Chapter 4

Detailed Protocols for the Selection of Antiviral Human Antibodies from Combinatorial Immune Phage Display Libraries

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Additional information is available at the end of the chapter

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Abstract

Broadly, neutralizing antiviral antibodies holds great promise for improving treatment opportunities for patients suffering from viral infections (e.g., human immunodeficiency virus [HIV], hepatitis B virus [HBV], cytomegalovirus [CMV], Rabies, Ebola, Zika) leading to serious health disorders or even to death without effective antiviral treatment. The potential of antibodies in host protection against lethal viral infections has been demonstrated in numerous animal models and is best exemplified by the protection conferred to neonates by maternal antibodies. Over the past few decades, virus-neutralizing human monoclonal antibodies (nAbs) have been isolated from humans successfully cured of disease using a wide range of recently developed antibody isolation technologies. In this chapter, we present an approach for isolating recombinant human nAbs from combinatorial gene libraries being cloned from individuals who have recovered from viral infections. The presented protocols describe the selection and screening of antiviral single-chain antibody fragments (scFvs) from phage display immune libraries. This technology represents a well-established, high-throughput approach allowing fast selection of broadly neutralizing, antiviral antibodies. The protocols for generating and selecting antigen-specific scFvs can be applied for the selection of scFvs against any target.

Keywords: recombinant human monoclonal antibodies, broadly neutralizing antiviral antibodies, antibody phage display, combinatorial immune libraries, high-throughput screening

1. Introduction

Antibodies are powerful tools for the prophylaxis and treatment of viral infections. The use of antibodies against severe, life-threatening infections began in the 1890s when Robert Koch

demonstrated that administration of sheep antiserum against diphtheria toxin to a girl dying from diphtheria infection led to her rapid recovery and survival [1]. On that basis, numerous attempts to treat potentially deadly viral diseases such as influenza, severe acute respiratory syndrome (SARS), or Ebola by administrating sera from survivors have successfully been undertaken [2]. Further improvements led to the development of polyclonal hyperimmunoglobulin G (IgG) preparations, consisting of purified antibodies from seropositive donors [3]. Several hyperimmunoglobulins against human cytomegalovirus (CMV), hepatitis B virus (HBV), rabies, and other viral infections are available on the market. Although polyclonal preparations provide strong antiviral activity, the major disadvantage of such hyperimmunoglobulin preparations is that they include a high amount of nonspecific antibodies and only a low proportion of neutralizing antibodies. The development of hybridoma technology by Köhler & Milstein in 1975 revolutionized science and medicine and led to the isolation of numerous monoclonal antibodies [4]. Since the commercialization of the first therapeutic monoclonal antibody product in 1986, this class of therapeutics has grown significantly [5]. In 2016, 5 of the top 20 pharmaceuticals were therapeutic antibody drugs [6]. The vast majority of monoclonal antibodies is approved for the treatment of cancers, multiple sclerosis, or rheumatoid arthritis [7]. However, numerous potent human or humanized antiviral antibodies against H5N1 influenza virus, human immunodeficiency virus (HIV), herpes simplex virus (HSV), human cytomegalovirus (CMV), hepatitis C virus (HCV), Ebola virus, severe acute respiratory syndrome (SARS) virus, and other viral infections are in preclinical development, clinical studies, or even approved for antiviral treatment [2, 7–12]. Antibodies mostly neutralize free viruses by targeting the initial stages of virus infection described as the binding of free virions to permissive target cells followed by entry and replication [2]. Neutralizing antibodies not only provide new tools for prophylaxis and therapy of viral diseases, but also identify conserved epitopes that may be used to design new vaccines capable of conferring broader protection [11]. However, enveloped viruses such as HIV-1, HSV-1/2, CMV, and measles can also move between adjacent cells without diffusing through the extracellular environment (cell-to-cell spread). This mechanism facilitates rapid viral dissemination, promotes immune evasion from the host's immune response, and enhances the progression of disease [13]. We recently described a murine monoclonal antibody (mAb) capable of inhibiting the cell-to-cell spread of HSV. This antibody proved to be highly effective in the prevention of drug-resistant HSV infections in highly immunodeficient NOD/SCID mice indicating the enormous potential of antibody blocking mechanisms crucial for the virus spread [12]. Due to these unique features, this antibody was humanized for clinical applications and is now being tested in phase I and II trials. Besides the generation of antiviral antibodies by humanization approaches, neutralizing antibodies can also be isolated from humans cured of viral infections, such as SARS or Ebola [9, 14]. Various methods have been developed for the isolation of antibodies from humans including B-cell immortalization and single-cell expression cloning [15]. Among these approaches, high-throughput screening of phage-displayed antibody libraries has become one of the leading technologies for generating human therapeutic antibodies (**Figure 1**) [16]. Nowadays, very large phage display libraries from naive (IgM) B-cell repertoires (>10^{10} independent clones) are widely used for the selection of human antibodies against a broad panel of targets including human self-antigens to identify high-affinity binders. However, these antibodies are not affinity-matured by the human

