^{15} peptide, four clones were selected. However, despite MAb recognition of the C-terminally KLH-coupled phospho-Ser^{15} peptide, there was no detectable binding to any of the N-terminally biotinylated *BOX-1* peptides (data not shown). The proximity of the N-terminally coupled biotin moiety sterically occluded antibody access to the phospho-Ser^{15} site. Therefore, to characterize these antibodies, a panel of free *BOX-1* peptides (i.e., with no biotinylation) was coupled to BSA. Following direct capture of the BSA-coupled peptides to ELISA wells, phospho-Ser^{15} MAb binding could be measured. Using this approach, three MAbs (one clone is shown; FPS15-1) out of four selected clones exhibited specific binding to only the phospho-Ser^{15} peptide (**Fig. 3D**).

3.3. Fine Mapping of Phospho-Specific Epitopes Using More Sophisticated ELISA Techniques

The p53 *BOX-1* domain contains three phospho-acceptor residues within a six-amino acid sequence and, therefore, has the potential to be phosphorylated at multiple sites in vivo. In support of this, Chk2 has been shown to phospho-

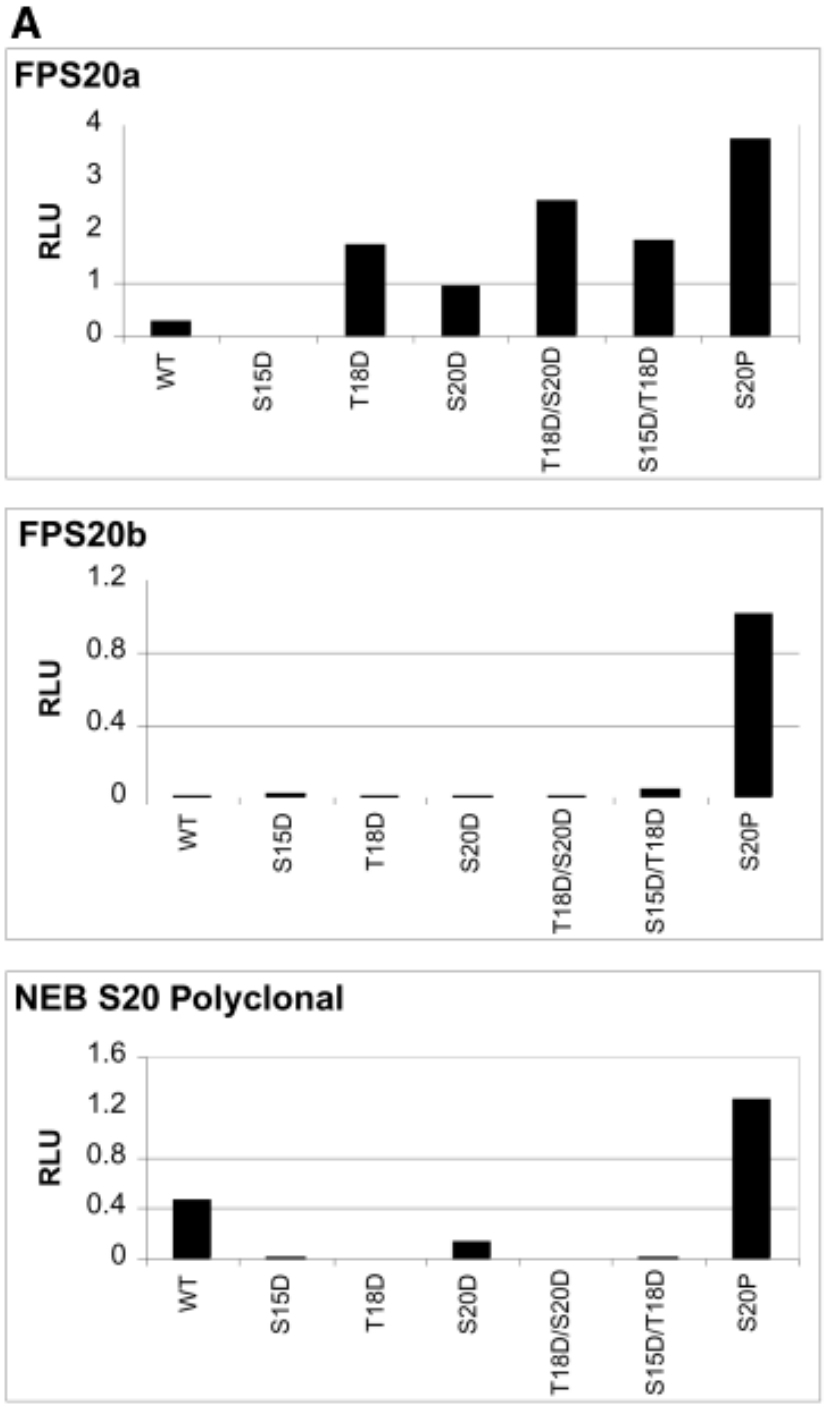
rylate both Ser^{15} and Ser^{20} residues in vitro ***(23)***. Firstly, we tested the possibility that dual site phosphorylation could preclude binding of the phospho-Thr^{18} and phospho-Ser^{20} reagents using a doubly phosphorylated phospho-Thr^{18}/Ser^{20} peptide. Secondly, the relative contribution of charge and sequence context towards the recognition elements of individual MAbs was defined using panels of aspartate-substituted or alanine-scanned peptides. We have used these analyses to define the epitopes of two FPS20 MAbs ***(11)*** and the only commercial phospho-p53 (Ser^{20}) polyclonal antibody available ***(23)*** and to rationalize the observed restricted applicability of these reagents.

3.3.1. Examples of Steric Hindrance by Adjacent Phosphate

Peptide-ELISA data showed that an adjacent peptide at Ser^{20} caused only a slight reduction in FPT18 binding compared to a single phosphate at Thr^{18} (**Fig. 3E, top**). However, double-site Thr^{18}/Ser^{20} phosphorylation severely impaired the ability of both FPS20a and FPS20b to recognize their epitopes. In the presence of an additional phosphate at Thr^{18}, FPS20a binding to phospho-Ser^{20} was reduced by 82% (**Fig. 3E, middle**), and FPS20b binding was almost completely blocked (**Fig. 3E, bottom**). These data indicate that lack of reactivity of phospho-specific IgG to phospho-Ser^{20} site can be explained by either dephosphorylation of p53 at Ser^{20}, or a double-site phosphorylation at Thr^{18}/Ser^{20}. Apparent increases in Ser^{20} phosphorylation observed with the Ser^{20} phospho-specific IgG may actually result from dephosphorylation at Thr^{18} and constitutive maintenance of the Ser^{20}-phosphate. As such, studies where phosphorylation changes at Ser^{20} on endogenous p53 may actually reflect changes in Thr^{18} phosphorylation have been published.

3.3.2. Aspartate Cannot Mimic a Phospho-Ser for all Phospho-Ser^{20} Antibodies

The addition of a phosphate moiety to a protein confers a bulky negative charge, which may classically and theoretically be mimicked by an aspartate or glutamate residue. We synthesized a panel of peptides based on the p53 *BOX-1* sequence with single or double aspartate substitutions at the Ser^{15} (S15D), Thr^{18} (T18D), and Ser^{20} (S20D) positions and tested the ability of two FPS20 MAbs and a commercial αphospho-Ser^{20} polyclonal antibody to bind these peptides. The phospho-Ser^{20}-specific MAb FPS20a, surprisingly, bound to the mutant peptides T18D and S20D, but not to the S15D peptide (**Fig. 4A, top**). Enhanced binding to the double T18D/S20D peptide was observed, suggesting that this antibody recognizes a broad negative charge distribution. In contrast, neither MAb FPS20b (**Fig. 4A, middle**) nor commercial phospho-p53 (Ser^{20}) polyclonal antibody (**Fig. 4A, bottom**) could detect any of the mutant peptides, indicating that aspartate could not mimic phospho-Ser for these antibod-


A
FPS20a
RLU
4
3
2
1
0
WT
S15D
T18D
S20D
T18D/S20D
S15D/T18D
S20P
FPS20b
RLU
1.2
0.8
0.4
0
WT
S15D
T18D
S20D
T18D/S20D
S15D/T18D
S20P
NEB S20 Polyclonal
RLU
1.6
1.2
0.8
0.4
0
WT
S15D
T18D
S20D
T18D/S20D
S15D/T18D
S20P

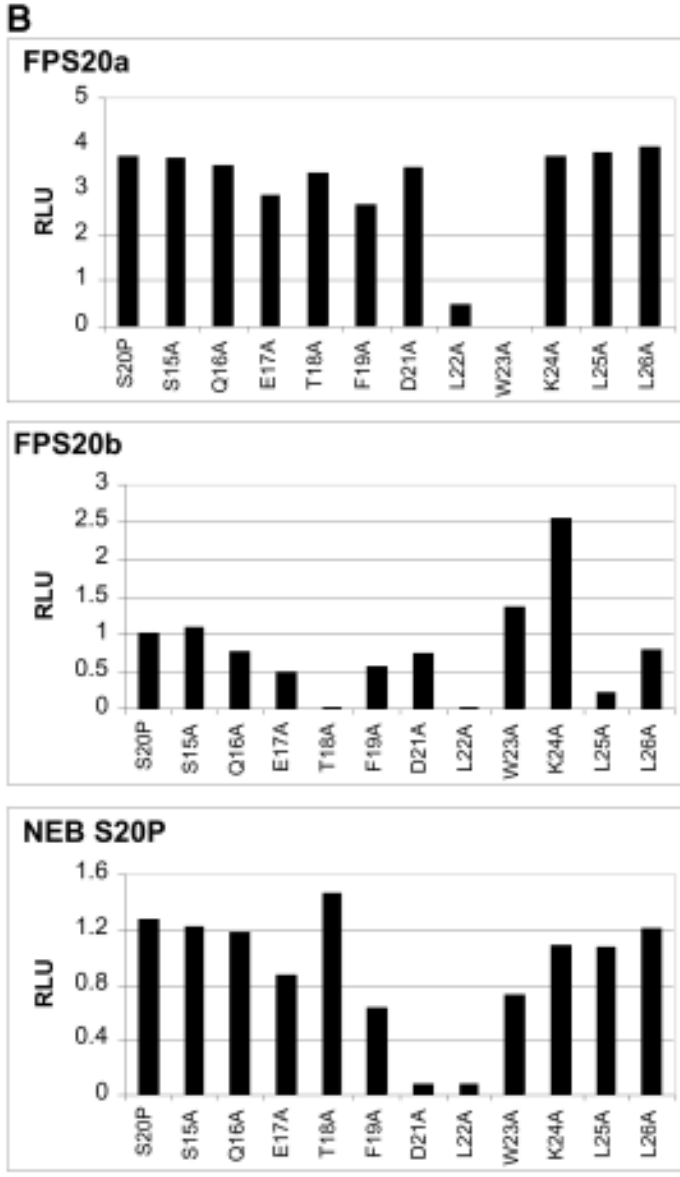


Fig. 4. The effect of mutant residues on the binding of phospho-Ser^{20}-specific antibodies to synthetic p53 *BOX-1* N-terminally biotinylated peptides. (**A**) Aspartate substitution of the *BOX-1* domain using FPS20a (top), FPS20b (middle), and commercial polyclonal anti-phospho-Ser^{20} sera (bottom). (**B**) Alanine substitution of the *BOX-1* domain using FPS20a (top), FPS20b (middle), and commercial polyclonal anti-phospho-Ser^{20} sera (bottom).

ies, possibly due to an insufficient negative charge or shape parameters. However, the phospho-specific Ser^{20} polyclonal antibody displayed some affinity for the nonphosphorylated epitope (**Fig. 4A, bottom**), and its use to detect Ser^{20} phosphorylation is therefore questionable.

3.3.3. Alanine-Scanned Phosphopeptides Can Define MAb Epitopes

The sequence requirements for binding of the three αphospho-Ser^{20} antibodies were determined using a set of alanine-scanned peptides based on the p53 *BOX-1* phospho-Ser^{20} sequence with single alanine substitutions at every residue. Mutation of Lys^{22} or Trp^{23} strikingly reduced MAb FPS20a binding (**Fig. 4B, top**), indicating that the epitope was very short, consisting of amino acids $^{20}S(P)XLW^{23}$. In contrast, mutation of residues Gln^{16}, Glu^{17}, Phe^{19}, Asp^{21}, and Leu^{26} reduced binding moderately, and mutations at Thr^{18}, Leu^{22}, and Leu^{25} severely decreased binding of FPS20b to the phosphopeptide (**Fig. 4B, middle**). The marked requirement of this antibody for Thr^{18} can explain the inhibitory effect of phospho-Thr^{18} found with the double Thr^{18}/Ser^{20} phosphopeptide (**Fig. 4B, bottom**). Also, neutralizing the positively charged Lys^{24} increased binding to this antibody. It is clear that FPS20b has a relatively broad epitope and is sensitive to the size of the negative charge. Finally, the commercially available phospho-p53 (Ser^{20}) polyclonal antibody ***(23)*** appeared to consist of a dominant clone with the narrow epitope $^{20}S(P)\ DL^{22}$ (**Fig. 4B, bottom**). This is consistent with the relative scarcity of specific clones in polyclonal sera (*see* **Subheading 3.2.1.**).

3.4. Use of BIAcore to Characterize p53 BOX-1 Phospho-Specific MAbs

An analysis of the real-time kinetics of antibody–antigen interactions for phospho-specific reagents is possible using surface plasmon resonance (SPR) technology. Briefly, this assay can detect changes in mass caused by transient protein–protein interactions at the surface of a metal chip by measuring changes in the angle of light reflected from the chip. By chemically attaching single peptides to the metal surface, and allowing antibodies to flow over the surface of the chip, transient interactions can be recorded as a shift in angle of reflected light. Thus, SPR allows a sensitive and direct measurement of real-time biospecific interactions, without the need for labeling strategies. For the characterization, synthetic biotinylated peptides were individually captured onto streptavidin chips, and the real-time kinetics of association and dissociation of the phospho-specific antibodies to individual peptides was measured. The data captured for all four peptides was combined in a single graph for each antibody, with the Y-axis representing resonance units (RU) of antibody binding, and the X-axis representing time in seconds.

1. Dock two sensor SA chips into a 2000 upgrade and prime with 200 μL priming buffer using a flow rate of 20 μL/min.
2. Dilute MAbs in HBS to a final concentration of 100 n*M* and inject consecutively over the primed flow cells at 5 μL/min to determine nonspecific binding. Regenerate flow cells between MAbs using 20 μL stripping buffer.
3. Dilute four biotinylated p53 BOX-1 peptides to 500 ng/mL in HBS and inject 50 μL/flow cell using a flow rate of 20 μL/min. This procedure binds the peptides individually to the streptavidin surface of each of four flow cells on the two sensor SA chips, resulting in a baseline shift of approx 500 resonance units (RUs) (1000 RU ≅ 1 ng).
4. Pass MAbs consecutively over each flow cell as before, regenerating the flow cells between antibodies using stripping buffer. Analyze individual peptide binding data using the dedicated software package (Biacore) to generate composite graphs for each MAb.

As an example, the kinetics of the Ser^{20} phospho-specific reagents were subject to analysis. For FPS20a, high levels of antibody bound the phospho-Ser^{20} peptide, and there was no dissociation within 350 s (**Fig. 5A**). However, some significant binding to both phospho-Ser^{15} and phospho-Thr^{18} peptides did occur, although the off-rates were rapid. No binding to the nonphosphorylated sequence was detected. The FPS20b MAb reagent bound only to the phospho-Ser^{20} peptide, and there was no dissociation within 350 s (**Fig. 5B**). In this case, no binding to the remaining three peptides occurred. In general, therefore, N-terminal phospho-specific MAbs can be characterized for specificity and affinity using peptides coupled to a solid phase which places significant confidence in their use as reagents to study signaling to p53.

3.5. Phage–Peptide Display to Define MAb Epitopes

In contrast to the N-terminal antibodies, the C-terminal MAbs towards Ser^{315} and Ser^{392} were characterized by phage display experiments in order to provide an alternate example of how to characterize the specificity of phospho-specific MAbs. Two phospho-antibodies specific for phospho-Ser^{315} (FPS315) or phospho-Ser^{392} (FPS392) were raised against either the KLH-conjugated phosphopeptide $NNTSSS^{PO4}PQPKKKPLDG^{325}$ (corresponding to amino acids containing the CDK site on human p53) or phosphopeptide $^{376}STSRJKKLMFKTEGPDS^{PO4}\,D^{393}$ (corresponding to amino acids containing the CK2 site on human p53), respectively, and their use in a Cdk2/Cdc2 kinase or CK2 kinase assay has been reported ***(24)***. Purification of the MAb from hybridoma tissue culture supernatants using Protein-G sepharose (**Subheading 3.1.**) was required to obtain pure IgG devoid of contaminating phosphatases found in serum supernatant, and this highly purified IgG was used in the in vitro assays described below. Phage–peptide display libraries were used to define the essential residues within the FPS315 and FPS392 epitopes.

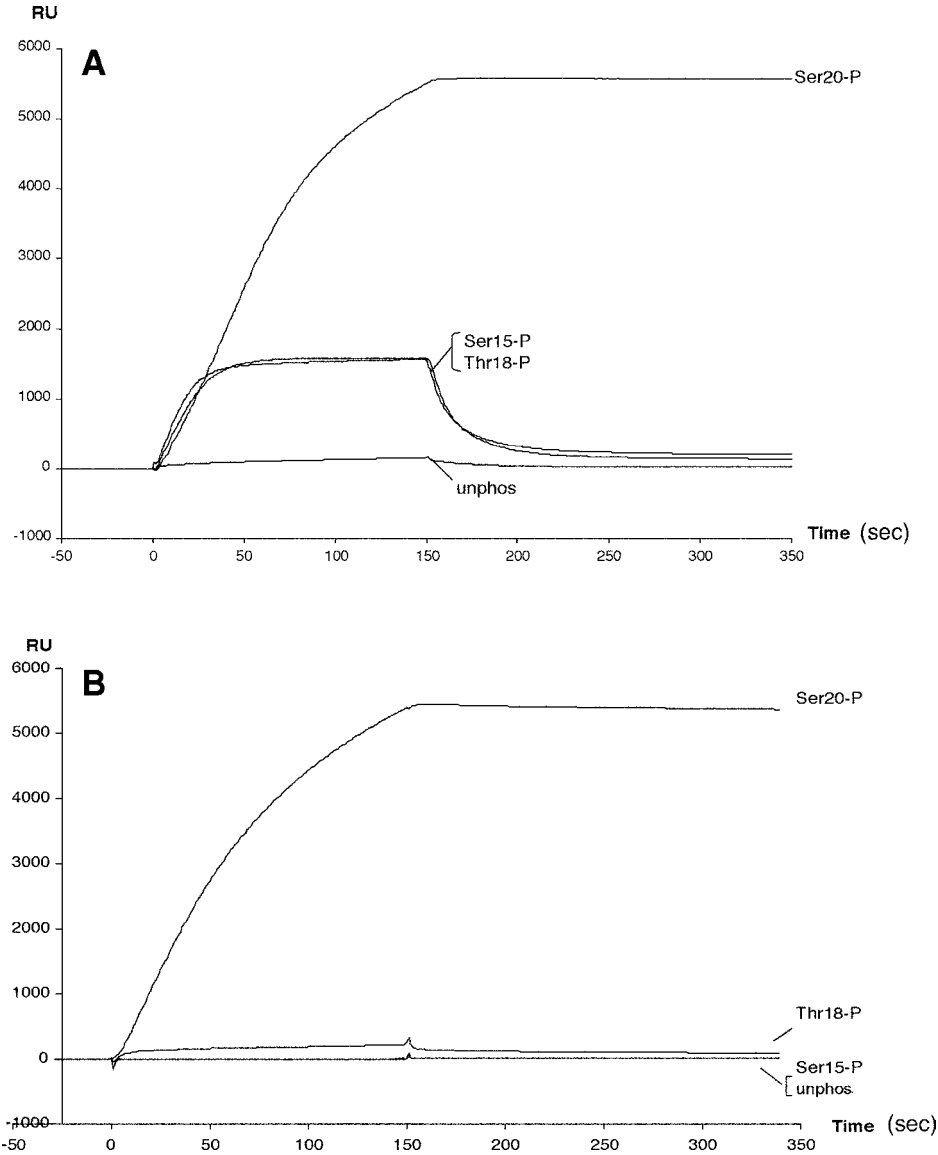


Fig. 5. Determination of real-time kinetics of peptide–antibody interactions using surface plasmon resonance. For the measurement of peptide–MAb interactions, nonphosphorylated or singly phosphorylated synthetic N terminally biotinylated p53 *BOX-1* peptides (unphos, nonphosphorylated; S15-P, phospho-Ser[15]; T18-P, phospho-Thr[18]; and S20-P, phospho-Ser[20]) were attached to individual streptavidin chips, and MAbs were passed over the chip surfaces. Binding data for two different phospho-Ser[20]-specific clones is shown: one has a high on-rate for any phospho-peptide (**A**) and a second has complete phospho-Ser[20] specificity (**B**).

1. Coat protein G affinity-purified MAbs onto microtitre wells at a concentration of 5 μg/mL in 50 μL of ELISA coating buffer, and incubate at 37°C overnight.
2. Block nonspecific binding sites on MAbs with 200 μL/well 3BPBST.
3. Select bacteriophage from libraries containing random peptides inserted within the phage III coat protein according to the manufacturer's protocol. Two separate libraries were screened: PhD12 contains random linear 12-mer peptides; C7C contains 7-mer cyclic peptides, the structure of which is constrained by a disulfide bridge between two cysteine residues at either end of the insert peptide. After two cycles of selection and amplification, individual phage clones were screened for antibody binding by ELISA, and the inserts of strongly positive clones were sequenced.