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immune system. Hence, the preparation of combinatorial immune libraries from immunized or infection cured donors can deliver a broader range of highly functional antibodies, even from smaller libraries (i.e., potent antiviral antibodies with broadly neutralizing efficacies). For instance, the selection of combinatorial immune repertoires (>10^8 independent clones) from HSV seropositive donors resulted in a broad panel of various high-affinity binders with partly HSV-neutralizing properties [17, 18].

Although various other *in vitro* selection platforms have been developed during the last decades (e.g., ribosome display and yeast display), phage display using filamentous phages is predominantly used for library generation since it is robust, inexpensive, and allows the automation of the selection and screening process. Due to the limitation in the production of full-length IgG antibodies in *Escherichia coli*, only smaller antibody fragments (e.g., scFvs or Fabs) can be efficiently expressed within the *E. coli* periplasm as functional proteins. Several systems

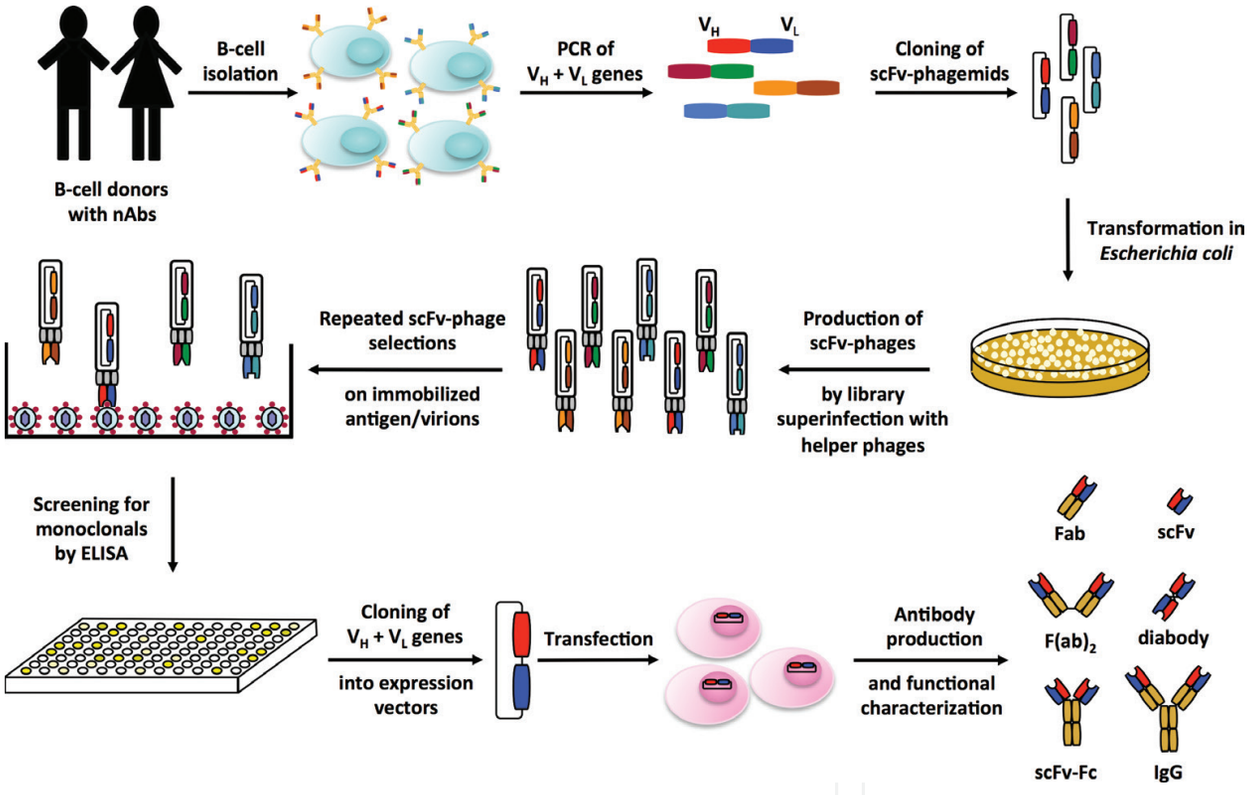

Figure 1. Isolation of human neutralizing antiviral antibodies (nAbs) by phage display technique. Lymphocytes comprising B-cells from humans harboring neutralizing antibodies with unique features, e.g., Ebola disease survivors are isolated from blood, spleen, lymph nodes, or bone marrow by standard techniques (e.g., PBMCs by Ficoll density gradient centrifugation). Lymphocytes RNA is prepared and transcribed into single-stranded cDNA that is used as the source for PCR amplification of the variable heavy (VH) and light chain (VL) genes. Variable genes are randomly cloned into phagemid vectors as scFv antibody fragments prior to electroporation of phagemids into *E. coli* bacteria to produce combinatorial immune libraries. Library glycerol stocks are then used for the generation of a bacterial culture that is superinfected with a helper phage to produce phages presenting different scFvs on their surface. Specific binding scFv-phages are enriched over several selection rounds by stringent washing and elution using antigen/virions immobilized on immunotubes. After screening for monoclonal binders on ELISA plates, the best specific binders are directly produced as monovalent scFvs in bacteria cultures or cloned into appropriate expression vectors for the production of Fab, or various bivalent antibody formats prior to functional analysis (e.g., virus neutralization capacity and affinity).

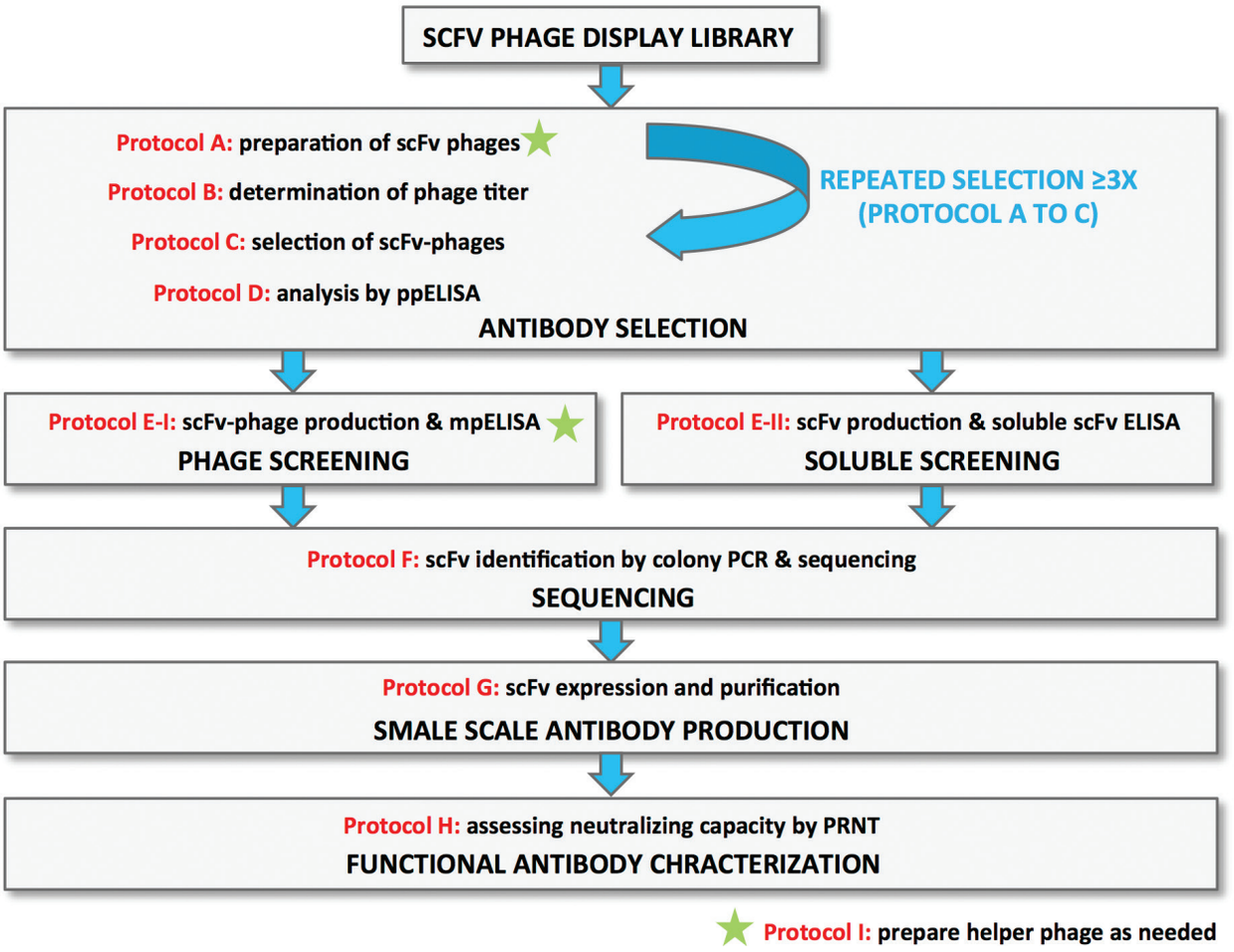

Figure 2. Workflow of the selection and screening procedure. For the selection of antigen-specific scFv-phages, log-phase library cultures are packed by superinfection with helper phages (for the preparation of helper phages, see Protocol I) that provide all proteins necessary for phage propagation (see Protocol A). After IPTG induction, expressed scFv-pIII fusions are inserted within the produced phages leading to the presentation of scFv antibody fragments on the phage surface. After determination of phage titer (see Protocol B), specific binding scFv-phages are enriched over several selection rounds by stringent washing and elution on recombinant antigen/virions that have been immobilized onto immunotubes (see Protocol C). Successful enrichment of specific binding scFv-phages can be analyzed by polyclonal phage ELISA (ppELISA) (see Protocol D) prior to screening of monoclonal antibodies as scFv-phages by monoclonal phage ELISA (mpELISA) (see Protocol E-I) or as soluble scFvs (see Protocol E-II). After the identification of bacterial colonies encoding for full-length scFvs by colony PCR and sequencing (see Protocol F), soluble scFvs can be produced in the periplasm of bacteria (see Protocol G) or variable antibody genes can be cloned into mammalian expression vectors to produce Fab or various bivalent antibody fragments. Finally, antibody fragments can be analyzed for their neutralizing activity in functional assays like the plaque reduction neutralization test (PRNT) (see Protocol H).

for displaying antibody fragments on the phage surface have been developed over time using different vectors and phage coat proteins for display. The most common type (3 + 3 system) is based on phagemid vectors where the antibody gene fragments are cloned as fusions with the pIII phage gene. Cloning of the antibody gene repertoires can be done by using different strategies in one, two, or three independent steps where the variable light and heavy chain genes are PCR-amplified and randomly combined into reliable phagemid vectors. In the-one step cloning strategy, the VH and VL genes are separately amplified with an overlapping, additional linker sequence and combined by assembly PCR [19]. In the two-step cloning strategy, mostly the VL gene repertoire is cloned first into the phagemid followed by insertion of