3.5.1. Phage–Peptide Display of FPS315

Since the peptide library was not phosphorylated prior to its use, it was not obvious that phage–peptide display would select peptides representing an epitope for a phospho-specific antibody, which has an absolute requirement for the phosphate. However, positive peptide–phage clones were selected by two rounds of biopanning that bound to the MAb FPS315, and all contained an invariant PQP motif corresponding to $Pro^{316}Gln^{317}Pro^{318}$ on human p53 of $^{313}SSSPQPKKK^{321}$ ***(6)***. However, differences were observed in amino acids flanking the PQP motif, depending upon the combinatorial library utilized. The linear 12-mer peptide library gave rise to a predominant set of clones with a stabilizing glutamate (negatively charged phosphate mimetic) at the Ser^{315} position and two other clones with a glycine or serine at the Ser^{315} position (data not shown). In contrast, the cyclic 7-mer peptide library selected clones without the expected stabilizing aspartate at the Ser^{315} position, but yielded a stabilizing lysine at position 320, which was not observed using the linear combinatorial library. Although the differences in the affinity between each of these peptide–epitope clones are not known, these experiments demonstrate that an important recognition feature of the MAb FPS315 involves its specificity for amino acids flanking the Ser^{315} phosphorylation site of p53 and that this antibody is therefore not just detecting a phospho-serine moiety. This antibody can be used uniquely to measure the stoichiometry of phosphorylation on the p53 tetramer (*see* **Subheading 3.6.1.**).

3.5.2. Phage–Peptide Display of FPS392

Phage-peptide display was also used to define the FPS392 consensus epitope. ELISA wells were coated with purified FPS392 and used to select bacteriophage from libraries containing random peptides inserted within the phage III coat protein. After two cycles of amplification, antibody-specific peptide–phage were identified by ELISA, and the inserts were sequenced. Although two dif-

ferent libraries were screened (a cyclic 7-mer and a linear 12-mer), only the 12-mer peptide library selected peptide–phage populations, which expressed epitopes with detectable affinity for FPS392 ***(17)***. A general peptide consensus of Phe-His-x-x-Trp-Pro was observed, which exhibited homology to the C-terminal region of human p53 protein that was used as the original antigen (^{385}FKTEGPDSD^{393}; Phe^{385}-x-x-x-x-Pro^{390}). FPS392 can also be used to study PKR/CK2-site signaling to the CK2/PKR site of p53 protein in species other than human, suggesting that the conserved amino acids F-x-x-x-GPDS^{PO3}D are critical in the recognition of denatured antigen by this MAb.

3.6. Electrophoretic Mobility Shift Assay (EMSA)

p53 protein is tetrameric, thus raising the relatively unique issue of determining the number of monomers modified by a particular enzyme. Most immunoblotting data do not address the stoichiometry of p53 modification in vivo, which is an important milestone in determining the significance of an enzyme pathway interacting with p53 in cells. p53 protein bound to DNA can be supershifted by an MAb, such as DO-1, to produce four discrete IgG:p53:DNA complexes that represent integers of the number of IgG bound per monomer ***(25)***. Of all the phospho-specific MAbs described in this review, only FPS315 (recognizing the phospho-CDK site) can be used to determine the stoichiometry of modification ***(6)***.

1. Prepare polygrip consensus p53 dsDNA radiolabeled probe. Add 2 μg of each oligomers 5381 and 5382 to 0.7 μL of 10X Klenow buffer (Promega, Madison, WI, USA), 5 μL ^{32}P-γ-ATP and 0.5 μL T4 kinase (Promega). Make up the vol to 10 μL with H_2O, and incubate at the following temperatures: 37°C for 45 min; 80°C for 3 min; 65°C for 5 min; 37°C for 15 min; then allow to come to room temperature over 20 min. Purify labeled double-stranded (ds)DNA probe using a chroma-spin-10 column and adjust the final concentration to 10 ng/μL in TE (*see* **Note 5**).
2. Preparation of p53 samples. CyclinB/CDK2-phosphorylated bacterially expressed recombinant p53 (*see* **Fig. 6A**) and endogenous p53 from UV-C irradiated tumor cells (*see* **Fig. 6C**) were analyzed for Ser^{315} phosphorylation ***(6)*** (*see* **Note 6**).
3. DNA-p53 binding reaction: incubate 10 μL DNA-binding (DB) buffer, 2 ng ^{32}P DNA probe, 100 ng sheared salmon sperm competitor DNA, 1 μL FPS315, and 1–5 μL kinase reaction products (*see* **Note 7**) or 5 μg nuclear lysate on ice for 15 min.
4. Load 15 μL samples onto a 4% polyacrylamide nondenaturing gel and electrophorese at 200 V for 2 h at an ambient temperature of 4°C, using prechilled apparatus and running non-denaturing gel buffer.
5. Dry gel at 80°C for 1 h, and expose to photographic film at –70°C for autoradiography.

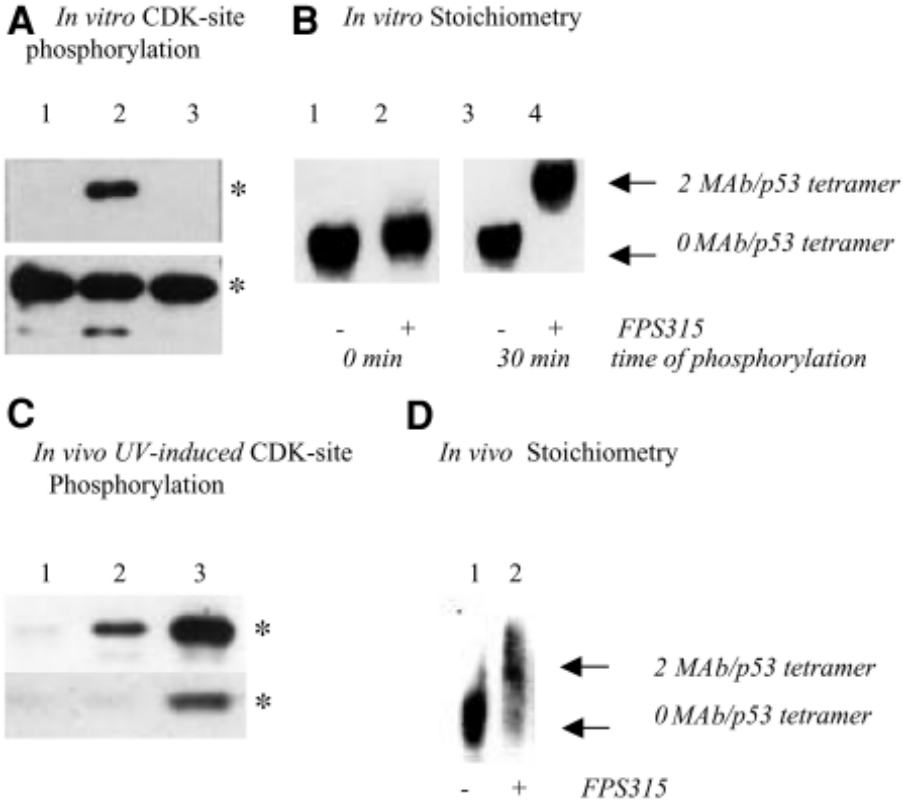


Fig. 6. In vivo Ser^{315} phosphorylation detected using the FPS315 MAb. p53 phosphorylation was analyzed by: (**A**) immunoblotting after phosphorylation with cyclin-cdk2 kinase in vitro: lane 1, no kinase; lane 2, cyclinA-cdk2; lane 3, casein kinase 2. The top panel is immunoblotting with FPS315 after immunoprecipitation of total p53, and the bottom panel is the total p53 protein levels. Asterisks mark the position of p53. (**B**) Shifting native p53-DNA complexes in a DNA binding reaction without or with FPS315 added (as indicated) to determine stoichiometry of in vitro cdk2-phosphorylated p53 (lane 1, no kinase; lane 2, with kinase phosphorylation at t = 0; lane 3, no kinase; lane 4, with kinase phosphorylation at t = 30 min). (**C**) Immunoblotting after immunoprecipitation of total p53 protein from crude lysates with FPS315: lane 1, no IgG; lane 2, nonirradiated MCF7 cells; lane 3, irradiated MCF7 cells. The top panel is blotted with DO-1, and the bottom panel is blotted with FPS315. Asterisks mark the position of p53. (**D**) Shifting native p53-DNA complexes in a DNA-binding reaction to determine stoichiometry of in vivo phosphorylated p53 (lane 1, irradiated MCF7 cells without FPS315; and lane 2, irradiated MCF7 cells with FPS315).

3.6.1. Native Gel Electrophoresis Using Phospho-Specific Antibodies Can Define PhosphoStoichiometry of the Tetrameric p53

The phosphorylation of p53 by CDK2 results in the progressive supershifting of the phospho-p53 by the FPS315 IgG until the maximum phosphorylation of the tetramer where no unmodified p53 protein remains (**Fig. 6B**, **lane 4 vs lane 1**). When p53 protein from irradiated MCF7 cells is phosphorylated (**Fig. 6C**), the majority of p53 protein is similarly shifted (**Fig. 6D**), indicating that more than 50% of the tetramers are modified more than twice in vivo after damage ***(6)***.

3.7. Immunoblotting

The utility of immunoblotting with phospho-specific MAbs to demonstrate changes in damaged-induced phosphorylation of p53 or differential phosphorylation in breast cancers is discussed in this section (*see* **Note 8**).

1. For tissue culture cells, prepare extracts from cells seeded in 10-cm diameter petri dishes. Scrape cells into ice-cold PBS, pellet by centrifugation, and snap-freeze in liquid nitrogen. For tumor samples, prepare extracts from small tissue chips taken from snap-frozen tumor samples, stored at –70°C.
2. Add 2 to 3 vol of urea lysis buffer to tissue chips or cell pellets. Tissue chips are lysed using a microtube pestle, and cells are lysed by pipeting. Incubate for 15 min on ice.
3. Clarify the lysates by centrifugation at 11,000*g* for 10 min at 4°C.
4. Determine the protein concentration by Bradford assay, using a BSA titration curve. Boil equivalent amounts (*see* **Note 9**) in Laemmli buffer before loading onto a 10% SDS-polyacrylamide gel.
5. Transfer the protein to nitrocellulose following electrophoresis, and block the membranes in 5MPBST containing 10 m*M* β-GP for 1 h at room temperature (*see* **Note 3**).
6. Probe the blots with the affinity-purified primary antibody diluted 1/1000 in 5MPBST containing 10 m*M* β-GP.
7. Detect the antigen using HRP-conjugated secondary antibody, and visualize using ECL.

3.7.1. Phosphorylation of p53 Derived from Breast Cancers at Ser^{15} and Thr^{18}

The use of polyclonal antibody reagents to measure damage-dependent changes in p53 phosphorylation at Ser^{15} and Ser^{20} has been reported previously ***(26)***. However, the specificity of many of these reagents is unproven,

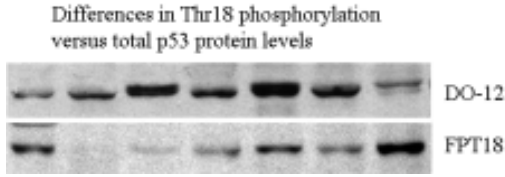


Fig. 7. Thr[18] phosphorylation on p53 in breast cancers in vivo detected using MAb. FPT18 p53 phosphorylation was analyzed by immunoblotting lysates for: p53 protein (top panel, DO-12) or p53 phosphorylation at Thr[18] (bottom panel) FPT18. Many patients displayed different levels of total p53 protein comparative to Thr[18] phosphorylation. Since all the samples have mutant p53 (data not shown), these data suggest there are significant differences in the activity of the Thr[18] kinase in different breast cancers.

and their sensitivity to nearby phosphates has not been published. It is most likely that polyclonal preparations of phospho-specific IgG are not mono-specific, and we have focused on using MAbs in their place. However, this places significant limitation on a comprehensive study of signaling to p53, as it has become evident that each phospho-specific MAb can only be used for a subset of assays that are summarized in this text. The phospho-specific MAbs specific for Ser[15] (data not shown) and Thr[18] (**Fig. 7**) can be used in clinical material to show phosphorylation of stabilized p53 protein at these two sites, suggesting their importance in modulating p53 activity, especially since phosphorylation at these sites can either block MDM2-binding or promote p300 binding to p53.

3.7.2. Phosphorylation of p53 Derived from Breast Cancers at Ser[20]

The phospho-specific Ser[20] MAb and the only commercially available polyclonal antibody both have a very low affinity for Ser[20]-phopshorylated p53 and are best used for in vitro kinase assays (*see* **Subheading 3.9.**). Further, the anti-phospho-Ser[20] polyclonal was originally described in coupled immunoprecipitation–immunoblotting assays. However, since DO-1 antibody is specifically blocked by Ser[20] phosphorylation, this assay is a more reliable measure of Ser[20] phosphorylation via epitope masking when normalized to total p53 using DO-12 (or other antibodies that are not sensitive to posttranslation modification) ***(14,16)***.

3.7.3. Phosphorylation at the CDK2 Site at Ser[315]

Denaturing immunoblots of human recombinant p53 protein demonstrate that FPS315 only recognizes p53 phosphorylated at Ser[315] (**Fig. 6A**, **top panel,**

lane 2), but does not recognize nonphosphorylated p53 (**Fig. 6A**, **top panel, lane 1**) or Ser^{392} phosphorylated p53 (**Fig. 6A**, **top panel, lane 3**) ***(6,24)***. A duplicate immunoblot probed with antibody DO12 shows that p53 protein levels are equal in all three lanes (**Fig. 6A**, **bottom panel**). However, the FPS315 antibody, though specific, has a relatively low affinity for its epitope and cannot always be used to detect endogenous p53 protein phosphorylation from cell lysates by direct immunoblotting.

3.7.4. Phosphorylation at the CK2 Site at Ser^{392}

Immunochemical blotting can be used to demonstrate that FPS392 binds specifically to human p53 protein in denaturing immunoblots only after in vitro Ser^{392} phosphorylation of p53 by CK2 ***(17)***. In addition, lysates from normal cells demonstrate an increase in steady-state phosphorylation at Ser^{392} of endogenous p53 after exposure to UV or ionizing radiation ***(17,18)***. Further, FPS392 can be used in clinical material to define the levels of Ser^{392} site phosphorylation in formalin-fixed sections when p53 protein is denatured (*see* **Subheading 3.8.2.**). As FPS392 is one of the few phospho-specific antibodies we have developed that can be used on either human or mouse p53 (discussed in **Subheading 3.5.2.**), it has been possible to demonstrate an increase in CK2-site phosphorylation of p53, coincident with elevated p53 protein levels, in both the spleen and thymus of animals exposed to whole body ionizing radiation ***(27)***. FPS392 has also been a valuable probe to examine the p53-activation mechanism by combined drug treatment. A375 cells exposed to X-irradiation produce a stabilized p53 protein of low specific activity. The addition of the kinase inhibitor roscovitine to X-irradiated cells can reactivate the p53 response, and this results in hyper-phosphorylation at the Ser^{392} site that is not seen with X-irradiation or drug treatment alone ***(17)***. Thus, recruitment of the CK2 kinase pathway may play an important role in p53 activation after damage.

3.8. Immunohistochemical Applications to Study Kinase Activity In Vivo

1. Dry 4-μm-thick formalin-fixed and paraffin-embedded tissue sections onto glass slides and take down to water, using standard methodology.
2. Block endogenous peroxidase activity by incubating in 1.5% hydrogen peroxide for 10 min. Wash in running tap water for 5 min.
3. Perform antigen retrieval as required using a microwave-based technique.
4. Block slides with 10% normal goat serum containing 2.5% avidin for 5 min.
5. Incubate with primary antibody diluted in PBS containing 2.5% biotin overnight at 4°C in a humidified chamber. Primary antibody dilutions are: 1/50000 for CMI antiserum; 1/100 for DO-1 tissue culture supernatant; 1/2000 for protein

G-purified FP392; 1/200 for anti-phospho-p53 Ser^{15} polyclonal. Wash sections in PBS for 5 min.

6. For antigen detection, use a biotin–streptavidin immunoperoxidase kit and DAB in 0.03% hydrogen peroxide as a chromogenic agent. Incubate slides with 1/66 dilution anti-mouse IgG-biotin (for MAbs) or anti-rabbit IgG-biotin (for polyclonal serum) antibody in PBS for 30 min at room temperature. Wash in PBS for 5 min.
7. Incubate with ABC solution for 30 min at room temperature. Wash in PBS for 5 min.
8. Incubate with DAB for 5 min. Wash in PBS for 5 min.
9. Counterstain slides with Mayer's hematoxylin. Wash briefly in water and dehydrate through graded alcohols before clearing with Histoclear. Mount slides using DPX mountant.

Controls: sections known to stain positively in each batch replace the primary antibody with PBS for negative controls.

3.8.1. Ser^{386} (CK2 Site) Phosphorylation on Murine p53 Increases after X-Irradiation In Vivo

Although original studies have shown that ionizing radiation does not induce phosphorylation of p53 at the CK2 site ***(28,29)*** using polyclonal antibodies generated to the phospho-CK2 epitope (Ser^{392} on human p53 or Ser^{386} on murine p53) ***(28,29)***, the CK2 site has been shown to be targeted by ionizing radiation in cell lines and in murine tissue using the phospho-Ser^{392}-specific MAb characterized above ***(17,27)***. Methods have been developed for demonstrating strain differences in the activation of the CK2 pathway in vivo providing a model to measure how the CK2 pathway is regulated ***(27)***. However, the MAbs are not very useful for staining murine tissue, since a very high background is often obtained due to cross-reactivity of mouse blood in tissue with the second antibody to murine IgG. Also, direct cross-linking of the MAbs to peroxidase to counteract this problem usually inactivates the IgG (data not shown).

3.8.2. Differential Ser^{15} and Ser^{392} Phosphorylation in Clinical Material

For cell and tissue staining, the strict requirement for high specificity and high affinity of the IgG generally rules out the use of this first generation of phospho-specific MAbs reported in this review for staining clinical material. Two notable exceptions include the commercially available polyclonal antibody to phospho-Ser^{15} and MAb FPS392 (**Fig. 8**). UV-damaged human skin can show induction of p53 protein using the polyclonal antibody CM1 or the MAb DO-1, as well as phosphorylation of p53 at Ser^{15} or Ser^{392} (**Fig. 8A–D**).

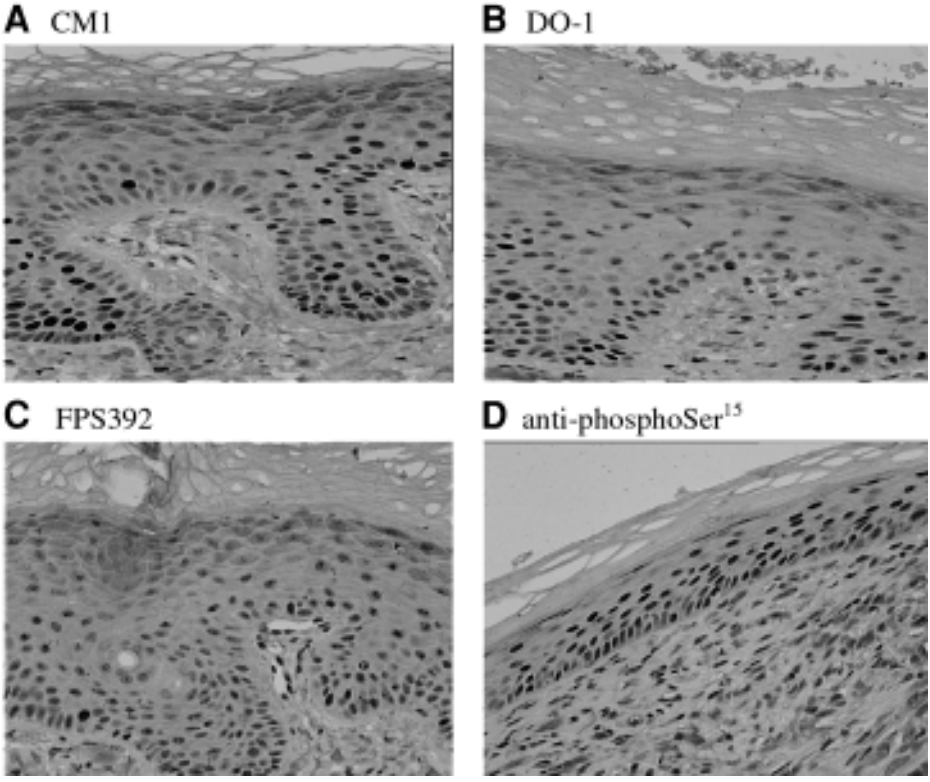


Fig. 8. IHC of p53 phosphorylation on UV-treated human skin. UV-irradiated skin archive was processed for IHC, and p53 levels or phosphorylation were examined using: (**A**) CM1; (**B**) DO-1; (**C**) FPS392; and (**D**) anti-phospho-Ser^{15} polyclonal antisera.

Interestingly, the Ser^{15} phosphorylation shows cytoplasmic staining (**Fig. 8D**), consistent with the cytoplasmic localization of ATM family members. However, the drawback of immunohistochemistry (IHC) is that the stoichiometry is unclear, and the possibility that staining comes from cross-reaction cannot be ruled out.