the VH repertoire into the VL phagemid [17, 18, 20–22]. In the three-step cloning strategy, two separate VH and VL libraries are prepared before exercising one repertoire and including it into the phagemid containing the other repertoire [23]. After electroporation of the phagemids into electrocompetent *E. coli* bacteria, the antibody libraries are grown on selection plates and stored as frozen bacterial glycerol stocks. Prior to PCR amplification of the variable antibody genes, B-cells from respective donors need to be isolated. In the case of viral infection and depending on when the infection occurred, the isolation of the short-lived plasmablast pool during the early antibody response or the long-lived memory B-cell and plasma cell pool may be preferred. Although the isolation of peripheral blood mononuclear cells (PBMC) from whole blood (the main source for plasmablasts) is often described for retrieving antiviral antibodies, B-cell sources such as spleen, lymph nodes (many memory B-cells), or bone marrow (the main source of plasma cells) might be considered for library construction. Good protocols for the cloning of combinatorial scFv phage display libraries including the primer sets for PCR amplification of antibody genes can be found elsewhere [17, 20–22] and are out of the scope of this chapter.

Here, we present a methodology for the recovering of potential therapeutic antibodies with unique antiviral properties from human B-cell repertoires (for workflow see **Figure 2**). This strategy has been successfully used for generating neutralizing human antibodies against HSV as a proof-of-principle [18]. The following protocol will systematically describe the procedure of generating broadly neutralizing antiviral antibodies from isolated human B-cells by a phage display technique.