3.9. Detection of Site-Specific Kinase Activity In Vitro

Despite problems in the detection of endogenous modified p53, our phospho-specific MAb reagents have proven exceptional in enabling the development of immunochemical assays for studying the regulation of the physiological

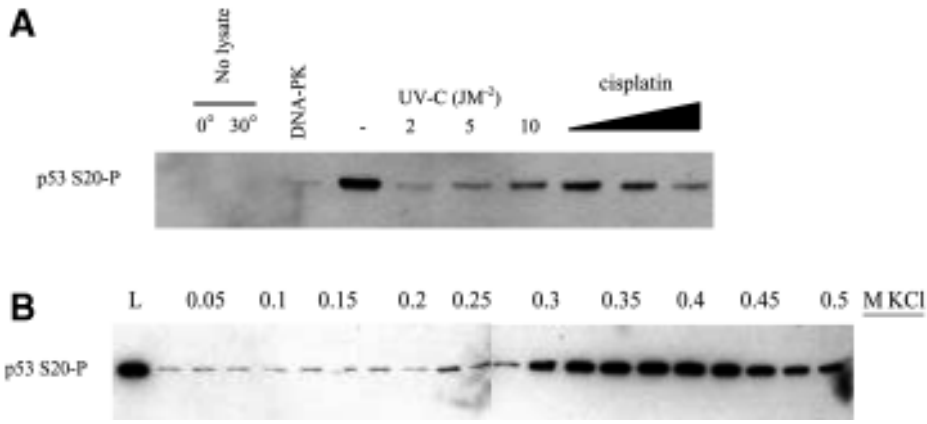


Fig. 9. In vitro kinase assays. (**A**) Full-length purified p53 was incubated either alone at the 0°C (lane 1) or at 30°C in the absence (lane 2) or presence of 50 U of purified DNA-PK (lane 3), or with 10 μg A375 HSE prepared from untreated cells (lane 4) or from cells treated with 2 J/m^2 (lane 5), 5 J/m^2 (lane 6), or 10 J/m^2 UV-C (lane 7), 33 μL dimethylformamide (DMF) (lane 8), or cisplatin at 5 μg/mL (lane 9), or 25 μg/mL (lane 10). Ser^{20} site-specific phosphorylation of p53 was detected by immunoblotting of reaction products with FPS20. (**B**) Lysates from A375 cells were fractionated on an anion exchange column using a 0–0.5 *M* KCl gradient. Fractions containing p53 Ser^{20} site-specific kinase activity were detected by immunoblotting reaction products with FPS20. The peak of Ser^{20} kinase activity eluted at 0.35–0.40 *M* KCl.

kinases that target p53 *BOX-1* sites in vivo. Kinases from lysates were incubated with p53 protein substrate in the presence of ATP, and site-specific phosphorylation is detected by immunoblotting. Immunochemical assays have a clear advantage over standard radioactive kinase assays, which rely on the incorporation of ^{32}P-orthophosphate into a peptide substrate, usually mutated to contain only one phospho-acceptor to measure specific kinase activity. Non-radioactive assays are relatively unaffected by the presence of contaminating kinases, enabling the activities of several different kinases targeting distinct sites within the p53 molecule to be assayed from a single crude extract preparation. Additionally, the use of a native wild-type p53 substrate facilitates the development of assays for kinases (e.g., the Ser^{20} kinase assays described in the following example) that cannot phosphorylate a peptide containing the target site due to the necessity for other distal docking-site sequences, as has been reported for cyclinA-CDK2 on p53 ***(24)***. These small molecules permit the development of specific kinase inhibitor as a first step in drug development.

The site-specific in vitro kinase assay is based on the method of Achari and Lees-Miller ***(30)***.

1. Treat cells as required and harvest according to **Subheading 3.7.1.** Add 100 µL LSE buffer to each frozen cell pellet and lyse cells by pipeting. Incubate on ice for 5 min and snap-freeze in liquid nitrogen. After at least 15 min, thaw frozen extracts on ice, and add 11 µL HSE buffer per 100 µL cell extract. Incubate on ice for 5 min.
2. Spin these high salt extracts (HSEs) at 10,000*g* for 3 min at 4°C, and remove the supernatant. Add 50 µL SWB to the pellet, mix, and centrifuge as above. Add the second supernatant to the first, snap-freeze, and store at –70°C in 20-µL aliquots.
3. Add 100 ng bacterially expressed p53 tetramers to 10 µL HK, 2 µL 10X MED, 100 µ*M* ATP, and either 2 µg A375 HSE or 1 µL A375 Q-Sepharose fraction in a final vol of 20 µL (*see* **Notes 2** and **10**).
4. Incubate reactions at 30°C for 30 min, then stop by the adding 30 µL SDS-SB. Resolve 10-µL samples (containing 20 ng p53) by 10% SDS-PAGE, and immunoblot with the phospho-specific antibodies to determine site-specific phosphorylation. p53 protein levels are normalized by reprobing blots with MAb DO-12.

3.9.1. Phospho-Specific MAbs Can Be Used to Assay Site-Specific Kinase Activity in Crude Extracts

HSEs prepared from a melanoma cell line treated with low dose UV-C non-ionizing radiation or the DNA cross-linker cisplatin were assayed for Ser^{20} kinase activity (**Fig. 9A**). Low dose UV-C (2 J/m^2) inhibits S20K activity (measured using FPS20a reactivity following immunoblotting of the kinase reaction products), and this effect is reduced with increasing doses of UV-C (**Fig. 9A, lanes 5–7**). Similarly, high dose cisplatin inhibits S20K activity (**Fig. 9A, lanes 8–10**). The specificity of FPS20a on p53 protein (as opposed to p53 BOX-1 peptides, as described in **Subheading 3.2.3.**) is demonstrated by its inability to detect DNA-PK-treated p53 (**Fig. 9A, lane 3**) that targets residues Ser^{15} and Ser^{37}.

3.9.2 Purification of Site-Specific Kinase Using Phospho-Specific MAbs

It is possible to use this methodology to purify physiological p53 kinases by column chromatography. Four milligrams of undamaged A375 HSE was filtered (0.22 µ*M*) and fractionated on a MonoQ anion exchange column using a 0–0.5 *M* KCl gradient in Buffer A. The site-specific kinase assay was used to show elution of the major S20K activity in (**Fig. 9B**) from 0.3–0.5 *M* KCl. In this way, column chromatography can be used to purify site-specific p53 kinases to sufficient homogeneity to allow their identification by mass spectroscopy.

4. Notes

1. It is important to point out that several studies have used polyclonal phospho-specific sera that are uncharacterized with respect to specificity, and therefore, conclusions based on the use of these reagents may be unreliable.

2. The general phosphatase inhibitors, NaF and βGP, block phosphatase function by competing for binding with phosphoprotein substrates to the phosphatase enzyme, i.e., they are competitive inhibitors. Although they are interchangeable in most assays, NaF may also be toxic to some enzymes. For this reason, βGP is the preferred inhibitor used in kinase assays. Okadaic acid is a marine toxin produced by dinoflagellates and is a potent inhibitor of types 1 and 2A protein phosphatases.
3. Primary screening of secreted MAbs by dot blotting with BSA-coupled peptides facilitated the selection of individual clones that bound to the antigenic peptide and eliminated KLH-specific clones.
4. All blocking buffers contain βGP to inhibit potential phosphatases present in BSA or fat-free milk powder.
5. Labeled probe can be stored at –20°C and used for up to 4 wk.
6. CDK kinase assay: incubate 100 ng full-length bacterially expressed p53 protein with 1 μL baculovirus co-expressed recombinant cyclin B and CDK2 with 250 μ*M* ATP in 15 μL total vol CDK kinase buffer at 30°C for 30 min. Process immediately or snap-freeze in liquid nitrogen and store at –70°C (*see* **Note 10**).
7. Kinase reaction products must be titrated into the DNA binding reaction, as the efficiency of p53 phosphorylation will vary with each batch of cyclinB/CDK2 and p53 preparations.
8. Despite their specificity, the N-terminal p53 MAbs generated in our laboratory have not generally proven useful for detecting endogenous p53 in cell lines. This is presumably due to their low affinity for p53 protein.
9. The amount of lysate required to achieve a good signal will vary depending on cell type and antibody affinity, but use 10 μg per sample to start.
10. The addition of phosphatase inhibitors when assaying for kinase activity using whole cell extracts is optional, as some kinases are activated by dephosphorylation.

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13

Detection of Mitochondrial Localization of p53

Motohiro Mihara and Ute M. Moll

Summary

p53 is a master regulator of cell death pathways and has transcription-dependent and transcription-independent modes of action. Mitochondria are major signal transducers in apoptosis and are critical for p53-dependent cell death. Recently, we discovered that a fraction of stress-induced wild-type p53 protein rapidly translocates to mitochondria during p53-dependent apoptosis. Suborganellar localization by various methods shows that p53 predominantly localizes to the surface of mitochondria. Moreover, bypassing the nucleus by targeting p53 to mitochondria is sufficient to induce apoptosis in p53-null cells, without requiring further DNA damage. Here, we describe subcellular fractionation as a classic technique for detecting mitochondrial p53 in cell extracts. It consists of cell homogenization by hypo-osmotic swelling, removal of nuclear components by low-speed centrifugation, and mitochondrial isolation by a discontinuous sucrose density gradient. p53 and other mitochondrial proteins can then be detected by standard immunoblotting procedures. The quality of mitochondrial isolates can be verified for purity and intactness.

Key Words

apoptosis, p53, mitochondria, p53 localization, mitochondrial targeting, stressed cell, mitochondrial fractionation

1. Introduction

p53 kills tumor cells by transcriptionally activating gene products that have various roles in the cell death pathway. However, it has long been observed that p53 has a transcription-independent way of killing cells, although no mechanism was known ***(1–3)***. Mitochondria are major signal transducers in apoptosis and are critical for p53-dependent cell death. Mitochondria can trigger cell death by releasing cytochrome c and other potent apoptosis activators stored in their intermembraneous space that, once released, activate effector

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caspases and break down nuclear chromatin ***(4,5)***. We recently discovered that a fraction of stress-induced wild-type p53 protein rapidly translocates to mitochondria during p53-dependent apoptosis induced by, e.g., γ-irradiation (IR), topoisomerase inhibitors, and hypoxia. This phenomenon is widespread and occurs in human and mouse cultured cells ***(6–8)*** (*see* **Note 1**). The translocation of p53 to mitochondria is rapid (within 1 h after p53 activation) and precedes changes in mitochondrial membrane potential, cytochrome c release, and procaspase-3 activation. In contrast, p53 does not translocate during p53-independent apoptosis or p53-mediated cell cycle arrest. Suborganellar localization by various methods shows that the predominant amount of p53 is located at the surface of mitochondria. This association can be reproduced in vitro with purified baculoviral p53 and isolated mitochondria. Importantly, bypassing the nucleus by targeting p53 to mitochondria is sufficient to induce apoptosis in p53-null cells without further DNA damage. Similar results were seen with various p53 mutants, including the transcriptionally inactive hotspot mutant R175H. This indicates that p53 protein is sufficient to launch apoptosis directly from the mitochondria.

Subcellular fractionation for mitochondrial enrichment is a major analytical tool for studying this event. While this method can also be adapted to isolate mitochondria from rat or mouse liver ***(9,10)***, bovine heart, skeletal muscle ***(11)***, or yeast ***(12)***, we focus here on cultured tumor cells, because they are easy to grow and respond well to various treatments with drugs, IR, UV, hypoxia, or transfection. The protocol for isolation of mitochondria is a discontinuous sucrose gradient and follows largely the method described by Bogenhagen and Clayton ***(13)*** and others ***(14,15)***.

2. Materials

1. Confluent suspension or adherent cultured cells, treated or untreated with drugs, IR, hypoxia, and/or transfection.
2. TD washing buffer: 135 m*M* NaCl, 5 m*M* KCl, 25 m*M* Tris-HCl, pH 7.6, ice-cold. To make a 10X solution: 39.15 g NaCl, 1.85 g KCl, 15.15 g Tris-HCl, 3.485 g Tris-base, 0.5 g Na_2HPO_4. Add water up to 500 mL. Store at 4°C.
3. CaRSB buffer: 10 m*M* NaCl, 1.5 m*M* $CaCl_2$, 10 m*M* Tris-HCl, pH 7.5, ice-cold. Mix together 1 mL 5 *M* NaCl, 750 µL 1 *M* $CaCl_2$, 5 mL 1 *M* Tris-HCl, pH 7.5. Add water up to 500 mL.
4. MS buffer: 210 m*M* mannitol, 70 m*M* sucrose, 5 m*M* ethylenediamine tetraacetic acid (EDTA), 5 m*M* Tris, pH 7.6, ice-cold. To make a 2.5X solution: 38.26 g mannitol, 23.96 g sucrose, 5 mL 1 *M* Tris-HCl, pH 7.6, 20 mL 0.25 *M* EDTA, pH 7.4. Add water up to 400 mL. Store at 4°C.
5. Sucrose buffer: 1 *M* or 1.5 *M* sucrose, 2 m*M* dithiothreitol (DTT), 5 m*M* EDTA, 10 m*M* Tris, pH 7.6. Mix together 34.24 or 51.34 g sucrose, 200 µL 1 *M* DTT, 2 mL 0.25 *M* EDTA, 1 mL 1 *M* Tris-HCl, pH 7.6. Add water up to 100 mL. Store at 4°C.

6. Protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN, USA). Should be made fresh and added to each solution. Store as recommended by company.
7. Glass Dounce homogenizer (Kimble/Kontes, Vineland, NJ, USA), fit A.
8. Low-speed centrifuge with swinging bucket rotor.
9. Ultracentrifuge with swinging bucket rotor (e.g., Rotor type SW41; Beckman Coulter, Fullerton, CA, USA: cat. no. L8-80M).
10. 10% sodium dodecyl sulfate (SDS) acrylamide gel.
11. Tris-buffered saline with Tween® 20 (TBST) buffer. For 8 L of stock solution: 80 mL 1 *M* Tris-HCl, pH 8.0, 20.4 g NaCl, 4 mL 20% Tween 20. Add distilled water (dH_2O) up to 8 L. Store at room temperature.

3. Methods

3.1. Isolation of Mitochondria

3.1.1. Suspension Culture Cells

3.1.1.1. Prepare Cells

1. Pellet suspension cells by spinning for 5 min at 1000–3000*g* at 4°C in low-speed centrifuge with swinging rotor (*see* **Note 2**).
2. Aspirate media completely.
3. Resuspend cells from 100-mL culture into 50 mL 1X TD buffer, ice-cold (*see* **Note 3**).
4. Pellet cells by spinning for 5 min at 1000–3000g at 4°C.

3.1.1.2. Homogenize Cells

1. Discard supernatant completely and resuspend cells into CaRSB buffer containing protease inhibitors. Use approx 10X vol of packed cells.
2. Leave on ice for 10 min to let cells swell; monitor swelling under microscope.
3. Transfer cells to ice-cold glass Dounce homogenizer and homogenize on ice with frequent monitoring under microscope (*see* **Note 4**).
4. Quickly add 2 mL of 2.5X MS buffer containing protease inhibitor per 3 mL homogenate. Transfer an aliquot to another tube for Western blotting to check for nuclear contamination. Another aliquot is used for protein determination (*see* **Note 5**).
5. Transfer the rest of the homogenate to centrifuge tubes and spin down nuclei for 5 min at 1000–3000*g* at 4°C. Decant supernatant into new clean tube and repeat again (*see* **Note 6**).

3.1.1.3. Isolate Mitochondria

1. Make a discontinuous sucrose gradient in a ultracentrifuge tube (Polyallomer tubes; Beckman Coulter; cat. no. 331372) by carefully layering 3 mL of 1 *M* sucrose buffer on top of 3 mL of 1.5 *M* sucrose buffer (each containing protease inhibitors) (*see* **Note 7**).

2. After spinning, take supernatant of homogenate (from **Subheading 3.1.1.2.**, **step 5**) and apply carefully on top of the sucrose gradient.
3. Ultracentrifuge the gradient in a SW41 swinging bucket rotor for 35 min at 26,000 rpm (85,000*g*) at 4°C.
4. Mitochondria will collect at the 1–1.5 *M* interphase of the sucrose gradient and appear as a clearly visible white band. Carefully aspirate the band with an 18-G needle using a 3-mL syringe. Be careful not to suck off buffer. Mix with 4× the vol of 1X MS buffer containing protease inhibitors (*see* **Note 8**).
5. Aliquot into microcentrifuge tubes and spin them for 10–15 min at 16,000*g* at 4°C in high-speed Eppendorf® microcentrifuge.
6. Aspirate supernatant and pool all pellets in 100 μL of 1X MS (with protease inhibitors) and spin again (*see* **Note 9**).
7. Resuspend in a final 200 μL of 1X MS buffer (with protease inhibitors). This sample will be used for all subsequent assays (*see* **Note 10**).
8. Measure protein concentration. Add SDS/β-mercapto-ethanol sample buffer for electrophoresis.

3.1.2 Adherent Cells

3.1.2.1. Prepare Cells

1. Aspirate media completely and rinse quickly once with phosphate-buffered saline (PBS).
2. Scrape cells into PBS, and pellet for 5 min at 1000–3000*g* at 4°C.
3. Aspirate media completely. Resuspend cells into 50 mL of 1X TD buffer, ice-cold.
4. Pellet cells for 5 min at 1000–3000g at 4°C.
5. Follow the steps in **Subheading 3.1.1.1.**, **steps 1–4**, **Subheading 3.1.2.2.**, **steps 1–5**, and **Subheading 3.1.1.3.**, **steps 1–8**.

3.2. Western Blot Analysis

1. Prepare a 10% SDS polyacrylamide gel.
2. The same amount of mitochondrial and crude lysates are run (1–10 μg protein per lane).
3. Transfer proteins to nitrocellulose membrane with semi-dry transfer apparatus at 200 mA for 17 min (depends on area of membrane).
4. Block in 50 mL 5% nonfat dry milk in TBST for at least 20 min at room temperature.
5. Incubate for 2 h at room temperature with a cocktail of anti-p53 antibody, anti-proliferating cell nuclear antigen (PCNA) antibody (nuclear contamination marker), and anti-mitochondrial (mt) heat-shock protein (hsp)70 antibody (mitochondrial enrichment marker) properly diluted in blocking buffer (*see* **Note 11**).
6. Wash with TBST 3X for 15 min.
7. Incubate for 1 h at room temperature with secondary antibody.
8. Wash with TBST 3X for 15 min.

9. Develop with enhanced chemiluminescence reagent (Luminol Reagent; Pierce Chemical, Rockford, IL, USA).

4. Notes

1. A death stimulus is required to induce endogenous wild-type p53 to translocate to mitochondria. Mitochondria from unstimulated cells have undetectable levels of mitochondrial p53. γ-IR, DNA damaging agents (e.g., 5 μ*M* camptothecin for 6 h) or hypoxia (125 μ*M* desferoxamine for 5 h or GasPak pouches; Becton Dickinson, Franklin Lakes, NJ, USA) induce mitochondrial p53 ***(6,7)***. Cancer cell lines with mutant p53 exhibit mitochondrial p53 constitutively without prior DNA damage ***(8)***. When mitochondrially targeted p53 fusion protein is transfected (the LFp53wt plasmid used in **ref. *6***), mitochondrial p53 is clearly detectable by immunofluorescence analysis.
2. First resuspend pellet in about 5 mL of CaRSB buffer by pipeting up and down, then add the rest of the buffer. For some cell types, it is difficult to ascertain whether they undergo swelling within 10 min. In that case, it is acceptable to keep cells in TD buffer or in growth medium and only remove small aliquots to try out the proper swelling time. Compare with cell shape prior to swelling and observe enlargement under the microscope. ML-1 cells (human chronic myelogenous leukemia line) are the standard cell line for verifying swelling.
3. How many strokes are needed? It really depends on the type of cells. Homogenize to the point where ideally 70–90% of the cells are broken, but the nuclei are still intact (they appear as smooth round small spheres under the phase optic microscope). The nuclei of some cell line are very fragile. Some cell lines needs 100 strokes, others need only 10. To determine the proper number of strokes, visually monitor after every 5 strokes.
4. Usually 100 μL of crude cell extract is enough.
5. To avoid nuclear contamination, the second centrifugation is critical. A third spin is even recommended if the second spin still produces a significant nuclear pellet. Although 5 min is enough for centrifugation, it can be extended until 15 min. Do not disturb the pellet when removing the supernatant. Decanting is better than pipeting. Although pure mitochondria will be isolated with the following sucrose gradient, washing the nuclear pellet to obtain a higher yield of mitochondria is not recommended, because some cell lines have very fragile nuclei that may release nuclear proteins.
6. In preparation for the experiments, the rotor and rotor buckets should be placed at 4°C and –20°C, respectively.
7. Do not disturb the mitochondrial shelf. Another white ring on top of the sucrose gradient contains smaller organelles (e.g., lysosomes, peroxisomes).
8. Pipet very gently up and down as to not break the mitochondria.
9. After this step, the mitochondria can also be resuspended in cell lysis buffer for subsequent co-immunoprecipitation experiments.
10. An example of the antibody combination is given in **Table 1**. To verify the absence of nuclear contamination, we found that PCNA is a highly sensitive

Table 1
Antibody Combinations

	Name	Origin	Dilution	Company
anti-p53	DO-1 (Ab-6)	mouse $IgG_{2a}\kappa$	1:1000	Oncogene Research Products (Cambridge, MA, USA)
anti-PCNA	PCNA (PC10)	mouse IgG_{2a}	1:1000	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
anti-mt hsp70	mitochondrial heat-shock protein 70	mouse IgG_3	1:1000	Affinity Bioreagents (Golden, CO, USA)
anti-mt hsp60	mitochondrial heat-shock protein 60	mouse IgG_{2a}	1:1000	Affinity Bioreagents

Ig, immunoglobulins.

marker. PCNA is a very abundant small soluble nuclear protein, and excellent antibodies exist for its detection. As an anti-mitochondrial protein, we routinely use anti-mt hsp70 or anti-mt hsp60 (matrix proteins). Other combinations can be made as needed.

11. For all the antibodies described in **Table 1**, we use sheep anti-mouse immunoglobulin (Ig)G (peroxidase-linked, species-specific whole antibody) (Amersham Pharmacia Biotech, Piscataway, NJ, USA) Use 1:2000 dilution in blocking buffer.

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14

Analysis of Nuclear and Cytoplasmic Degradation of p53 in Cells after Stress

Troy W. Joseph and Ute M. Moll

Summary

Until recently, the accepted model held that p53 degradation occurs exclusively on cytoplasmic proteasomes and, hence, has an absolute requirement for nuclear export of p53 via the CRM1 pathway. However, proteasomes are abundant in both cytosol and nucleus. We recently analyzed HDM2-mediated degradation of endogenous p53 in the presence of various CRM1 blockers. We found that significant HDM2-mediated degradation takes place despite nuclear export blockade, indicating that endogenous p53 degradation occurs locally in the nucleus, in parallel to cytoplasmic degradation.

Here, we describe how subcellular fractionation can be used to monitor nuclear and cytoplasmic degradation of endogenous wild-type p53 during the recovery phase after a stress stimulus. The fractions are then analyzed by immunoblotting in a time-dependent fashion. Vimentin and lamin A proteins are used to monitor the purity of the cytosolic and nuclear fractions, respectively, and to control for equal loading.

Key Words

p53, HDM2, ubiquitination, proteasomes, degradation, cytoplasm, nucleus

1. Introduction

The principal negative regulator of p53 stability and function is the HDM2 protein, which functions as an E3 ubiquitin ligase for p53 in vivo ***(1–5)***. (The mouse homolog is named Mdm2.) The E3 ligase activity of HDM2/Mdm2 maps to a cysteine and histidine rich Zinc-coordinated RING finger motif at the C terminus, which ubiquitinates p53 on multiple lysine residues throughout its C terminus ***(5)***. p53 and HDM2 are linked through an autoregulatory feedback loop, in which activated p53 stimulates HDM2 transcription, while induced HDM2 in turn inhibits p53 function ***(6)***. The significance of this auto-

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regulatory feedback loop becomes dramatically apparent in MDM2-deficient mice, which die early in embryogenesis due to unchecked p53 activity ***(7,8)***. Simultaneous ablation of p53 in MDM2–/– p53–/– double null mice, however, fully rescues embryonic lethality. Thus, HDM2 is responsible both for the low steady-state levels of p53 in unstressed cells and for switching off a p53 stress response after cell damage is repaired. Consistent with p53 inactivation by HDM2, the HDM2 gene is amplified or overexpressed in the absence of p53 mutations in some human tumors including sarcomas, leukemias, and lymphomas ***(9,10)***.

The principal regulator of p53 stability is HDM2, which is an E3 ligase that mediates p53 degradation via the ubiquitin-26S proteasome pathway. Proteasomes are abundant in both cytosol and nucleus. We have recently shown that p53 degradation occurs in the nucleus and the cytoplasm during down-regulation of the p53 response after DNA damage ***(11)***. The fraction of p53 that undergoes cytoplasmic degradation is subject to nuclear export of p53 via the CRM1 pathway, utilizing the p53 nuclear export signal (NES) in the C terminus ***(12)***. HDM2 also can undergo nuclear export via its NES signal. One can utilize Leptomycin B (LMB), a noncompetitive nuclear CRM1 inhibitor to prevent the export of p53. Due to the presence of a nuclear export blockade, we can determine the ability of HDM2 to degrade endogenous p53 in the nucleus. CRM1/exportin 1 is a karyopherin essential and specific for nuclear export of NES-containing proteins of the human immunodeficiency virus (HIV)-1 Rev type, such as p53 and HDM2 ***(13–19)***. CRM1 binds directly to their leucine-rich NES ***(13)***, in addition to binding to Ran-GTP and nucleoporins ***(18)***. CRM1 is the cellular target of LMB in vitro ***(20)***, and CRM1 is the only protein detectable to bind to LMB in vivo at nanomolar concentrations ***(21)***. LMB is a fast acting noncompetitive and highly specific inhibitor of CRM1 ***(15,17,20)*** that acts within 10–30 min ***(22)***. It abolishes association of CRM1 with NES by direct binding to CRM1, thereby inhibiting nuclear export of NES proteins ***(14,15,17)***. LMB covalently binds to the sulfhydryl group of cysteine 529 in the central conserved region of CRM1 via its α, β-unsaturated δ-lactone group ***(21)***. It does not significantly interfere with general mRNA nuclear export ***(15)***. Of particular importance for our studies, LMB does not nonspecifically interfere with proteasome activity, as demonstrated by undisturbed degradation of β-catenin, which is a prototype for cytoplasmic proteasomal degradation ***(23)***. Also, LMB does not nonspecifically affect steady state levels of export-independent proteins, such as proliferating cell nuclear antigen (PCNA) or UsnRNP Sm ***(24)***, nor does it induce gross morphologic changes ***(11)***. Therefore, LMB has been an important tool in studying nuclear export requirements of Rev-like proteins.

Studies on compartmental degradation of p53 and HDM2 also use cycloheximide, which is a protein synthesis inhibitor. The addition of a protein syn-

thesis inhibitor is important, since its usage prevents new p53 from being made, while the degradation and/or stabilization of p53 and HDM2 are being studied. Experiments can be done to monitor the kinetics of p53 degradation and/or its stabilization in the nucleus and or cytosol. This chapter describes a rapid and reliable protocol for cleanly separating the nucleus from the cytoplasm and for observing under what physiological circumstances p53 and HDM2 degradation might occur in both compartments. The analysis is done by immunoblots. Vimentin is used as a cytosolic marker, and lamin A is used as a nuclear marker to check for the purity of the fractionation procedure.

2. Materials

2.1. Cell Preparation

1. Cultured mammalian cells.
2. 60-mm plates.

2.2. Harvesting the Cells

1. Gloves.
2. Blanked Dulbecco's modified Eagle medium (DMEM).
3. Cell scraper.
4. Protease inhibitors cocktail (Calbiochem-Novabiochem, San Diego, CA, USA).
5. 1.5-mL Eppendorf® tubes.
6. Microcentrifuge.

2.3. Testing the Purity of Cytoplasmic and Nuclear Purification

1. p53 antibody (clone DO-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA).
2. HDM2 antibody (clone IF-2; Chemicon International, Temecula, CA, USA).
3. Vimentin antibody (Chemicon International).
4. Lamin A antibody (Chemicon International).
5. Mouse secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA).
6. Nuclear and cytoplasmic extraction kit (Pierce Chemical, Rockford, IL, USA; cat. no. 78833). The kit contains 2 cytoplasmic and 1 nuclear extraction buffers: CER1, CER2, and NER.

3. Methods

3.1. Cytoplasmic and Nuclear Extraction Protocol

1. Grow cells in 60-mm plates to 70–90% confluency (e.g., human U2OS or RKO cells) (*see* **Note 1**).
2. Treat cells with DNA damaging drug to induce p53 response (e.g., camptothecin, 5 μ*M* for 2.5 h).
3. Aspirate media that contains DNA damaging drug. Wash cells with new media.
4. Allow cells to recover for 4 h in fresh media (3 mL for a 60-mm plate).

5. Treat cells with LMB, 20 n*M* (Sigma, St. Louis, MO, USA) for 1 h.
6. Treat cells with cycloheximide (10 mg/mL) for 15 h.
7. Aspirate off media completely and add 100 µL of ice-cold CER1 lysis buffer to cells.
8. Scrape cells carefully in the CER1 buffer to one edge of the plate.
9. Place cells and CER1 buffer in 1.5-mL Eppendorf tube and vortex mix vigorously on the highest setting for 15 s.
10. Incubate the tube on ice for 10 min.
11. Add 5.5 µL of ice-cold CER2 to the tube and vortex mix for 5 s on the highest setting (*see* **Note 2**).
12. Incubate the tube on ice for 1 min.
13. Vortex mix for 5 s on the highest setting. Centrifuge the tube for 5 min in a cold microcentrifuge (16,000*g*) (*see* **Notes 2–4**).
14. Immediately transfer the supernatant (cytoplasmic extract) to a clean prechilled 1.5-mL Eppendorf tube. Place the tube on ice.
15. Resuspend the insoluble pellet fraction produced in **step 11**, which contains nuclei, in 100 µL of ice-cold NER (*see* **Notes 5** and **6**).
16. Vortex mix on the highest setting for 15 s. Return the sample to ice and continue vortex mixing every 10 min, for a total of 40 min (*see* **Note 7**).
17. Centrifuge the tube at full speed (16,000*g*) in a cold microcentrifuge for 10 min.
18. Immediately transfer the supernatant (nuclear extract) (*see* **Note 3**) fraction to a clean prechilled tube. Place on ice (*see* **Note 8**).
19. Store all extracts at –80°C until needed.

3.2. Cytoplasmic and Nuclear Fractionation Verification Protocol by Immunoblot

1. Measure protein concentrations of the cytoplasmic and nuclear fractions.
2. Load equal amounts of protein from the cytoplasmic and the nuclear fraction on two 8% acrylamide gels and transfer to membranes.
3. Blot one membrane with vimentin antibody as a marker for cytosolic proteins.
4. Reblot this membrane with lamin A antibody as a marker for nuclear proteins.
5. Blot the other membrane first with DO-1 antibody for p53 detection, followed by IF-2 antibody for HDM2 detection (*see* **Note 9**).
6. A pictorial representation of how the experiment should be done is outlined in **Fig. 1**, followed by some actual results from U2OS cells (human osteosarcoma cell line harboring wild-type p53) (T. W. Joseph and U. M. Moll, unpublished results).

4. Notes

1. A 60-mm plate of cells should yield about 150–200 µg of proteins in the cytoplasmic fraction and 20–30 µg of proteins in the nuclear fraction.
2. If there is low cytoplasmic yield, increase the amount of CER2 buffer (e.g., 1.5–2× as much).
3. Cytoplasmic and nuclear extracts are now ready for use in other downstream assays. If large vol are required in subsequent applications or if problems occur

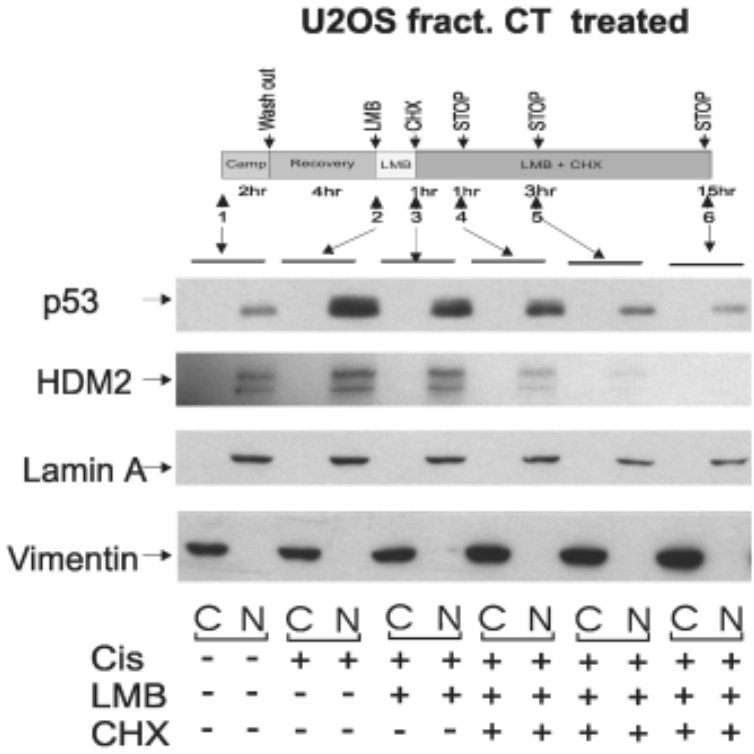

Fig. 1. Cytoplasmic and nuclear fractionation verification immunoblots.

with functional downstream assays, the nuclear extract can be dialyzed prior to use to remove salts and detergents.

4. If proteins are not properly compartmentalized, carefully remove all cytoplasmic extract prior to nuclear lysis.
5. The detergent in NER buffer is not dialyzable, however. For dialysis, the Slide-A-Lyser MINI dialysis unit (Pierce Chemical) is recommended.
6. If more concentrated nuclear extracts are desired, the vol of NER used in the extractions can be decreased two- to four-fold with no adverse effects on protein recovery or compartmentalization.
7. If nuclear proteins are low, vortex mix more thoroughly.
8. If there is low protein activity, do all work at 4°C. Use fresh protease inhibitors.
9. A good starting concentration for primary antibodies is 1:1000. The mouse secondary can be used at 1:3000–10,000 (Femto Chemiluminescence developer; Pierce Chemical). If there is too much background, decrease the concentration of the primary antibody to 1:3000. Always wash nitrocellulose membrane thoroughly and dilute all antibodies in 5% skim milk–phosphate-buffered saline (PBS).

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15

Mutational Analysis of p53 in Human Tumors

Direct DNA Sequencing and SSCP

Susan Erster, Neda Slade, and Ute M. Moll

Summary

This chapter describes the techniques necessary to identify p53 genomic mutations in either frozen or paraffin-embedded tumor samples. DNA is extracted from the tumor samples and then used as a template to amplify the p53 coding sequences by polymerase chain reaction (PCR). The PCR products are characterized by agarose gel electrophoresis. Subsequently, mutations can be detected by direct DNA sequencing of the PCR products, followed by acrylamide gel electrophoresis and autoradiography. Alternatively, PCR products containing mutations can be identified by their aberrant mobility on denaturing acrylamide gels, using single-stranded conformation polymorphism (SSCP).

Key Words

p53, tumors, mutations, PCR, SSCP, DNA sequencing

1. Introduction

1.1. PCR Amplification and DNA Sequencing

Apoptosis ***(1)***, or programmed cell death, is an essential cellular response to stresses, such as DNA damage, hypoxia, and oncogene deregulation (reviewed in **refs. *2*,*3***). The p53 protein mediates the apoptotic response, and loss of p53 function is implicated in tumor progression. Not surprisingly, the *p53* gene is either lost or mutated in greater than 50% of human tumors ***(4*,*5)***. To determine whether a tumor has undergone mutation of the *p53* gene, one can extract genomic DNA from a fixed or frozen tumor specimen, amplify the *p53* coding sequences by polymerase chain reaction (PCR), and subject the PCR products to DNA sequencing. The DNA from archived samples is often highly degraded,

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but should be adequate for short-length PCR amplification. The PCR products are then the templates for the sequencing reaction. They can be recovered and purified by extraction from low-melt agarose gels or through commercially available PCR clean-up kits. In many cases, adequate template can be prepared simply by nucleolytic digestion of the PCR. The PCR products are sequenced, and the sequence is compared with that of wild-type *p53* to identify mutations.

Another method to study *p53* missense mutations is single-stranded conformational polymorphism (SSCP) analysis. This method detects DNA sequence changes as a shift in electrophoretic motility ***(6)***. DNA is amplified by PCR, denatured followed by rapid chilling, and run on polyacrylamide electrophoresis gels under nondenaturating conditions. Under such conditions, single-stranded DNA takes on a folded conformation that is stabilized by intrastrand interactions. The radioactive amplicons can be detected by phosphoimager analysis or by exposing the dried gels to X-ray film. This simple, rapid, and sensitive method enables us to detect even single-base changes or point mutations, which represent the overwhelming majority of *p53* mutations in tumors.

2. Materials

2.1. Extraction of DNA from Frozen or Paraffin-Embedded Tumor Specimens

1. Proteinase K. Dissolve powder at 20 mg/mL in 50 m*M* Tris, pH 8.0, aliquot, and store at –20°C.
2. Phenol–chloroform–isoamylalcohol (25:24:1). Liquefy phenol (work in fume hood, as phenol is toxic) and add an equal vol of 0.5 *M* Tris-HCl, pH 8.0. Mix well and allow the phases to separate. Test pH of phenol phase with pH paper and repeat equilibration if pH is still below 7.8. Store equilibrated phenol under Tris overlay at 4°C in a dark bottle. For 100 mL phenol–chloroform–isoamyl alcohol, combine 50 mL phenol, 46 mL chloroform, and 4 mL isoamyl alcohol. This solution should also be stored in a dark bottle at 4°C.
3. 95% Ethanol.

2.1.1. Paraffin Samples Only

1. Xylene (work in fume hood, as xylene is toxic).
2. Isopropanol.

2.1.2. Frozen Samples Only

1. Lysis buffer (SNET): 20 m*M* Tris-HCl, pH 8.0, 5 m*M* ethylenediamine tetraacetic acid (EDTA), 400 m*M* NaCl, 1% sodium dodecyl sulfate (SDS). Filter-sterilize and store at room temperature.
2. 3 *M* sodium acetate, pH 5.2.
3. TE, pH 8.0: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* Na_2 EDTA.

2.2. PCR Amplification of p53 Coding Sequences

1. Polymerase (i.e., *Taq*) plus 10X PCR buffer.
2. 25 m*M* $MgCl_2$ (often included in 10X buffer or supplied separately).
3. dNTP mixture. 10 m*M* for each nucleotide in PCR-grade water (filter-sterilized). Store frozen in small aliquots and avoid repeated freeze-thawing.
4. p53-Specific exon primers for exons 5–8 and the size of the expected product are given below. Primers need not be purified for PCR, but should be purified by high-performance liquid chromatography (HPLC) or electrophoresis for sequencing.
   5F: 5'-ctcttcctgcagtactcccctgc-3', 5R: 5'-gccccagctgctcaccatcgcta-3', product length: 211 bp.
   6F: 5'-gattgctcttaggtctggcccctc-3', 6R: 5'-ggccactgacaaccacccttaacc-3', product length: 185 bp.
   7F: 5'-gtgttgtctcctaggttggctctg-3', 7R: 5'-caagtggctcctgacctggagtc-3', product length: 139 bp.
   8F: 5'-acctgatttccttactgcctctggc-3', 8R: 5'-gtcctgcttgcttacctcgcttagt-3', product length: 200 bp.

2.3. Analysis and Purification of PCR Products

1. Agarose (preparative grade, low-melt if desired).
2. Agarose gel electrophoresis buffer.
3. TBE (10X): 890 m*M* Tris-borate, 20 m*M* EDTA, pH 8.0.
4. Ethidium bromide (10 mg/mL in water). Handle carefully, as this is carcinogenic. Store at 4°C, wrapped in aluminum foil.
5. Agarose gel 6X sample buffer (e.g., 30% glycerol, 0.25% each bromophenol blue and xylene cyanol) (for additional recipes *see* **refs. *7*,*8***).
6. Low molecular weight (i.e., less than 1 kb) DNA size ladder.
7. Exonuclease 1.
8. Shrimp alkaline phosphatase.

2.4. Dideoxy-Sequencing

2.4.1. Sequenase Protocol with α-35S dATP

1. Sequenase® (version 2) enzyme (USB, Cleveland, OH, USA).
2. dNTPs and ddNTPs stock solutions are at 0.5 m*M* in water, stored in small aliquots at –20°C.
3. 5X Labeling mixture: 7.5 m*M* each dCTP, dGTP, dTTP.
4. Extension-termination mixture: 160 μ*M* of each dNTP, and 160 μ*M* of either ddATP, ddGTP, ddCTP, or ddTTP.
5. α-35S dATP (1000 Ci/mmol, 10 mCi/mL) Caution: this is a radiation hazard.
6. These buffers are typically supplied with Sequenase enzyme: sequenase dilution buffer: 10 m*M* Tris-HCl, pH 7.5, 5 m*M* dithiothreitol (DTT), 500 μg/mL bovine serum albumin (BSA).
   Sequenase reaction buffer: 200 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$, 125 m*M* NaCl.

2.4.2. Sequenase Protocol with End-Labeled Primers or Cycle-Sequencing Protocol

1. γ-^{32}P ATP, or γ-^{33}P ATP (radiation hazard) (10 mCi/mL, 5000 Ci/mmol).
2. T4 polynucleotide kinase.
3. Ammonium acetate (10 *M*). Filter-sterilize. Do not autoclave.
4. ddNTP mixtures: 20 μ*M* each of all four dNTPs plus either ddATP (600 μm), ddCTP (400 μ*M*), ddGTP (200 μ*M*), or ddTTP (800 μM) in TE, pH 8.0. Store in small aliquots at –20°C.
5. *Taq* CS or other thermostable polymerase.
6. 5X Cycle-sequencing buffer: 200 m*M* Tris-HCl, pH 8.8, 25 m*M* $MgCl_2$.

2.4.3. Denaturing Acrylamide Gel of Sequencing Products

1. 45% Acrylamide: bis-acrylamide (neurotoxin) solution: dissolve 434 g of acrylamide and 16 g of N,N-methylene bisacrylamide in approx 600 mL water by heating on stir plate to about 37°C. Adjust vol to 1 L, filter-sterilize, and store at room temperature in dark bottle.
2. Urea.
3. Ammonium persulfate (APS): 10% in water, store at 4°C, prepare weekly.
4. N,N,N',N'-Tetramethylethylene diamineTEMED (this is toxic).
5. Formamide (this is toxic) loading dye buffer: 95% formamide, 10 m*M* EDTA, 0.2% xylene cyanol, and bromophenol blue.

2.4.4. Fixation

1. Fix solution: 10% acetic acid, 10% methanol (this is toxic) in water.

2.4.5. SSCP of Human p53

1. PCR device.
2. Primer sense, primer antisense.
3. dCTP ^{32}P, 10 μCi/μL.
4. dNTP, 10 m*M*.
5. PCR buffer: 100 m*M* Tris-HCl, pH 8.8, 500 m*M* KCl, 15 m*M* $MgCl_2$, 1% non-ionic detergent Triton® X-100.
6. *Taq* DNA polymerase.
7. Chloroform–water saturated.
8. 0.1% SDS, 10 m*M* EDTA, pH 8.0. Store at room temperature.
9. Stop solution for SSCP: formamide 80%, TBE 0.5X , xylene cyanol 0.5%, bromophenol blue 0.5%, water up to 1 mL.
10. Polyacrylamide gel electrophoresis (PAGE) device.
11. 6% Polyacrylamide gel, 0.35-mm thick: 6 mL acryl–bis acrylamide (40% solution: 38 g acrylamide plus 2 g bisacrylamide up to 100 mL), 1 mL 20X TBE, 33 mL water, 40 μL TEMED, 160 μL 25% APS.

3. Methods

3.1. Extraction of DNA from Frozen Tumor Specimens (see Note 1)

1. Place tissue in lysis buffer. Typically, 10 mL of lysis buffer is sufficient for 1 g of tissue. Add proteinase K to 500 μg/mL.
2. Incubate at 55°C overnight with gentle constant mixing. An end-over-end rotator is ideal, but excellent lysis can also be achieved if tubes are placed horizontally in a shaker or on a rocking platform.
3. Add an equal vol of phenol–chloroform–isoamyl alcohol to the lysate. Ensure that the tubes are sealed tightly and begin mixing. Mixing should be gentle, but sufficient to form an emulsion between the organic and aqueous phases. Allow the sample to incubate at room temperature for 10–30 min with either constant or intermittent gentle mixing. Excessive vortex mixing will shear high molecular weight DNA and is not recommended.
4. Centrifuge at room temperature for 5 min. Small samples can be spun in a microfuge at full speed. Larger samples can be spun in a table-top centrifuge at approx 1000*g*.
5. Carefully remove the upper aqueous phase to a fresh tube. Try to avoid disturbing the interface.
6. Add an equal vol of isopropanol and centrifuge at maximum speed (approx 13,000*g*) for 15 min.
7. Remove isopropanol carefully, without dislodging the pellet. Wash the pellet by gently vortex mixing in a small vol of 70% ethanol, and centrifuge again.
8. Remove the 70% wash and semidry the pellet in a lyophilizer. Do not dry completely, as pellet will be extremely difficult to resuspend.
9. Redissolve DNA in TE, pH 8.0. Dissolution may require overnight incubation, with gentle agitation, at 4°C or a brief incubation at room temperature.
10. If desired, DNA may be further purified by re-extraction with phenol–chloroform. Precipitate the DNA by adjusting the aqueous phase to 0.3 *M* Na-acetate, pH 5.2, and then adding 2.5 vol 95% ethanol. Mix by inverting the tube several times and then store at –20°C for at least 30 min. Centrifuge, wash, dry, and resuspend as above. This additional extraction helps remove residual SDS and other tissue contaminants, which may inhibit subsequent enzymatic reactions.