2. Preliminary notes

Before starting antibody phage display, please be aware that phages are highly stable and decontamination of workspace and consumables is hard to achieve. It is best to do phage work in a special lab keeping equipment/material separated from the common bacterial workspace, especially when antibody library construction is performed. If not possible, phage work should be carried out in at least a separate workspace including a separate hood, shaker, and centrifuge. Inactivation of phage solutions can be done by incubation with diluted bleach (caution, always wear personal protection during handling) and/or sterilizing workspaces with UV light. For decontamination of tubes and Erlenmeyer flasks, bleach can be added to water-filled tubes and incubated overnight before washing, rinsing, and autoclaving. In common, single-use material is preferred for phage work. Collect phage-contaminated solutions in glass flasks and inactivate by adding bleach before dumping. Only use polypropylene (PP) tubes since phages might stick to other kinds of plastics. To prevent contamination to pipettes, always use barrier tips.

Presented protocols are intended for the selection and screening of antibody libraries based on the scFv antibody format being cloned in phagemid vectors as pIII fusion with an intrinsic amber stop codon and under the *lac* promoter (inducible by IPTG, repressible by glucose). Many current antibody phage display libraries are constructed in phagemid vectors with listed features (e.g., most derivatives of pHEN, pComb3X, pHAL, and pCANTAB), although

other selection relevant features such as signal peptides, molecular tags, etc., might differ. Please check features of your antibody library used prior to selection and screening and change protocols accordingly if necessary. If using libraries based on phagemid vectors with *lac* promoter, **always add ≥2% glucose** to the media to repress scFv-pIII protein expression as long as antibody phages are not produced. Lower amounts of glucose results in background expression of pIII fusions and clones with growth advantages (e.g., truncated scFvs) might overgrow leading to a reduced library diversity. More detailed information about antibody phage display [24] and commonly used phagemid vectors can be found elsewhere [25].

Independent of the source of your antibody library, always try to package antibody libraries from primary bacteria stocks, never from secondary stocks or using phage-packaged libraries to infect bacteria. Only correctly stored (−80°C) primary glycerol bacteria stocks guarantee highest initial antibody diversity. For novel libraries or if not familiar with antibody phage display, perform test selection and subsequent screening using not relevant proteins such as bovine serum albumin.

3. Materials

A. Preparation of scFv-phages

- 2xTY medium: 16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, dissolved in ultrapure water, autoclaved, and stored at room temperature (RT).
- 2xYT-GA: 900 ml 2xYT medium, supplemented with 100 ml glucose stock solution and 1 ml ampicillin stock solution right before use.
- Kanamycin stock solution (2000×): 100 mg/ml kanamycin sulfate dissolved in ultrapure water, filter-sterilized, stored at −20°C.
- Ampicillin stock solution (1000×): 100 mg/ml ampicillin sodium salt dissolved in ultrapure water, filter-sterilized, stored at −20°C.
- 20% (w/v) glucose stock solution (10×): 200 g/l D-glucose dissolved in ultrapure water, filter-sterilized, stored at 4°C.
- Helper phage VCSM13 (Agilent Technologies) or M13K07 (e.g., NEB Biolabs), Kanamycin-resistant.
- Optional for oligomeric display: hyperphage M13 K07ΔpIII (Progen Biotechnik).
- 2xYT-GK agar plates (100 mm): 16 g tryptone, 10 g yeast extract, 5 g NaCl, and 15 g agar dissolved in 900 ml ultrapure water, autoclaved, cooled down to 50°C, and supplemented with 100 ml glucose stock solution and 500 μl kanamycin stock solution right before pouring, stored at 4°C.
- 1 M IPTG stock solution (20,000×): 2.38 g isopropyl β-D-1-thiogalactopyranoside dissolved in 10 ml ultrapure water, filter-sterilized, stored at −20°C.