3.1.1. Paraffin Blocks

1. Transfer a portion of the paraffin block to a microfuge tube and add approx 100 μL of xylene, followed by 100 μL of ethanol.
2. Mix well, and pellet tissue by centrifuging at full speed for 10 min at room temperature.
3. Remove xylene–ethanol, and dry briefly.
4. Resuspend pellet in 250 μL Tris, pH 8.3, containing 200 μg/mL proteinase K.
5. Incubate overnight at 37°C.

6. Transfer tubes to boiling water bath for 5–10 min, then to an ice bath until completely chilled.
7. Store at 4°C.

3.2. PCR Amplification of p53 Coding Sequences (see Note 2)

1. Prepare a reaction mixture containing all reagents (except template): 10X PCR buffer, 2 m*M* $MgCl_2$ (only if PCR buffer does not contain Mg), dNTPs (usually 100–200 μ*M* each), primers (10–20 pmol each), *Taq* DNA polymerase (1 U), and water.
2. Dispense aliquots into reaction tubes on ice. Add template (10–50 ng) to each tube, overlay with mineral oil if required, mix, and place in thermal cycler.
3. Cycle as follows: (*i*) 95°C for 1 min; (*ii*) 35 cycles of 95°C for 1 min, 55–60°C for 1 min, 72°C for 1 min; (*iii*) 72°C for 7 min; and (*iv*) 4°C hold.

3.3. Analysis and Purification of PCR Products

1. Prepare a 1.8–2.0% agarose gel in either 1X TAE or TBE buffer. Add agarose to buffer in microwave-safe flask, dissolve agarose in microwave, and allow to cool.
2. Add ethidium bromide (this is a carcinogen) to 1 μg/100 mL.
3. Pour gel in horizontal electrophoresis apparatus and add comb.
4. Mix approx 10% of PCR sample with one-fifth vol sample buffer.
5. Load in wells and apply current. Also load molecular weight markers (100–1000 bp ladder is ideal).
6. View and photograph gel. If a distinct band of the appropriate size is observed, treat the remainder of the PCR as follows. If no product is available, *see* **Note 3**.
7. Add exonuclease 1 and shrimp alkaline phosphatase (as per unit definition provided by manufacturer).
8. Incubate at 37°C for 15–30 min.
9. Incubate at 80°C for 15 min.
10. Store at –20°C or proceed to sequencing.

3.4. Dideoxy-Sequencing (see Note 4)

A number of sequencing protocols are available, offering options regarding template preparation, incorporation of radioactive label, and polymerase. For detection of mutations in relatively short templates, accurate unambiguous data is crucial. The most straightforward approach is to follow the Sequenase protocol. Sequenase is a modified T7 polymerase, which lacks 3'-5' exonuclease activity. This protocol can accommodate kinase-labeled primers and cold nucleotides or unlabeled primers and labeled dATP. A cycle sequencing protocol using end-labeled primers is also presented.

3.4.1. Sequenase Protocol

1. Anneal template and primer: combine in a 500 μL reaction tube: template (100 ng–1 μg), primer (2.5 ng), 5X Sequenase buffer, and water to 10 μL.

2. Incubate at 65–70°C in a water bath for 2 min. Remove the tubes to room temperature to slow-cool. Ideally, stand the tubes in a small beaker containing water from the water bath. It should take approx 30 min to cool to room temperature.
3. Add one of each of the four ddNTP mixes to an appropriately labeled (A, C, G, or T) microfuge tube. Dispense the aliquot at the upper wall of the tube. Prewarm to tubes to 37 °C.
4. Dilute 5X labeling mixture in ice cold water and store on ice.
5. Dilute Sequenase to approx 3 U/2 μL in ice-cold Sequenase dilution buffer, and store on ice.
6. Add to annealed template primer: 2 μL of diluted labeling mixture, 1 μL of 100 m*M* DTT, 0.5 μL of radiolabeled dATP (this is a radiation hazard), and 2 μL of diluted Sequenase. Mix by tapping gently.
7. Incubate at room temperature for 2–5 min.
8. Add 3.5 μL of labeling reaction to each of the ddNTP-containing tubes, quickly spin in a microfuge, and return to 37°C for 3–5 min.
9. Stop reaction by adding 4 μL of formamide loading buffer. At this point the reaction may be stored at –20°C. Alternatively, it may be denatured by placing the tubes in a boiling water bath for 2 to 3 min, then quick-chilling on ice.

3.4.2. Cycle Sequencing with End-Labeled Primer

End-label primers (same as PCR primers described in **Subheading 2.2.**) as follows:

1. Mix: primer (10 pmol), 10X T4 polynucleotide kinase buffer, γ–^{32}P ATP (10 pmol), T4 polynucleotide kinase (1 μL = 10 U), and water to vol (typically 20–25 μL).
2. Incubate at 37°C for 1 h.
3. Heat-inactivate kinase at 65°C for 10 min.
4. Add ammonium acetate to 2.5 *M* (from 10 *M* stock) and 2.5 vol ethanol.
5. Incubate at –20°C at least 30 min.
6. Recover pellet by centrifugation in microfuge at maximum speed for at least 15 min.
7. Remove supernatant carefully. (This is a radiation hazard.) Wash pellet 2× with 70% ethanol.
8. Dry slightly, dissolve in 10 μL of TE, pH 7.6, and store at –20°C or continue with sequencing.
9. To PCR tubes labeled A, C, G, and T, add 4 μL of the appropriate ddNTP mixture.
10. Prepare a mixture containing: double-stranded template DNA (i.e., PCR product) 10–100 fmol, 1 μL of γ-ATP-labeled primer (at 1.5 pmol/μL), 2 μL of 5X cycle sequencing buffer, and water to 5 μL.
11. Add 5 μL to each of the 4 tubes.
12. Add 1 μL of AmpliTaq® CS (Applied Biosystems, Foster City, CA, USA) (or any other cycle-sequencing polymerase), diluted to 1 U/μL.
13. Mix, spin in microfuge, place in thermal cycler, and amplify as follows: denature at 95°C for 1 min; 30 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 60 s; then 4°C hold.

14. Add 5 μL of formamide loading buffer per tube, store at –20°C, or proceed to electrophoresis.

3.4.3. Denaturing Polyacrylamide Gel Electrophoresis

1. Clean and dry gel plates.
2. If desired, treat one plate with siliconizing solution such as Sigmacote (Sigma, St. Louis, MO, USA), following the manufacturer's instructions.
3. Assemble gel plates with spacers, seal with electric tape, and clamp tightly with binder clips.
4. Mix in a side armed flask:17.8 mL of acrylamide solution, 10 mL of 10X TBE buffer, 42 g of urea, and 36.9 mL of water.
5. Heat to 55°C, with swirling, to dissolve urea.
6. Cool to room temperature.
7. Attach to vacuum line, apply vacuum to degas. Swirl gently and continue until bubbling ceases (1 to 2 min).
8. Add 0.5 mL of 10% APS.
9. Add 50 μL of TEMED. Swirl gently.
10. Position gel plates at a slight elevation, i.e., by positioning a 2- to 3-in. object (pipet tip rack, Eppendorf® tube rack, "lead pig," etc.) under the top of the plate sandwich.
11. Draw the gel mixture into a 60-mL syringe, position the syringe in one of the upper corners, and gently but steadily apply pressure, so that gel mixture streams between plates. As the gel mixture enters, it gradually lift plates, so that trapped air rises. When the gel mixture reaches the top, lay the plates down again, and insert a comb. Clamp comb in place with binder clips.
12. Let polymerize for 15–30 min.
13. Remove tape from bottom with razor blade, position in vertical gel apparatus, and proceed with electrophoresis.
14. Electrophoresis until bromophenol blue is about 1 to 2 in. from the bottom.
15. Remove tape from sides, lay sandwich, such that nonsiliconized plate is on the bottom, and separate plates by wedging spatula in one corner and lifting. Gel should stick to nonsiliconized plate.
16. Immerse gel (on plate) in fix solution (10% methanol, 10% acetic acid in water) for 15 min.
17. Remove from fix, lay on bench, press Whatman paper (Whatman, Clifton, NJ, USA) over gel, press hard, and begin lifting at one corner. Gel should stick to paper.
18. Cover with plastic wrap. Dry briefly on gel-dryer, or proceed directly to autoradiography.

3.5. SSCP of Human p53 (see Note 5)

The sequence which has to be analyzed should be amplified and labeled by PCR using labeled nucleotide. The generated fragments should be denatured

and analyzed by polyacrylamide gel electrophoresis. The DNA strands are visualized by autoradiography ***(9)***.

1. Prepare the reaction as follows: 1 μL DNA (0.5 μg/μL), 16.7 μL water, 1.5 μL primer I (20 pmol), 1.5 μL primer II (20 pmol), 2.5 μL buffer 10X, 0.5 μL 10 m*M* dNTP, 0.8 μL (8 μCi) dCTP–^{32}P, 10 mCi/mL, and 0.5 μL *Taq* DNA polymerase for a total of 25 μL.
   Run the 35 cycles of PCR as follows: predenaturation at 94°C for 3 min, denaturation at 92°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min plus 2 s extension per round. Elongation at the end at 72°C for 10 min.
2. After run, take out 25 μL, and add 100 μL of chloroform–water saturated.
3. Spin down for 2 min at maximum speed.
4. Take off aqueous supernatant and load 2 μL for agarose gel to visualize the products.
   Take 4 μL of supernatant and add 40 μL of 0.1% SDS, 10 m*M* EDTA, pH 8.0.
5. Take out 4μL of this mixture and add 7 μL Stop solution. Boil for 10 min at 95°C and chill on ice.
6. Run on 6% polyacrylamide gel at 30 W, constant power (let bromophenol blue run to bottom except for exon 7: only three-fourths).
7. Expose autoradiograph overnight at –80°C.

4. Notes

1. DNA extraction. The lysis buffer used to extract DNA from frozen samples contains SDS, which lyses cells and nuclei, and proteinase K, which degrades proteins. The EDTA chelates Mg ions, reducing nuclease activity. DNA is then recovered by phenol extraction, followed by alcohol precipitation. DNA can be recovered from paraffin blocks using commercially available kits, or various protocols which involve: (*i*) dewaxing, to remove paraffin; (*ii*) proteinase K digestion, with or without SDS and EDTA; and (*iii*) inactivation of proteinase K, which can be achieved either by boiling or by phenol extraction, followed by ethanol precipitation. An excellent side-by-side comparison of these methods is presented in ***(10)***. The DNA will be sheared and degraded, but should be suitable for PCR analysis, particularly if the PCR primers are designed to amplify relatively small products (<500 bp).
2. PCR amplification. Mutations in the *p53* gene can be characterized by PCR amplification, followed by DNA sequencing. In this approach, PCR primers anneal to the intron sequences immediately upstream and downstream of each exon. As most inactivating mutations occur within the DNA binding domain, researchers typically limit their analyses to the exons encoding that region of the protein, which would include exons 5 through 8 (aa 110–307) ***(11)***. Primers designed to amplify each of these exons were at one time commercially available (Clontech Laboratories, Palo Alto, CA, USA), but the kit has been discontinued. The primer sequences and PCR product sizes are included above.

All PCRs must include a "no template" control, to ensure that your solutions, plasticware, pipetmen, etc., are not contaminated with DNA that may serve as a template.

3. Characterization and purification of PCR products. After completion of the reaction, remove an aliquot (typically 5–10%) for electrophoresis. Store the remainder of the reaction at 4°C, as –20°C will freeze the sample and inactivate the polymerase. If there is little to no product, allow the reaction to proceed for an additional 3–5 cycles. Be sure to reamplify your no template control as well. (A PCR product generated from purified template DNA is only valid if there is no product in the no template control.) If the reaction must be stored frozen prior to reamplification, fresh polymerase must be added to the reaction tubes. If there is still no product after 40 cycles, repeat the PCR using more (i.e., 100 ng) template. Additional positive clones would include human genomic DNA (25–100 ng) or cloned p53 fragments, if available in your laboratory. Exercise caution when using cloned p53 as a template, as the resulting amplicons are an ideal source of contamination of future reactions. Use only 1–5 ng and avoid contaminating gloves, etc., with PCR products. If PCR products are observed in positive controls, but not your tumor-derived sample, then the tumor sample may contain compounds which are inhibiting *Taq* DNA polymerase. Dilute the DNA in TE, pH 8.0, re-extract with phenol–chloroform–isoamyl alcohol, and precipitate with one-tenth vol 3 *M* Na-acetate and 2.5 vol 95% ethanol. If DNA concentration is low, add carrier (i.e., glycogen) to facilitate precipitation.

   If there is a product in the no template control, consider the multiple possible ways template may have been accidentally introduced into your reaction. This is extremely important if you routinely generate amplicons using a given primer pair and/or work with plasmid clones containing these sequences. To avoid future contamination, always wear gloves, use barrier pipet tips, autoclave all plasticware, dissolve primers, nucleotides, etc. in PCR-grade (sterilized, filtered) water. Many investigators set up PCRs in a hood, using designated pipetmen and solutions. Regardless, it is good practice to avoid handling pre- and post-PCRs simultaneously.

   Prior to sequence analysis, the PCR products must then be recovered free from the PCR primers, as these primers could compete with the sequencing primer, resulting in multiple simultaneous sequencing reactions, and a thoroughly ambiguous gel. The PCR products may be purified by commercially available PCR cleanup kits, and/or digestion with exonuclease I and shrimp alkaline phosphatase, which digest single-stranded DNA (i.e., primers and incomplete PCR products) and unconsumed dNTPs. Alternatively, PCR products may be purified by extraction from a low-melt agarose gel. Protocols for recovering DNA from agarose gels are presented in ***(5,6)*** This approach is recommended if the PCR contains either more than one readily visible PCR product or if there is a single band with significant "smearing."
4. Sequencing. The sequencing protocols are straightforward and optimized to avoid many of the major artifacts associated with sequencing. The p53 coding

sequences are devoid of troublesome regions, such as tandem repeats and GC rich regions. In addition, the templates are fairly small (<300 bp), so they should be resolved on a single gel. They can also be sequenced in both directions (i.e., with forward and reverse primers) to resolve any ambiguities.

As loss of heterozygosity (LOH)/loss and/or mutation of p53 is common in tumors, a tumor may contain two normal alleles, one normal allele and one mutant allele, two alleles bearing different mutations, or only one mutant allele. Ideally, a known wild-type sequence should be run alongside the tumor samples for comparison. In a true heterozygote, there should be two bands of roughly equal intensity at the same position. If the band corresponding to the wild-type nucleotide is substantially weaker then the mutant, it could be due to residual normal (stromal) tissue within the tumor.

5. SSCP of human p53. For SSCP, the primers need to be as stringent as possible. The calculated melting point for SSCP primes (for all primers) should be at least 60°C, and average 64°C (use 21- to 24-mers). PCR primers should be adjusted to a final concentration of 20 μ*M* (20 pmoles/μL).

   Optimum fragment length for best resolution is about 150 bp. The longer they are, the less optimal the resolution is.

   Some PCR products have to be run on PAGE under specific conditions. p53 exon 5 should be run at room temperature with a fan blowing at the plate to dissipate the heat, with 6% glycerol added into PAGE. p53 exons 6, 7, and 8 should be run in the cold room with 2 fans blowing at the plate from both sides.

   Controls that can be used for p53 SSCP include nondenatured DNA (2 μL/25 μL without stop solution to get nondenatured band) and human cell lines with mutations for p53, specifically exon 8: SW 480 colon carcinoma at codon 273, exon 7: S 164 at codon 245, exon 7: ATCC HT-3 cervical carcinoma at codon 245, exon 6: ATCC T 47 D/ HT 13 133 ductal breast CA at codon 194, and exon 5: ATCC HOS TE 85 at codon 156.

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Mutational Analysis of p53 in Human Tumors

Immunocytochemistry

Neda Slade and Ute M. Moll

Summary

Mutations of the *p53* gene are the most common genetic changes in human malignancies; therefore their detection is of practical importance. In contrast to wild-type p53 in resting normal cells, mutant p53 proteins are easily detectable by immunocytochemical methods due to their abnormally extended half-life. Several methods of immunocytochemistry can be used to analyze the presence and localization of p53 protein in cells or tissues. The most important is immunocytochemical p53 staining of sections from paraffin embedded tissues. This method is used as a relatively reliable surrogate marker for p53 mutations and has the advantage of being easy, fast, and suitable for mass screening of large archival tissue banks. Frozen sections can also be used to detect and localize the p53 proteins. p53 can also be detected in tissue culture cells. p53 can be detected *in situ* through a secondary antibody coupled to a fluorescent dye or an enzymatic activity that reacts with certain chromogens.

Key Words

p53 mutation, immunoperoxidase staining, immunofluorescence, frozen sections, paraffin sections, cytological specimens

1. Introduction

Immunocytochemistry is a method that enables the identification of a tissue constituent *in situ* by means of a specific antigen–antibody interaction with a labeled antibody. With this simple, cheap, and rapid method, it is possible to demonstrate both the presence and location of a particular molecule of interest ***(1,2)***. In addition, a semiquantitative estimate about the relative amount of antigen can be made (undetectable, weak, moderate, strong), and the antigen

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content from cell to cell and among different cell types within a tissue can be compared.

Missense mutations of the *p53* gene are the most common genetic change in human malignancies ***(3)***. The DNA binding region of p53 is targeted in 90% of all cases. Biochemically, p53 can act as a transcription factor that controls key genes involved in cell death and cell cycle regulation. p53 mutations result in a loss of tumor suppressor functions.

Normal wild-type p53 protein has such a short half-life (30 min) that it is undetectable by immunocytochemical methods. In contrast, mutant p53 proteins frequently have greatly extended half-lives (up to 9 h), leading to stable nuclear accumulation in all cells that carry the mutation. Hence, their easy detectability provides a practical surrogate marker for the presence of missense mutations. While this formula is true for a wide variety of tumor types, including those from bladder, colon, breast, brain, and lung, it is important to be aware of a distinct subset of human cancer types that harbor nuclear p53 overexpression without genetic alteration. These include testicular cancers ***(4–8)***, head and neck tumors ***(9)***, childhood liver tumors ***(10)***, the usual type of endometrial carcinoma ***(11)***, uterine papillary serous carcinoma ***(12)***, primary peritoneal carcinoma ***(13)***, and acute myelogenous leukemia ***(14)***. Similarly, in some breast and colon cancers and most undifferentiated neuroblastomas, abnormally stable wild-type p53 protein (due to HDM2-resistence) is hyperactively exported from the nucleus and, thus, accumulates in the cytoplasm, causing an impaired nuclear function ***(15,16)***. These wild-type but functionally impaired nuclear or cytoplasmic p53 phenotypes can also be detected by immunocytochmistry in cancer tissues.

In normal unstressed cells, p53 is a short-lived protein (a half-life of 20–30 min), which is maintained at very low levels through continuous degradation. Conversely, a hallmark of all cellular stress pathways that signal to p53 is the rapid stabilization of p53 via a block in its degradation. HDM2, through its complex with p53, is the principal regulator that destabilizes p53 protein levels ***(17,18)*** and promotes p53 degradation through a ubiquitin-dependent pathway on nuclear and cytoplasmic 26S proteasomes ***(19,20)***. HDM2 harbors a p53-specific E3 ubiquitin ligase activity ***(21)***, housed at the C-terminal RING finger domain ***(22,23)***, which transfers multiple ubiquitin tags onto lysine residues, mainly in the C terminus of p53. Biochemical evidence exists that *mdm2*-mediated p53 ubiquitination takes place in the nucleus in a complex with the large p300/CBP protein, possibly serving as a scaffolding ***(24)***. Thus, HDM2 is largely responsible for the rapid p53 turnover in undamaged cells ***(25)***. By the same token, mutant p53 proteins in tumor cells are stable, because they are deficient in transactivating HDM2; hence, they have a defective negative feedback loop.

The very low levels of wild-type p53 in unstressed cells and tissues render them undetectable by immunocytochemical methods. However, when cells with functional p53 undergo DNA damage, a short-lived physiological damage response is evoked, leading to transient (up to 48 h) nuclear p53 stabilization that can be detected by immunocytochemistry ***(26)***. Increased amounts of active p53 lead to either G1 arrest or apoptosis, thereby protecting the affected cells from proliferating without repairing their damaged DNA and, thus, preserving genetic stability (reviewed in **ref.** ***27***). Importantly though, the p53 overexpression pattern in tissues under such circumstances is transient and heterogenous in both proportion and intensity, due to the stochastic nature of the DNA damage event and a possible dependence of the p53 response on cell cycle stage ***(26,28)***.

To analyze the presence and localization of p53 protein in cells or tissues, several methods of immunocytochemisty are used. The anti-immunoglobulin antibodies labeled with fluorescent dye are widely used in immunofluorescence to detect the p53 protein in tissue culture cells grown on cover slips. Instead of fluorescent dye, the antigen can also be detected through a secondary antibody coupled to an enzymatic activity. The most common enzymes that have been used in immunocytochemistry are horseradish peroxidase (HRP) and alkaline phosphatase. For each enzyme, a permanent, water-insoluble chromogenic substrate exists that identify the antigen *in situ*.

2. Materials

2.1. Immunostaining of Paraffin Sections

2.1.1. Immunoperoxidase Staining

1. Gloves.
2. Forceps.
3. Staining racks and dishes for solution incubation.
4. Xylene.
5. Absolute ethanol.
6. 95% Ethanol.
7. 70% Ethanol.
8. Microwave oven.
9. Plastic coplin jar for microwave.
10. 1% Zinc sulfate–water or citric acid buffer (0.01 *M* citric acid in distilled water [dH_2O], pH 6.0)
11. Phosphate-buffered saline (PBS) (*see* **Note 1**).
12. Humid chamber–level tray.
13. 30% H_2O_2.
14. 10% Normal serum from the species in which the secondary antibody was generated (usually goat serum; Zymed Laboratories, South San Francisco, CA, USA).

15. Bovine serum albumin (BSA), prepare 2% BSA in PBS.
16. Primary p53 antibody (*see* **Table 1**).
17. Histostain SP kit: SA System kit for immunohistological staining for mouse antibodies (Zymed Laboratories; cat. no. 95-6543B) or biotinylated secondary antibody and streptavidin–HRP diluted in PBS.
18. 3',3'Diaminobenzidinetetrachloride (DAB) (Sigma, St. Louis, MO, USA): 0.06% DAB in 0.05 *M* Tris buffer, pH 7.6 (*see* **Note 2**).
19. Hematoxylin.
20. Mounting medium.
21. Cover slips.
22. Light microscope.

2.1.2. Alkaline Phosphatase Labeling for Immunocytochemistry

1. As for **Subheading 2.1.1.**, **steps 1–12**, **14–16**, **19**, and **22**.
2. Streptavidin–alkaline phosphatase concentrate (BioGenex Laboratories, San Ramon, CA, USA; cat. no. HK 321-UK) use as a 1:20 dilution (supersensitive category).
3. 0.05% Triton® X-100 (0.5 mL/1 L PBS).
4. Substrate Pack kit (BioGenex Laboratories; cat. no. HK-182-5K) contains Fast Red tablets and Naphtol Tris dilution buffer.
5. Lavamazole (inhibitor of all other alkaline phosphatase, but not intestinal alkaline phosphatase), 6 mL stock solution (30 mg/mL PBS), use 2 drops/5 mL of buffer (BioGenex Labortories; cat. no. HK-113-5K).
6. Crystalmount (30 mL) (Fisher Scientific, Pittsburgh, PA, USA; cat. no. BM-MO2).

2.2. Immunostaining of Frozen Sections

2.2.1. Tissue Preparation for Frozen Sections

1. Gloves.
2. Metal pan.
3. Aluminum foil.
4. Forceps.
5. 2-Methylbutane (Fisher Scientific).
6. Styrofoam bucket.
7. Liquid nitrogen.
8. Embedding media: optimal cutting temperature (OCT) or TISSUE TECH.
9. Poly-L-lysine (Sigma) (*see* **Note 3**).
10. Superfrost slides.
11. Acetone.

2.2.2. Immunoperoxidase of Frozen Sections

1. Humid chamber.
2. PBS (*see* **Note 1**).

Table 1
Recommended p53-Specific Antibodies for Analysis of Human Tumor Tissues

Species name	Working dilution frozen	Working dilution paraffin	Source	Reference
mouse PAB 1801	0.5–1 μg/mL	0.5 μg/mL needs Ag-retrieval	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	***29***,***30***
mouse DO-1	0.5 μg/mL	0.5 μg/mL needs Ag-retrieval	Santa Cruz Biotechnology	***31***
mouse DO-7	1:100–1:200	1:100–1:200	Vector Laboratories (Burlingame, CA, USA)	***31***
rabbit CM-1	no	1:1000–1:1500	Vector Laboratories	***32***

See **Note 11**.

3. 10% Normal serum from the species in which the secondary antibody was generated (usually goat serum).
4. BSA, prepare 2% BSA in PBS.
5. Primary p53 antibody (*see* **Table 1**).
6. Histostain SP kit: SA System kit for Immunohistological staining for mouse antibodies or biotinylated secondary antibody and streptavidin–HRP diluted in PBS.
7. DAB: 0.06% DAB in 0.05 M Tris buffer, pH 7.6 (*see* **Note 2**).
8. Hematoxylin.
9. Xylene, absolute ethanol, 95% ethanol.
10. Mounting medium.
11. Cover slips.
12. Light microscope.

2.3. Immunostaining of Cytological Specimens

2.3.1. Immunofluorescence

1. Gloves.
2. Falcon® Culture Slide (8 chamber slide; Becton Dickinson Labware, Bedford, MA, USA; cat. no. 354108) or cover slips coated with poly-L-lysine (*see* **Note 3**) and 6-well plate.
3. PBS (*see* **Note 1**).
4. BSA, prepare 2% BSA in PBS.
5. Methanol:acetone (1:1), ice-cold, keep at –20°C.
6. 10% Normal serum from the species in which the secondary antibody was generated (usually goat serum).
7. Primary p53 antibody (*see* Table 1) diluted in 2% BSA/PBS.
8. Secondary antibody, fluorescein isothiocyanate (FITC)-labeled, diluted in 2% BSA/PBS.
9. Slow Fade Light Antifade kit (Molecular Probes, Eugene, OR, USA; cat. no. S-7461).
10. Fluorescence microscope.

2.3.2. Immunoperoxidase Staining

1. Gloves.
2. PBS (*see* **Note 1**).
3. BSA, prepare 2% BSA in PBS.
4. Methanol:acetone (1:1), ice-cold, keep at –20°C.
5. 10% Normal serum from the species in which the secondary antibody was generated (usually goat serum).
6. Primary p53 antibody (*see* **Table 1**).
7. Histostain SP kit: SA System kit for immunohistological staining for mouse antibodies or biotinylated secondary antibody and streptavidin–HRP diluted in PBS.
9. DAB: 0.06% DAB in 0.05 M Tris buffer, pH 7.6 (*see* **Note 2**).
10. Hematoxylin.
11. Mounting medium.

12. Cover slips.
13. Light microscope.

3. Methods

Important: never let cells or sections dry out! This will lead to a very strong background, preventing interpretation of the staining result. Always use controls (*see* **Note 4**).

3.1. Immunoperoxidase Staining on Paraffin Section

The use of immunocytochemical methods is the most important in the examinations of tissues. Since the paraffin-embedded tissue architecture and cell morphology remains well-preserved and the tissues are accessible for retrospective studies, they are preferred for immunohistochemical analysis. The tissue should be properly fixed and embedded into paraffin as soon as possible to be maximally preserved.

1. 5 μm Paraffin sections on Plus slides, dry at 37°C on hot plate (do not bake at 57°C).
2. Deparaffinize, then rehydrate slides up to water (*see* **Note 5**).
3. Antigen retrieval (*see* **Note 6**): microwave 2× for 5 min in 1% zinc sulfate or citric acid buffer. Let cool down in the solution.
4. Wash 2× in PBS for 5 min.
5. Quench endogenous peroxidase in 1% H_2O_2 in PBS for 15 min.Wash 3× in PBS for 5 min.
6. Block with 10% normal serum for 20 min (from the same species in which biotinylated secondary antibody is produced, usually goat).
7. Drain slides, but do not wash.
8. Overlay the tissue with primary p53 antibody (*see* **Table 1**), (50–100 μL/slide) diluted in 2% BSA/PBS, and incubate for 1 to 2 h at room temperature or at 4°C overnight (*see* **Note 7**).
9. Drain, quick rinse in PBS, then wash 3× for 5 min in PBS.
10. Apply biotinylated secondary antibody for 45 min at room temperature.
11. Drain, quick rinse in PBS, then wash 3× for 5 min in PBS.
12. Overlay the tissue with streptavidin–HRP in PBS for 15 min.
13. Wash 3× for 5 min in PBS. Hold in PBS until ready for chromogen.
14. Overlay the sections with chromogen (DAB plus H_2O_2) (*see* **Note 2**).
15. Stop the reaction at endpoint of 8–10 min by washing under tap water for 5–10 min.
16. Counterstain with weak hematoxylin, if desired.
17. Run through dehydration chain (*see* **Note 8**), embed in mounting media, cover slip, and blot on paper towel.

3.2. Alkaline Phosphatase Labeling for Immunocytochemistry

1. Repeat **steps 1–4** from **Subheading 3.1.** (Never ever quench with H_2O_2).
2. Repeats **steps 6–9** from **Subheading 3.1.**

3. Apply alkaline phosphatase-conjugated streptavidin, diluted 1:20 in PBS, incubate for 30 min at room temperature.
4. Wash 3× in PBS containing 0.05% Triton X-100 (0.5 mL/1 L PBS).
5. Substrate preparation: dissolve 1 tablet Fast Red in 5 mL Substrate Dilution Buffer containing 2 drops of Lavamazole stock. Vortex mix for 2 min until dissolved. Filter. Use within 2 h.
6. Incubate for 10 min at 37°C.
7. Wash under tap water for 3–5 min.
8. Counterstain with weak hematoxylin, if desired.
9. Embed in Crystalmount. Bake for 10 min at 50°C. No cover slip. (Never go through dehydration chain.)

3.3. Snap-Freezing of Freshly Harvested Tissue for Immunocytochemistry

This is a general snap-freezing protocol and works very well.

Sections of snap-frozen tissue can be used to detect the antigens that are destroyed during tissue fixation and processing of paraffin embedding.

1. Cut a slice of freshly harvested tissue (only about 10 x 10 x 5 mm, because bigger pieces do not freeze properly). Line a little metal pan with aluminum foil (avoid folds).
   Optional: fill with embedding media (OCT or TISSUE TECH) to 1 to 2 mm and push flat face of tissue gently down with forceps (optional: cover tissue with more embedding media).
2. Pour 40 mL of 2-methylbutane into thick-walled beaker and dip it into bucket (styrofoam) containing liquid nitrogen (N_2 level should be lower than beaker). When butane stops foaming and splattering, dip OCT-embedded tissue into chilled butane and submerse for 8–10 s while moving up and down (always submersed). OCT will freeze and appear white. The tissue should be stored at –20° or –80°C (ship on plenty of dry ice). The purpose of using butane as a direct freezing medium rather than liquid N_2 is that it cools only to –140°C, which avoids freezing artifacts and the tissue freezes more evenly.

3.4. Frozen Sectioning and Fixation for Immunocytochemistry

1. Cut 5 μm sections of snap-frozen tissue on cryostat, collect them on poly-L-lysine-coated Superfrost slides, and label with pencil (*see* **Note 3**).
2. Air-dry for 20 min and desiccate overnight at room temperature (all the moisture has to be out).
3. Fix for 10 min in 100% acetone (can be reused many times).
4. Air-dry for 40 min.
5. Wrap and label each slide in aluminum foil. Store at –80°C until needed. Before staining, let slides air-dry at room temperature for 30 min.

3.5. Immunoperoxidase Staining on Frozen Section

1. Take fixed slides from –80°C and let them equilibrate to room temperature before unwrapping.
2. Wash 3× for 3 min in PBS.
3. Repeat **steps 6–17** from **Subheading 3.1.**

3.6. p53 Immunofluorescence Protocol

The technique of immunofluorescence is used for frozen tissue sections or, more commonly, for tissue culture cells grown on cover slips or culture chambers. It provides a sensitive method for the detection and localization of specific antigens. Flouorescent molecules (fluorocromes) can be directly bound to primary p53 antibody (*see* **Table 1**) or indirectly to a secondary antibody. The fluorochromes most widely used are FITC (apple green emitting), rhodamine, and cyanines (Cy™3 and Cy5), red emitting.

1. Grow cells on Falcon culture slides. Cell density is crucial and should be about 50%. Too high density impedes with staining. An alternative is to grow cells on sterilized poly-L-lysine-coated glass cover slips in 6-well plates (*see* **Note 3**).
2. Rinse slides carefully (check cells to see if they are washing off) and quickly with PBS and fix in ice-cold solution of methanol:acetone (1:1) for 3 min. Air-dry well for 10 min (*see* **Note 9**).
3. Wash 3× for 3 min in PBS.
4. Overlay each well (or cover slip) with 50 µL of 10% goat serum to block, and arrange in humidified chamber. Incubate for 20 min at room temperature.
5. Quick rinse with PBS.
6. Overlay each well with 50 µL of primary p53 antibody (*see* **Table 1**) (diluted with in 2% BSA) in a humidified chamber. Incubate for 2 h at room temperature.
7. Wash 3× for 3 min in PBS.
8. Add 50 µL of anti-mouse secondary antibody FITC-labeled and incubate for 45 min at room temperature in the dark (*see* **Note 10**).
9. Wash 3× for 3 min in PBS.
10. Remove chamber seal and cover slip with Slow Fade Light Antifade kit. For cover slips: flip onto a drop of Antifade Kit on a glass slide.
11. Examine on the fluorescent microscope using appropriate filter.

3.7. Immunoperoxidase Staining of Tissue Culture Cells

1. Prepare the cells the same way as for immunofluorescence **Subheading 3.6.**, **steps 1–7**).
2. Incubate in biotinylated secondary antibody for 45 min at room temperature.
3. Wash 3× for 3 min in PBS.
4. Incubate with streptavidin–HRP in PBS for 15 min at room temperature.

5. Wash 3× for 3 min in PBS.
6. DAB staining for 5–10 min (depends on the cell system) (*see* **Note 2**).
7. Wash gently under tap water for 5 min.
8. Remove chamber.
9. Counterstain with weak hematoxylin, if desired.
10. Dehydrate all the way up to xylene (*see* **Note 8**), remove silicone gasket with forceps, and embed in mounting media, cover slip, and blot on paper towel.

4. Notes

1. Preparation PBS: prepare stock 1, 455 μ*M* Na_2HPO_4 (64.6 g/L); and stock 2, 85 μ*M* NaH_2PO_4 (11.7 g/L). Take 200 mL of each stock and 87.7 g of NaCl and add dH_2O up to 10 L.
2. Preparation of DAB solution: dissolve one 10-mg tablet in 16 mL of 0.05 *M* Tris buffer (pH 7.6) to get 0.06% DAB. Filter through Whatman paper. Freeze in aliquots at –20°C; no longer use when brown. To use, thaw in warm water in a light-protected place, and add 30% H_2O_2 (2 mL DAB plus 1.5 μL H_2O_2) and mix. Immediately use to overlay sections and observe reaction. DAB is a potential carcinogen, so it is necessary to handle with extreme precautions. The contaminated dishes should be cleaned up with 30% bleach. DAB is available in tablets what is better to reduce contact with chromogen.
3. Poly-L-lysine coating of glass slides: use precleaned slides or clean with 1% HCl in 70% ethanol. Allow diluted poly-L-lysine to come to room temperature before use. Place racks of clean slides into solution for 5 min. Drain slides and dry in 60°C oven for 1 h or at room temperature overnight. Working solution: dilute poly-L-lysine 1:10 with double-distilled water (ddH_2O). Filter after use and store at 4°C. Stable for at least 3 mo. Alternatively, the slides could be coated with silane, or "Plus" slides can be used. They are negatively charged glass slides that do not require coating with poly-L-lysine or silanization.
4. It is very important to use controls to be sure that the reaction is specific. Control slides of known reactivity should be run in each experiment, as well as the negative control. Possible negative controls are: (*i*) substitute primary antibody by mouse or rabbit normal immunoglobulin (IgG) at same concentration; (*ii*) substitute primary antibody by blocking (normal) serum (1:10 in 2% BSA in PBS); (*iii*) substitute primary antibody or secondary antibody or streptavidin–HRP by 2% BSA in PBS; and (*iv*) primary antibody pre-incubated with excess purified Ag to preabsorb.
5. Deparaffinize 3× for 3 min with xylene. Rehydrate 2× with absolute ethanol (1 min and 3 min, respectively), once with 95% ethanol for 1 min and then with 70% ethanol for 1 min.
6. Some antigens are altered, destroyed, or masked during the process of fixation and embedding of tissue, which may influence their detection. To increase the sensitivity, sometimes the pretreatment is necessary. Heat-induced epitope retrieval is the most effective in paraffin-section immunohystochemisty. The most popular retrieval buffers are 1% zinc sulfate and citric acid buffer (0.01 *M* citric acid in dH_2O, pH 6.0).

7. Drying danger during long incubation with primary antibody.
8. Dehydratation process: dip the slides 2× for 1 min into 95% ethanol, 2× for 1 min into absolute ethanol, and 3× for 3 min in xylene.
9. At this point, you can mail them with the cover on. Please pack them well in their original trays, because the chambers come off easily.
10. If primary antibody is made in rabbit, use anti-rabbit secondary. The secondary can be labeled with another fluorocrome, such as rodamine or cyanine to get red color.
11. Although some have suggested to use mouse monoclonal PAB 240 as an antibody that is capable of detecting mutant p53 "pan-mutant" antibody, it does not work reliably in tissue. Potentially use only in immunoprecipitations.

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17

Flow Cytometric Analysis of p53-Induced Apoptosis

Michael Berger and Ygal Haupt

Summary

The p53 protein is a key player in the cellular response to stress conditions. Activation of p53 induces growth inhibition in the form of cell growth arrest or apoptosis. The latter plays an important role in the tumor suppression function of p53. It is therefore of great interest to understand in detail the mechanisms by which p53 induces apoptosis. In this chapter, we describe a flow cytometric assay for the measurement of p53 apoptotic activity. This assay is applicable for exogenously expressed p53 by transfection as well as for endogenous p53. The p53 protein is detected by intra-cellular fluorescent staining of p53 or by tagging p53 with GFP. The extent of apoptosis in cells expressing p53 is determined by cell cycle distribution using flow cytometry. The protocol described here can be employed to study the regulation of p53-medaited apoptosis and can be broadly applied to other apoptotic proteins.

Key Words

apoptosis, p53, sub-G1, flow cytometry (FACS), GFP

1. Introduction

The p53 tumor suppressor protein is a transcription factor that plays a key role in the prevention of cancer development. In response to various stress signals, p53 is accumulated and activated to induce cell cycle arrest, thereby preventing the replication of damaged DNA. Under certain stress conditions, or in response to oncogenic events, p53 induces programmed cell death (apoptosis). These growth inhibitory activities are pivotal to tumor suppression by p53. The promotion of cell growth arrest permits the repair of the damaged DNA, whereas the induction of apoptosis ensures the elimination of cells with a potential to become cancerous. Intensive research over the past decade vastly improved our understanding of how p53 exerts these activities. In this chapter, we will describe a flow cytometric assay for studying p53-mediated

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apoptosis. We will discuss various approaches for performing this assay and will outline the pros and cons of this assay.

1.1. Measurements of p53-Mediated Apoptosis

Apoptosis is a suicide program that occurs naturally in multicellular organisms in response to physiological signals. The apoptotic process is essential for many different processes, as varied as the maintenance of the immune response, to proper embryonal development. The apoptotic activity of p53 is essential for the elimination of defective and potentially carcinogenic cells. p53 induces apoptosis by multiple mechanisms involving both transcription-dependent and -independent pathways. These include the generation of reactive oxygen species, depolarization of the mitochondrial membrane, and the activation of caspase cascades ***(1)***.

The apoptotic processes is characterized by morphological and biochemical features. These include blebbing of the plasma membrane, exposure of phosphatidylserine on the outer plasma membrane, condensation of the chromatin, and internucleosomal DNA fragmentation. We take advantage of the last two features in order to detect apoptotic cells in the assay described here. The DNA content in arrested diploid cells is 2N. During S (Synthesis) phase, the amount of DNA increases from 2N to 4N and remains 4N until mitosis, when the DNA content is divided to 2N for each daughter cell. A decrease in the total DNA content in apoptotic cells results from a number of events: (*i*) fragmented DNA leaves the apoptotic cells by diffusion; (*ii*) chromatic condensation reduces the accessibility of the DNA dye to the DNA; and (*iii*) some of the apoptotic cells fragment into apoptotic bodies, most of which contain less than the total DNA content. Cells with DNA content of less than 2N, referred to as sub-G1 DNA content, are dead cells and have been previously authenticated as apoptotic cells using other markers of apoptosis (e.g., ***2***).

The assay for the apoptotic activity of p53 described here is based on the transient transfection of cells using expression plasmids for p53. Cells are stained for p53 using a fluorescent marker, and the successfully transfected subpopulation is identified according to their fluorescent intensity. The transfected and nontransfected subpopulations are discriminated, and the cell cycle distribution of each is analyzed separately. This assay provides a measurement of the apoptotic fraction in each population. The detection of p53 and staining of DNA requires the prior permeabilization of the cells by fixation. This has two purposes: (*i*) to allow the entrance of antibodies and the DNA dye, propidium iodide (PI), into the cell and into the nucleus; and (*ii*) to allow the fragmented DNA to exit the apoptotic cells, a process that is essential for the detection of DNA loss. As discussed below, the type of fixation is important and can be critical for carrying out this protocol successfully.

2. Materials

Caution must be taken when using PI, which is a potentially mutagenic substance that can be absorbed through the skin or by inhalation. It is recommended to wear suitable protective clothing, including gloves and eye protection, when handling PI. The following materials are required for the assay.

1. Cells and suitable medium, serum, and other relevant supplements (depending on the cell type under study).
2. Tissue culture grade dishes (92 mm).
3. Trypsin solution (0.02% ethylenediamine tetraacetic acid [EDTA], 0.25% trypsin) for lifting adherent cells from the plates. Store the solution at 4°C and warm up to 37°C before use.
4. Polystyrene conic centrifuge tubes (15 mL) for centrifugation and 5-mL polystyerene round-bottom tubes (12 × 75 mm style) for the use in the flow cytometer.
5. Phosphate-buffered saline (PBS).
6. PI (5 μg/mL final), stored at 4°C at 5 mg/mL stock. Lyophilized PI should be dissolved in double-distilled water.
7. RNase I solution stored at 4°C as 10 mg/mL stock solution. Inactivate DNases by boiling for 15 min.
8. Nylon mesh (100 μ*M*).
9. Analytical grade methanol (100%) and cool to –20°C before use.
10. Anti-p53 antibodies, either directly conjugated to fluorescein isothiocyante (FITC) or nonconjugated. The antibody can be purified from ascetic fluid or a supernatant from hybridoma. For detecting human p53, we recommend using a mixture of two monolocal anti-p53 antibodies: DO1 and PAb 1801. For detecting mouse p53, we recommend using CM5 polyclonal antibody or PAb 421 monoclonal antibody. Other antibodies work well for both human and mouse p53.
11. A FITC-conjugated anti-mouse secondary antibody is required if the primary anti-p53 antibody is not directly conjugated to fluorescent marker.
12. Flow cytometer (e.g., FACScalibur™, FACScan™, FACSort, and FACS® Vantage), computer, and appropriate software (e.g., CellQuest or LYSYS™ II).

3. Methods

p53-transfected cells can be detected by intracellular staining of p53, using anti-p53 antibodies, or by using a tag, such as green fluorescent protein (GFP), either fused to p53 or co-expressed along with p53. In the following sections, we describe the three different options for detecting p53 in the transfected cells. In each subheading, the pros and cons of each approach will be outlined. While the protocol describes a transient transfection assay, it can easily be adapted for nontransfection experiments assessing the apoptotic activity of endogenous p53.

3.1. Apoptotic Assay for Transiently Transfected p53: Nontagged

The transient transfection apoptotic assay has several advantages over the stable transfection approach. First, as the name implies, the assay is short (between 1–3 d). Second, the assay is versatile and adjustable, allowing the assessment of various combinations of proteins at different ratios between the proteins assayed and under different experimental conditions. Third, there is no clonal selection of cells for certain growth or survival characteristics. Hence, the results represent the response of a heterogenous population. The major pitfall of transient transfection is that the transfected proteins are often overexpressed. This problem can be addressed in two ways: (*i*) the DNA may be introduced via a retroviral delivery system, which often results in a lower expression level of the recombinant protein, closer to the range of physiological expression levels; (*ii*) a weak promoter to drive p53 expression may be employed. In the analysis stage, region 1 (R1) may be adjusted to include only cells with lower p53 expression (*see* **Note 1**).

1. Plate out cells at semiconfluent density, 24 h before transfection.
2. Transfect cells with p53 expression plasmid (between 0.1–5 μg/sample; *see* **Note 2**) alone or together with an expression vector(s) encoding a desired protein(s). Calcium phosphate method or other transfection reagents, which do not cause much cell death and yet achieve high efficiency, are suitable. The use of electropotation, for example, is not suitable because of the high levels of cell death inflicted by the transfection itself (*see* **Note 3**).
3. At 24–72 h posttransfection, harvest the cells (*see* **Note 2**). In the case of adherent cells, collect the cells by combining the floating cells from the medium with the adherent cells. Adherent cells are lifted by trypsinization and are combined with the floating cells in the same tube. The dish is washed once with PBS, and the washed cells are added to the same tube. It is important to collect the floating cells, since these are often dying cells.
4. Wash cells by spinning at 1200 rpm (340*g*) in a tabletop centrifuge for 5 min at 4°C.
5. Discard the supernatant and resuspend cells in 300 mL of cold PBS. It is very important to bring the cells into a single-cell suspension before the next step of fixation. Any clumps of two or more cells will be fixed as such and can cause blockage in the flow cytomteric tubing during acquisition.
6. Fix the cells by adding 5 mL of cold (–20°C) methanol (*see* **Note 4**) in a dropwise manner, while the tube of cells are vortex mixed at medium speed. The slow addition of the fixative during agitation is done in order to fix single cells and avoid the fixation of cell clumps (*see* **Note 5**).
7. Incubate the sample at –20°C overnight. Methanol fixation permeates the cell membrane, and the long incubation in methanol allows for sufficient time for the fragmented DNA to leave the cells. This step facilitates the detection of cells with sub-G1 DNA content. The samples can be stored for longer periods (up to several

weeks) given that the sample does not dry out. This provides a convenient step for combining samples from kinetic studies having multiple harvest time points.

8. Following fixation, wash the cells by spinning at 2000 rpm (960*g*) in a tabletop centrifuge for 5 min at 4°C. Resuspend the cells in 1 mL of cold PBS. Leave the cells for 30 min at 4°C to allow for rehydration. Cells can be kept at 4°C at this stage for a longer period (up to several days).
9. Spin the cells as in **step 8**. Resuspend cells in 100 µL of antibody solution. In the case of purified antibody, whether directly conjugated to FITC or not, use between 2–10 µg/mL of antibody. The optimal concentration of the antibody must be determined empirically in advance. If the source of the antibody is from a hybridoma supernatant, resuspend the cells in 100 µL of undiluted supernatant.
10. Incubate the cells at room temperature for 1 h with occasional gentle mixing of the cells.
11. Rinse the cells by adding 5 mL of PBS and spin as in **step 8**.
12. If a nonconjugated primary antibody is used, resuspend the cells in PBS solution containing FITC-conjugated anti-mouse secondary antibody. As in the case of the primary antibody, the concentration should be determined in advance using a known concentration of primary antibody. Incubate in the dark for 30 min at room temperature with occasional mixing.
    Note: from this stage, keep the samples in the dark as much as possible.
13. Rinse the cells by adding 5 mL of PBS, and spin as in **step 8**.
14. Resuspend the cells in PBS (to achieve 1×10^6 cells/mL) (*see* **Note 6**).
15. Add RNase A to a final concentration of 50 µg/mL and incubate at room temperature for 20 min in the dark. RNase is used to degrade the RNA in the cells, which otherwise contributes to the overall staining by the PI.
16. Filter the sample through a nylon mesh (100 nm) to remove cell clumps and any debris in the sample.
17. Add PI to a final concentration of 5 µg/mL and acquire data in the fluorescence-activated cell sorter (FACS) (*see* **Subheading 4**).

3.2. Apoptotic Assay for Transiently Transfected GFP-p53 or Co-Expressed GFP

An alternative method for detecting p53-transfected cells is by using a tag. A variety of tags are available ***(3)***; however, most of the tags require a specific antibody to recognize them. Hence, the main advantage in using such a tag over direct detection of p53 is when there is a need to distinguish between exogenous p53 from the endogenous protein. The use of naturally fluorescent proteins, such as the GFP, provides an attractive alternative. There are two major advantages in using GFP to detect transfected cells over the direct staining of p53. First, fixed cells can be analyzed directly, bypassing the staining procedure, which saves time and expensive reagents. Second, it avoids the potential problem of background staining. Two approaches for the use of GFP as a marker can be used: (*i*) use a GFP-p53 fusion protein (e.g., ***4***) and detect

p53 according to the GFP fluorescence, since every GFP positive cell also expresses p53; and (*ii*) co-express p53 together with an expression plasmid for GFP. This alternative can be problematic, since there is a selection for cells expressing GFP alone, which presumably proliferate normally, whereas cells expressing both GFP and p53 either enter growth arrest or apoptosis. The longer the experiment is carried out, the greater the effect of the selection becomes. In order to minimize this selection, the ratio of expression plasmids for p53 and GFP should be 1:10, respectively. An additional problem with using co-transfected free GFP is the small size of the GFP protein (29 kDa), which tends to leave the cells following methanol fixation. For this purpose, a modified GFP construct, which can be anchored to the cell (e.g., ***5***) should be used. Regardless of whether the GFP is free or fused to p53, its effect on apoptosis in the experimental system under study should be determined. Hence, a negative control sample of GFP alone must be added to these apoptotic assays.

1. Follow steps 1–8 from **Subheading 3.1.1.**
2. Spin cells at 2000 rpm (960*g*) in a tabletop centrifuge for 5 min at 4°C.
3. Continue from **step 14** in **Subheading 3.1.1.**

3.3. Apoptotic Assay for Endogenous p53

The analysis of p53-mediated apoptosis in cells expressing endogenous p53, wild-type or mutant, does not require staining for p53, unless the expression level is heterogeneous among the population. In this case, follow the basic protocol in **Subheading 3.1.1.**, with the exception that transfection is not relevant, and the comparison is not within a sample, but rather between treated vs nontreated cells.

1. Plate out cells at a semiconfluent density 24 h before transfection.
2. At the desired time after treatment, collect cells by combining floating and adherent cells as described in **Subheading 3.1.1.**, **step 3**).
3. Follow **steps 4–8** as described in the basic protocol (**Subheading 3.1.1.**).
4. Spin cells at 2000 rpm (1660*g*) in a tabletop centrifuge for 5 min at 4°C.
5. Continue from **step 14** of **Subheading 3.1.1.**

3.4. Acquisition of Data

The apoptotic activity of p53 is analyzed by flow cytometry. The transfected cells are identified according to their p53 fluorescent intensity or according to their GFP fluorescent intensity, when appropriate. Each sample is viewed in a dot plot of forward scatter (which measures cell size) vs fluorescent intensity (of FL1) on a logarithmic scale (FL1 = FITC or GFP fluorescence) (**Fig. 1A**). Cells with high fluorescent intensity (in FL1) represent the subpopulation of successfully transfected cells, shown as black dots in **Fig. 1A**. The

nontransfected subpopulation, which represents cells with low fluorescent intensity, is shown as grey dots (**Fig. 1A**). A region is drawn around the transfected subpopulation and is defined as R1 in **Fig. 1A** (*see* **Note 7).** Note that the lower line of the region has a slope, which parallels the natural slope of the population relative to the y-axis. The nontransfected population is defined as all the cells except those belonging to R1 (not R1).

To eliminate doublets and larger aggregates of cells, a second region (R2) is defined. This discrimination (doublet discrimination module [DDM]) is based on PI staining of the cells. This discrimination is based on a comparison of two integral values of the electronic signal, the area and the width of the pulse, for each cell analyzed. Plot the FL3 area (FL3-A) vs the FL3 width (FL3-W), both on a linear scale. This plot provides a characteristic pattern of cell cycle distribution reflecting the DNA content of cells in different phases of the cell cycle. Events with excessively high FL3-A or FL3-W fluorescence represent the staining of two or more cell aggregates. A region is defined around the major population, excluding cell aggregates (R2 in **Fig. 1B**). It is crucial to include events with low fluorescent intensity, since these represent dead cells (marked as sub-G1 in **Fig. 1B**). Only cells falling within the R2 region will be collected. Now, the R1 and R2 regions are used to define the desired subpopulations for acquisition. These are as follows:

1. Transfected population equals R2 and R1 (cells falling within R1 and within R2).
2. Nontransfected population equals R2 and (not R1) (cells falling within R2, but outside R1). Equal number of cells (a minimum of 5000 cells) from each subpopulation is collected separately. The cell cycle distribution of each subpopulation is determined by profiling their DNA content. A histogram plot of Fl2, which measures the fluorescent intensity of PI on a linear scale, is used for this purpose. The cell cycle distribution, according to the DNA content of a diploid cell population, is marked in **Fig. 1C**. Cells falling into the sub-G1 region represent the relative fraction of apoptotic cells in the population (*see* **Note 7**).

3.5. Analysis of Data

The transfected and nontransfected subpopulations from each sample are analyzed (*see* **Fig. 2**). Calculate the percentage of cells with a sub-G1 DNA content by placing a marker encompassing the sub-G1 region (**Fig. 2B**). The marker should begin from the end of the left slope of the G1 peak to 50 channels to the right of the Y-axis. It is important not to draw the marker on the axis, because it will include electronic noise. To avoid this potential problem, choose the FL2 as the threshold, and set the channel value for 50. For each acquisition data, obtain statistical analysis for the number of events in the sub-G1 region (use the stat function in the application CellQuest or LYSYS II).

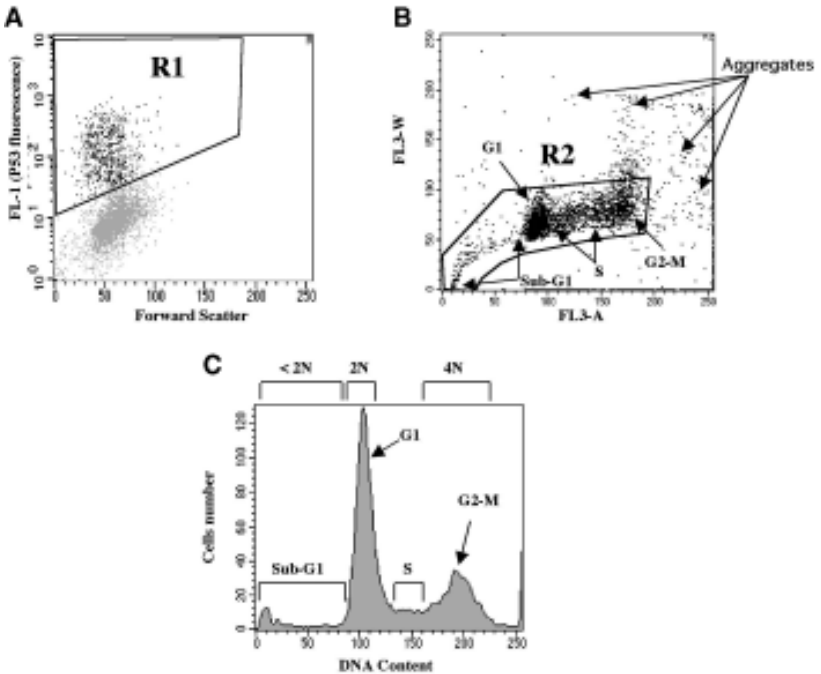

Fig. 1. Flow cytometric analysis of transiently transfected cells. Saos-2 lung adenocarcinoma cells (lacking p53 expression) were transfected with 3 μg of expression plasmid for wild-type human p53. Seventy hours posttransfection, cells were harvested, fixed, and stained with a mixture of anti-p53 antibodies, PAb 1801 and DO1, as described in the basic protocol (**Subheading 3.1.1.**). Cells were then stained with FITC-conjugated goat anti-mouse secondary antibody and simultaneously stained for DNA using PI (5 μg/mL). Cells were analyzed in the flow cytometer (4-color FACScalibur) with respect to their p53 fluorescent intensity in order to separate the brightly stained cells from the rest of the population. The fluorescent intensity of the FL1 is initially calibrated by measuring the non-transfected cells. Panel **A** shows a plot of p53 fluorescence (FL1 on logarithmic scale) vs forward scatter (x-axis). *(Continued on next page)*

The overall proportion of the apoptotic activity of p53 is calculated as the percentage of sub-G1 cells in the transfected population (**Fig. 2D**) minus the percentage of sub-G1 cells in the nontransfected population (**Fig. 2F**). In the example shown in **Fig. 2**, the transfected population had 21% apoptosis, while the nontransfected population had 5% apoptosis, hence p53-mediated in this sample was calculated to be 16%.

4. Notes

1. Certain proteins that regulate p53 activity, such as Mdm2 and HPV-E6, also promote its degradation through the proteasome ***(1)***. It is difficult to weigh the relative contribution of p53 degradation to the overall down-regulation of p53 by these inhibitors. The use of the assay described here allows us to measure the effect of a p53 inhibitor on p53 apoptotic activity in a manner independent of protein degradation. We compare equal numbers of cells, falling into the defined R1 region, from a sample of cells transfected with p53 alone to that of p53 plus an inhibitor. This approach has been used previously to show the effect of Mdm2 on p53 apoptotic activity independent of p53 degradation (e.g., *see* **refs. *7*** and ***8***). Further, it is sometime desirable to study the activity of p53 with relation to its expression levels. The selection of different p53 expression levels can be achieved by defining the R1 region around cells with high, medium, or low fluorescent intensity.
2. p53-mediated apoptosis is time- and dose-dependent. For any given experimental system, it is essential to calibrate both parameters. Kinetics studies using varying concentrations of p53 expression plasmids should be performed. The time window for p53-induced death in transient transfection is between 18 to approx 90 h, depending on the expression levels and the cell type. Kinetics experiments are facilitated by the ability to store the cells at the fixation step, and then continue the experiment at a convenient time. In such studies, it is expected to see a decrease in the transfection efficiency, as measured by the percentage of fluores-

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Fig. 1 *(continued from page 8)* The region R1 was drawn in such as away as to include the brightly stained cells. Cells falling within R1 (black dots) represent the p53-transfected cells, while the cells outside R1 are the nontransfected subpopulation (grey dots). Panel **B** demonstrates the use of the DDM to discriminate cell aggregates. A dot plot of FL3-A vs FL3-W is used to measure the DNA content of the population according to the PI staining. R2 is drawn around the single cells in this population. Cells outside this region are doublets or higher aggregates. R1 and R2 are now used to define the transfected and nontransfected populations as described in **Fig. 2**. The cell cycle distribution was determined by a histogram plot of FL2 measuring the DNA content according to PI fluorescence. Panel **C** shows the cell cycle distribution of the total population. The position of the G1, S, and G2/M peaks and the corresponding 2N–4N DNA content are marked. Cells with DNA content of less than 2N appear in the sub-G1 region to the left of the G1 peak. Cells falling in this region represent dead cells.

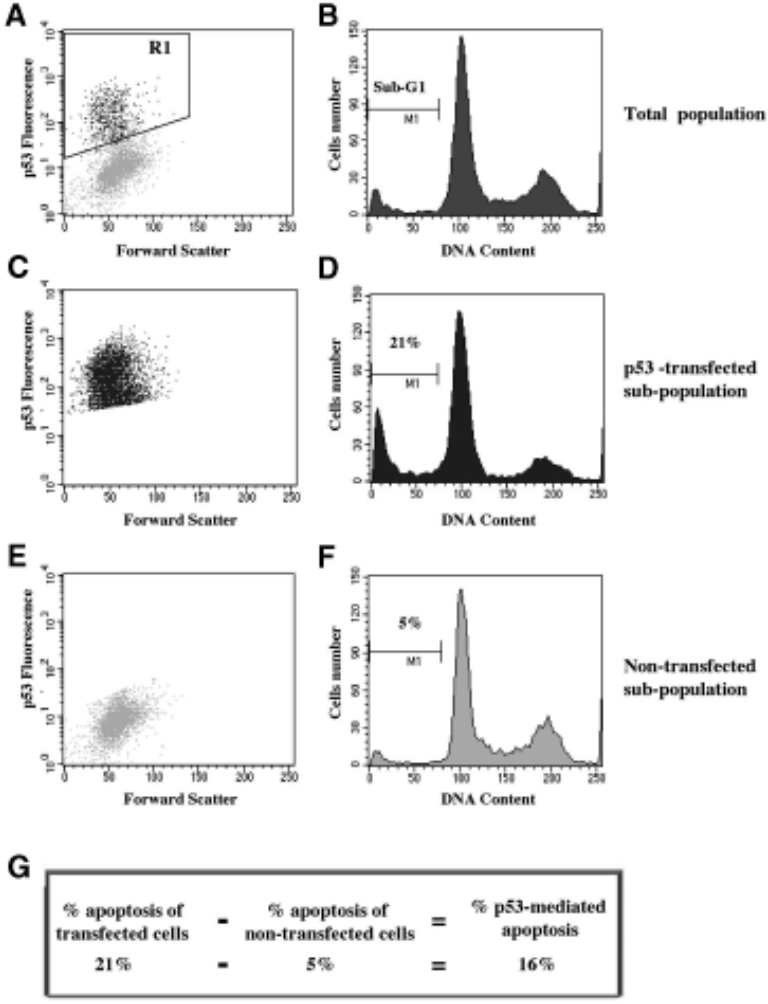

Fig. 2. Flow cytometric analysis of p53-mediated apoptosis. An analysis of the Saos-2 cells transfected with wild-type hp53 as described in the legend to **Fig. 1**. Although not shown here, the cells analyzed here fell within R2 as described in **Fig. 1**. (**A**) p53 fluorescent intensity of the total population. (**B**) The cell cycle distribution of the total population in panel **A**. The sub-G1 region is marked by bar. (**C**) Cells with high p53 fluorescence (R1) represent the transfected population (shown in black dots). (**D**) The cell cycle distribution of p53-transfected cells. The proportion of cells falling in the sub-G1 region is a percentage. (**E**) Cells with low fluorescent intensity represent the nontransfected population (shown in light grey). (**F**) Cell cycle distribution of the nontransfected population, with a percentage of cells with sub-G1 DNA content. The extent of p53-mediated apoptosis in Saos-2 cells in this sample is calculated by substracting the percentage of apoptosis in the nontransfected population from the transfected population (**G**).

cent positive cells, with longer incubation time and with increasing concentration of p53 expression plasmid. An important control for this apoptotic assay is an inactive p53 mutant, for instance hp53Gln179 ***(6)***.

3. Some cell types are very sensitive to the transfection procedure. It is thus recommended to change the transfection reagents or to use retroviral infection instead. In the case of adherent cells, the medium can be changed 24 h posttransfection in order to remove cells that died primarily due to the transfection procedure. Keep in mind that this change may also remove cells that were killed by p53 activity. Therefore, the extent of this loss can be estimated by comparing the percentage of cell death among the transfected vs the nontransfected cells. In the case of massive cell death due to the transfection procedure, e.g., by GFP alone, it would imply that this particular cell line is not suitable for this apoptotic assay.
4. In the protocol described here, methanol was used for fixation. The choice of fixative is influenced by the combinations of antibodies and antigens and is aimed to optimize accessibility of the antigen to the antibody. The assay described here is based on the exclusion of fragmented DNA from the apoptotic cells. Fixatives, such as formaldehyde, do not permeabilize the cells and, moreover, cause massive cross-linking within the cell. The combined effect is that the fragmented DNA remains within the apoptotic cells, hence the total amount of DNA in the cells does not decrease even if the DNA is fragmented. Therefore, it is strongly recommended to fix the cells with cold methanol. In our experience, all the anti-p53 antibodies tested work very well after this fixation.
5. During the procedure, caution should be taken at several steps. First, before fixation, cells must be brought into a single cell suspension with no visible clumps. This should be followed immediately by the fixation step, where cells are kept apart by constant vortex mixing. Excessive clumping is often problematic during acquisition. Second, following fixation, cells become more buoyant, hence, higher centrifugation speed is required in order to sediment the cells.
6. Although PI is in excess, the overall scale of the fluorescent intensity depends on the total amount of DNA in the sample. It is, therefore, important to adjust the vol of PBS in the final step before acquisition (**Subheading 3.1.1.**, **step 14**) to achieve approximately equal numbers of cells between the different samples.
7. It is important to keep in mind that not all the events that fall into the sub-G1 region represent intact cells. In fact, many of these events represent fragments of cells (apoptotic bodies). Therefore, the sub-G1 fraction represents a relative apoptotic activity rather than an absolute percentage of apoptotic cells in a population.

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Flow Cytometric Analysis of MDM2-Mediated Growth Arrest

Rebecca Frum and Swati Palit Deb

Summary

Although MDM2, the product of mouse double minute-2 (*mdm2*) gene, or its human homologue possesses the potential to confer tumorigenic properties, it induces G1/S arrest in nontransformed cells. Flow cytometry provides a way to determine the effects of MDM2 on the cell cycle by expressing the protein ectopically, immunostaining cells expressing MDM2 and analyzing their DNA content. The DNA histograms of MDM2-transfected and untransfected cells can then be used to visualize the effect of ectopically expressed MDM2 on the cell cycle. Fluorescence-activated cell sorter (FACS) analysis following bromodeoxyuridine (BrdU) incorporation can be used to determine whether MDM2-expressing cells are synthesizing DNA. Incorporation of BrdU during DNA synthesis or repair can be detected in partially denatured DNA with a BrdU-specific fluorescent antibody. Subsequent staining of transfected MDM2 with a different fluorochrome provides information about whether transfected cells make significant progression through S phase. Further analysis of the growth-regulatory properties of MDM2 will elucidate both its normal function and the ways in which its deregulation leads to tumorigenesis.

Key Words

MDM2, flow cytometry, cell cycle, bromodeoxyuridine, FACS

1. Introduction

The protein (hMDM2) coded by the human homologue of mouse double minute-2 (*mdm2*) gene frequently overexpresses in malignant human breast and other tumors ***(1–7)***. Artificial amplification of mouse *mdm2* gene derived from a transformed murine cell line enhances tumorigenic potential of murine cells ***(8,9)***. These evidences suggest oncogenic properties of human or mouse MDM2. The tumorigenic property of MDM2 is not unexpected, as MDM2

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interacts with several cell cycle regulatory proteins, the inactivation of which may contribute to its tumorigenic ability ***(10–18)***. Human or mouse MDM2 can inactivate several functions of the tumor suppressor p53 ***(19–21)***.

In view of all the oncogenic functions of MDM2, one would expect that overexpression of MDM2 would confer growth advantage in cultured cells. In contrast, MDM2 can only be stably expressed in cells harboring genetic defects. Overexpression of full-length MDM2 from its cDNA does not facilitate cell growth. Instead, it arrests the G1 to S phase transition of normal human or murine cells. Elimination of the growth inhibitory domains of the oncoprotein enhances the tumorigenic potential of NIH3T3 cells ***(22)***. Some cancer-derived cell lines are partially insensitive to MDM2-mediated growth arrest ***(22)***. Normal cells can induce MDM2 in response to oncogenic challenges, such as UV irradiation or estrogen treatment ***(23–29)***. It is possible that normal cells induce full-length MDM2 in response to oncogenic challenges to protect against premature cell cycle progression. If the cells are insensitive to MDM2-mediated growth arrest or if the oncoprotein is defective in growth arrest, premature progression of the cell cycle may lead to tumorigenesis. Since MDM2 seems to be a key member of cell growth regulation and possibly functions downstream of the p53 tumor suppressor, elucidation of its mechanism of action becomes essential.

The growth-inhibitory property of MDM2 poses restriction in exploring the growth-regulatory properties of MDM2 in normal cells, as the protein can only be expressed transiently. Also, it becomes essential to determine consequences of short-term MDM2 expression in nontransformed cells. Since MDM2 overexpresses in cancer cells and can be stably overexpressed in tumor-derived cells for several generations, nonspecific MDM2-mediated toxicity seems to be unlikely. To determine the growth regulatory function of MDM2 in normal cells, MDM2 needs to be expressed at a level detectable by Western analysis and immunofluorescence. Using different promoters and manipulating the amounts of expression plasmids levels of expression can be modulated if desired. However, while greater inhibition can be achieved through higher expression of MDM2 in tumor-derived cells, the growth-regulatory functions of MDM2 in normal cells do not seem to alter by increasing the levels of expression ***(22)***.

The basic protocol for flow cytometric analysis of MDM2-mediated growth regulation is similar to the flow cytometric analysis of p53-mediated growth regulation described in Chapter 17 of this text (Berger and Haupt). In this chapter, we will address some specific problems that we encountered in the case of MDM2. The basic strategy in this analysis is to express MDM2 in cells at a detectable level, separate or gate MDM2-expressing cells, and analyze the DNA content of the cells after staining.

To ensure that we are analyzing MDM2-expressing cells, we prefer to stain MDM2 directly for detection. Co-expression or tagging with other proteins, such as green fluorescent protein (GFP), perhaps can be used if the epitope-tagged protein is not functionally compromised. In the case of co-transfection, the cells that express GFP should express MDM2. If a significant portion of the GFP-expressing cells does not express MDM2, the inhibitory effect of MDM2 will be masked in the mixed population. Expression of MDM2 in GFP-expressing cells should be tested by isolating GFP-expressing and untransfected cells in a cell sorter and analyzing comparative MDM2 expression in these sorted cells by Western analysis. In our hands, co-transfected cell populations were a mixture of singly and doubly transfected cells with a higher population of singly transfected cells. We, therefore, recommend direct staining using an antibody against MDM2.

In this chapter, we will be assaying for presence of replicating cells by bromodeoxyuridine (BrdU) incorporation and suggesting methods for identification of MDM2-mediated G1 arrest by staining DNA to determine cell cycle compartments.

2. Materials

1. Phosphate-buffered saline (PBS) without calcium or magnesium. Use and store at 4°C (*see* **Note 1**).
2. Dulbecco's phosphate-buffered saline (DPBS) without calcium or magnesium. Use at room temperature. Store at 4°C (*see* Note 1)**.**
3. Trypsin 10X. Store at 4°C.
4. DPBS plus 10% heat-inactivated fetal bovine serum (FBS). Use at room temperature. Store at 4°C.
5. Absolute ethanol (ETOH). Use and store at –20°C.
6. PBS plus 0.5% bovine serum albumin (BSA). Use at room temperature. Store at 4°C.
7. For analysis involving BrdU incorporation, the following three items will be needed:
   a. 1 m*M* BrdU: dissolve in sterile PBS. Aliquot in the amount that will be needed per experiment. Store at –80°C in the dark. Do not thaw and refreeze.
   b. 2 *N* HCl.
   c. 0.1 *M* Sodium borate, pH 8.5. pH can be adjusted with HCl. Store at room temperature.
8. PBS plus 0.5% BSA plus 0.5% Tween(r) 20. Use at room temperature. Store at 4°C.
9. Syringes (3 mL) and needles (21-gauge)
10. Antibodies: to determine BrdU labeling in MDM2-expressing cells, two antibodies will be needed: one to detect MDM2 expression and the other to detect BrdU. For labeling with two different antibodies, we prefer antibodies directly coupled

to fluorescent dyes instead of indirectly labeling with dye-labeled secondary antibodies. Secondary antibodies could recognize both the primary antibodies without discrimination and yield high background. For this protocol, we used a fluorescein isothiocyanate (FITC)-labeled anti-BrdU antibody and an MDM2 antibody directly coupled to phycoerythrin (PE). Dye-coupled antibodies may be available commercially or can be prepared. Removal of uncoupled dye is very important to reduce background staining. If only staining with one antibody, a primary antibody and a FITC-labeled secondary antibody can be used.

11. 7-Aminoactinomycin D (7AAD) or propidium iodide (PI).
    a. 7AAD (1 mg/mL): 1 mg 7AAD. Add 50 μL absolute methanol. Mix well. Add 950 μL PBS. Mix. Store at 4°C in the dark (can be used for 1 mo).
    b. PI staining solution: sodium citrate (0.1%), Nonidet® P-40 (NP40) (0.1%), PI (50 μg/mL), DNase-free RNase A (100 μg/mL). The solution should be made fresh from stock solutions.
12. Sodium citrate (2%): filter and store at room temperature.
13. NP40 (2%): filter and store at room temperature.
14. PI (1 mg/mL): light sensitive. Store in dark at 4°C.
15. RNase A (10 mg/mL, heat-treated to inactivate DNase activity): store at –20°C.
16. Falcon(r) or Corning tubes (50 mL).

3. Methods (*see* Note 2)

3.1. Transfect Cells Using Calcium Phosphate Method

Cells should be seeded 20–24 h before transfection. Primary cells or cell lines can be used. Proper cell density is important to detect growth inhibitory effect of MDM2. Since the cells are seeded a day before transfection and are harvested 48 h after transfection, care should be taken so that the cells are not dense enough to show contact inhibition at the time of harvesting. The number of cells seeded may vary depending on the cell types. We seed 3×10^5 cells for NIH3T3 and 5×10^5 cells in the case of WI38 or MRC5 cells in a 10-cm dish.

Delivery of MDM2 by transfection of expression plasmids is comparatively inefficient to delivery by viral vectors such as adeno- or retroviral vectors. However, it is important to test whether the proteins coded by the viral vectors or the interaction of the viral membrane proteins with cellular receptors alters the growth regulatory properties of MDM2. For example, the adenoviral vector harboring MDM2 expresses a truncated form of MDM2 at a much higher level than the full-length protein. Thus, at present, transfection of the expression plasmids seems to be the method of choice, as this is less likely to generate secondary effects.

Transfected plates can be labeled with BrdU and/or harvested for MDM2 staining by trypsinization about 40 h after transfection, and the cells should be fixed. Methanol or ethanol fixation incurs some cell loss during the staining process, which can be circumvented by the use of BSA.

3.2. BrdU Labeling of Cells

1. After cells have been transiently transfected, remove old media.
2. Add 4 mL of normal media and allow to reach 37°C in cell culture incubator.
3. Add BrdU to each plate to a final concentration of 10 μ*M*.
4. Incubate for 40 min, tilting approx every 15 min.

3.3. Harvest Cells

1. Wash plates 2× in 8 mL DPBS.
2. Harvest cells by trypsinization and collect cells in DPBS plus 10% FBS.
3. Place cells in a 50-mL sterile plastic tube (*see* **Note 3**).
4. Take a small aliquot to count.

3.4. Fixation

1. Spin in 50-mL tubes at 400*g* for 11 min at 4°C. Aspirate.
2. For less than 10^7 cells, add 1.5 mL PBS. For more than 10^7 cells, add 3.0 mL PBS.
3. Suspend cells in PBS by vortex mixing.
4. Pass through a 21-gauge needle 5× (or until cells are suspended) to break up clumps.
5. While vortex mixing vigorously, add absolute ETOH that has been stored at –20°C drop-wise (*see* **Notes 4** and **5):** for less than 10^7 cells, add 3.5 mL of absolute ETOH, and for more than 10^7 cells, add 7 mL of absolute ETOH.
6. Store at 4°C for at least 18 h (*see* **Note 6).**

3.5. Wash

1. Spin cells at 2615*g* for 6 min at room temperature (*see* **Note 7**). Aspirate.
2. Resuspend pellet in 1 mL of PBS plus 0.5% BSA and transfer to microfuge tube (*see* **Note 8**).
3. Spin tube at 2615*g* for 6 min at room temperature. Aspirate.
4. Wash 2× in 1 mL of PBS plus 0.5% BSA. The washes remove extra ETOH and prevent clumping during the following steps. If necessary, break up clumps by passing through a 21-gauge needle.

3.6. Acid–Base Treatment for the Detection of BrdU Incorporation

1. After aspirating the final wash, incubate in 400 μL of 2 *N* HCl for 20 min at room temperature. Vortex mix well to break up clumps or pass through a 21-gauge needle (*see* **Note 9**).
2. Add 800 μL of PBS plus 0.5% BSA. Vortex mix.
3. Centrifuge at 2615*g* for 6 min at room temperature. Aspirate.
4. Incubate in 400 μL of 0.1 *M* sodium borate, pH 8.5, for 2 min at room temperature. Do not treat more than 5 tubes with sodium borate at the same time, due to the short incubation period.
5. Add 800 μL of PBS plus 0.5% BSA. Vortex mix.

6. Centrifuge at 2615*g*for 6 min at room temperature. Aspirate.
7. Wash 1× with 1 mL of PBS plus 0.5% BSA. Aspirate.

3.7. Staining for MDM2 and BrdU

1. Suspend the cells in PBS plus 0.5% BSA plus 0.5% Tween 20 and incubate with the FITC-coupled anti-BrdU antibody. The concentration of antibody for optimum staining should be determined experimentally. Excess antibody or uncoupled dye increases background staining and quenches detection of other staining (*see* **Notes 10–14**).
2. Incubate for 1 h at room temperature in the dark with tilting.
3. Spin at 2615*g* for 6 min at room temperature. Aspirate.
4. Wash the cells 1× with 1 mL of PBS plus 0.5% BSA.
5. Suspend the cells in PBS plus 0.5% BSA plus 0.5% Tween 20 and incubate with the PE-coupled MDM2 antibody.
6. Incubate for 2 h at room temperature in the dark with tilting.
7. Spin at 2615*g* for 6 min at room temperature. Aspirate.
8. Wash 1× with 1 mL of PBS plus 0.5% BSA. Aspirate. If necessary, the pellet can be stored on ice at 4°C in dark overnight and stained with 7AAD the next day.

3.8. 7AAD Staining (see Notes 15 and 16)

1. Add 7AAD at a concentration of 0.02 mg/mL.
2. Let cells incubate on ice for 90 min in the dark.
3. Spin at 2615*g* for 6 min at 4°C. Aspirate.
4. Dilute to 2×10^6 cells/mL in PBS plus 0.5% BSA to avoid clogging during analysis. It is best to analyze the same day, but if necessary, cells can be analyzed after more than 24 h.

3.9. Staining of MDM2 in Non-BrdU Labeled Cells

After transfection, follow the steps from **Subheadings 3.3.**, **3.4.**, and **3.5.** exactly. Then stain as follows.

1. Suspend cells in PBS plus 0.5% BSA plus 0.5% Tween 20. Vortex mix to suspend.
2. Add titrated amount of MDM2 primary antibody. Vortex mix lightly.
3. Incubate for 2 h at room temperature with tilting.
4. Spin at 2615*g* for 6 min at room temperature. Aspirate.
5. Wash 2× in 1 mL of PBS plus 0.5% BSA.
6. Suspend cells in PBS plus 0.5% BSA plus 0.5% Tween 20.
7. Add titrated amount of FITC-labeled secondary antibody against the primary antibody.
8. Cover the tubes in aluminum foil and incubate for 2 h at room temperature with tilting.
9. Spin at 2615*g* for 6 min at room temperature. Aspirate.
10. Wash 2× in 1 mL of PBS plus 0.5% BSA.
11. PI staining: if necessary, pellet can be stored on ice at 4°C in the dark overnight

Table 1
Comparison of Incorporating BrdU in MDM2 Expression Plasmid Transfected and Untransfected Populations

Quadrant	Percent of gated events	Percent of cells incorporating Brdu	
		MDM2+	MDM2-
Upper left (MDM2+, BrdU–)	14.46	—	—
Upper right (MDM2+, Brdu+	0.87	5.6	—
Lower left (MDM2–,BrdU–)	61.79	—	—
Lower right (MDM2–, BrdU+)	22.88	—	27

The table compares percentage of cells incorporating BrdU in MDM2 expression plasmid transfected and untransfected population as shown in Fig. 1D. Transfected cells are labeled as MDM2+, and untransfected cells are labeled as MDM2–.

and stained with PI the next day. Add 1 mL of PI staining solution for every 2 × 10^6 cells. Vortex mix. Let cells incubate in dark for at least 30 min. Analyze the same day (*see* **Notes 15** and **16**).

A representative result of an experiment to analyze cell growth regulatory property of MDM2 using the described method is shown in **Fig. 1** and **Table 1.** As evident from **Fig. 1D**, transfected NIH3T3 cells show 1.6 to 60-fold increased expression of MDM2, whereas most of the cells showed 10-fold overexpression. However, BrdU incorporation was inhibited at low or high levels (**Fig. 1D**, less number of BrdU-labeled cells in the upper right quadrant.) Comparison of the DNA histograms of PE-labeled (transfected, dotted line) and unlabeled (untransfected, bold line) cells suggests that the cells overexpressing MDM2 induced G1 arrest, reducing the number of cycling cells.

4. Notes

1. The time frame of this protocol consists of 6 d from start to finish: 1 d to plate cells, 2 d for transient transfection, 1 d to harvest and fix, 1 d to stain, and usually an additional 1 d for DNA staining and analysis.
2. Calcium and magnesium cause cells to clump; therefore, it is important to use buffers without calcium or magnesium for flow cytometry.
3. After harvesting, place cells in a 50-mL conical tube rather than a smaller one for easier fixation and to prevent clumping.
4. During fixation, the cells should be vortex mixed, and the ETOH should be added drop-wise to prevent the cells from being fixed together and causing subsequent clumping.
5. Precipitation by fixation with alcohols, such as ETOH and methanol, dehydrates the cells and permeabilizes the membrane. ETOH or methanol fixation is pre-

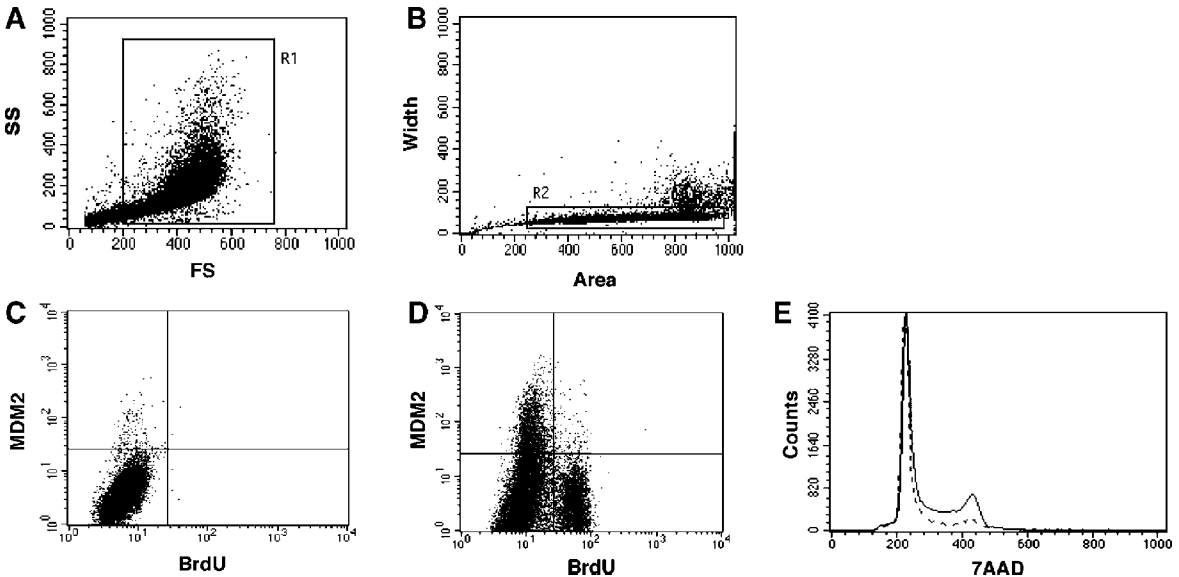

Fig. 1. Dot plot analysis of BrdU-labeled (or unlabeled) NIH3T3 cells transfected with MDM2 expression plasmid. Mock transfected or MDM2 expression plasmid transfected cells were fixed and stained with PE-labeled MDM2 antibody, FITC-labeled anti-BrdU antibody, and 7AAD. (**A**) Forward scatter (FS) vs side scatter (SS) plot, gated events (R1) are shown. (**B**) Area vs width plot used for doublet discrimination. Gated events (R2) are shown. (**C**) FITC vs PE plot of mock transfected, BrdU unlabeled cells stained with PE-labeled MDM2 antibody, FITC-labeled anti-BrdU antibody, and 7AAD. The plot has been used to determine the background staining. (**D**) FITC vs PE plot of MDM2 expression plasmid transfected, BrdU-labeled cells stained with PE-labeled MDM2 antibody, FITC-labeled anti-BrdU antibody, and 7AAD. (**E**) DNA histogram of transfected (dotted line) and untransfected (bold) cells sorted from MDM2 expression plasmid transfected cell population. Single color staining with PE-labeled MDM2 antibody, FITC-labeled anti-BrdU antibody, and 7AAD are not shown.

ferred over paraformaldehyde, which fixes through cross-linking. Cross-linking of the DNA results in low a quality DNA histogram with either PI or 7AAD.
6. After fixation, cells should be stored in ETOH for >18 h for better resolution.
7. Higher centrifugation speeds are required after fixation, because the fixation step causes the cells to become more bouyant.
8. BSA blocks nonspecific binding of the antibodies and also prevents loss of cells.
9. Treatment with HCl causes single-stranded nicks in the DNA and is necessary because the anti-BrdU antibody can only recognize BrdU in single-stranded DNA. Following the treatment of HCl with a treatment of sodium borate neutralizes the acid so that the DNA is only partially denatured.
10. Although ETOH fixation permeabilizes the cell membrane to allow the antibodies and dyes for staining DNA to have access within the cell, the addition of Tween 20 may help with this process during the incubation steps.
11. The amounts of antibodies used were determined through standardization. Excess PE- and FITC-coupled antibodies or uncoupled stains can cause quenching, which can be reduced by lowering the amounts of both antibodies.
12. The PI and 7AAD dyes and the PE and FITC antibodies are light-sensitive, so after their addition, the cells should be kept in the dark.
13. During staining with the antibodies, if the vol is small, it is better to manually mix the tube every 15 min by lightly vortex mixing instead of putting the tube on a tilter. Alternatively, the vol can be increased while keeping the same concentration, so that it is conducive to use with a tilter.
14. When staining with two antibodies in the same sample, it is necessary to have controls stained with only one of each antibody for setting up the flow cytometer.
15. ETOH-fixed cells that are treated with HCl do not stain well with PI. Conversely, 7AAD staining is more efficient for cells treated with HCl.
16. Cells stained with PE should only be stained with 7AAD because PE and PI overlap.

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