- Induction medium 2xYT-AKI: 2xYT supplemented with 1 ml ampicillin stock solution, 500 μl kanamycin stock solution, and 50 μl IPTG stock solution (IPTG concentration depending on phagemid vector used), no glucose (!), prepared right before use.
- Polypropylene (PP) centrifugation tubes: 2 and 50 ml (single-use), 250 ml (reusable).
- PEG/NaCl solution: 200 g/l polyethylene glycol 6000, 146.1 g/l NaCl, dissolved in ultrapure water, autoclaved, stored at 4°C.
- Optional for Western blot analysis: primary murine anti-pIII monoclonal IgG (MoBiTec, diluted 1:1000 milk in phosphate-buffered saline (MPBS)) and secondary goat anti-mouse HRP-conjugated antibody (Jackson ImmunoResearch, diluted 1:10,000 in MPBS).

B. Determination of the phage titer

- TG1 bacteria strain (e.g., Lucigen).
- M9 minimal stock solution (5×): 56.4 g/l M9 minimal salt (Sigma Aldrich) dissolved in ultrapure water, autoclaved, stored at 4°C.
- 1 M $MgSO_4$ stock solution: 6.02 g $MgSO_4$ dissolved in 50 ml ultrapure water, filter-sterilized, stored at 4°C.
- Thiamine stock solution: 50 mg thiamine hydrochloride dissolved in 50 ml ultrapure water, filter-sterilized, stored at −20°C.
- M9/+Thi minimal plates (100 mm): 15 g agar dissolved in 780 ml ultrapure water, autoclaved and supplemented with 200 ml M9 minimal salt stock solution, 20 ml glucose stock solution (see A), 1 ml $MgSO_4$ solution, and 1 ml thiamine stock solution.
- 2xYT-GA agar plates (100 mm): 16 g tryptone, 10 g yeast extract, 5 g NaCl, and 15 g agar dissolved in 900 ml ultrapure water, autoclaved, cooled down to 50°C, and supplemented with 100 ml glucose stock solution and 1 ml ampicillin stock solution right before pouring, stored at 4°C.

C. Selection of antigen-specific scFv-phages

- 5 ml Nunc MaxiSorp™ immunotubes (Thermo Fisher Scientific).
- Phosphate-buffered saline (PBS): 1.42 g/l Na_2HPO_4, 0.24 g/l KH_2PO_4, 0.2 g/l KCl, 8.0 g/l NaCl, dissolved in ultrapure water, adjusted to pH 7.4, autoclaved, stored at RT.
- PBST: phosphate-buffered saline supplemented with 0.1% Tween20.
- MPBS: 2% nonfat dry milk in phosphate-buffered saline, prepared right before use.
- TG1 bacteria strain (e.g., Lucigen).
- 2xYT-GA agar plates (100 mm round, 150 mm round, or 245 mm square): see B.
- Phage elution buffer: 0.1 M glycine-HCl, 0.5 M NaCl, dissolved in ultrapure water, adjusted to pH 2.2, filter-sterilized, stored at 4°C.

- Phage neutralization buffer: 1 M Tris-HCl dissolved in ultrapure water, adjusted to pH 9.5, filter-sterilized, stored at 4°C.
- Trypsin-PBS solution: 10 μg/ml trypsin (e.g., from porcine pancreas by Sigma Aldrich) dissolved in PBS, pH 7.4, prepared right before use.

D. Polyclonal phage ELISA (ppELISA)

- PBS and PBST: see C.
- 96-well microtiter ELISA plate (e.g., Nunc Maxisorp™ by Thermo Fisher Scientific).
- Murine anti-M13 monoclonal antibody HRP-conjugate (GE Healthcare), diluted 1:5000 in MPBS.
- TMB substrate solution (e.g., Thermo Fisher Scientific).
- Stop solution (2 M H_2SO_4): concentrated sulfuric acid dissolved 1:9 in ultrapure water.

E. Screening for monoclonal binders

(I) Monoclonal phage (mpELISA) screening:

- Sterile 96-well polypropylene round-bottom microplates (e.g., Greiner Bio-One).
- Breathable sealing membrane for microplates (e.g., Sigma Aldrich).
- 96-pin microplate replicator (Boekel Scientific).
- 8-channel pipettes (20 μl and 200 μl).
- 2xYT-glycerol: 2xYT dissolved in 50% glycerol and 50% ultrapure water, autoclaved, stored at RT.
- 2xYT-GA medium, ampicillin stock solutions, glucose stock solution, kanamycin stock solution, IPTG stock solution, helper phage VCSM13 or M13K07, and induction medium 2xYT-AKI: see A.
- PBS, PBST: see C.
- Maxisorb™ ELISA plates, anti-M13 monoclonal HRP-conjugate, TMB substrate, and stop solution: see D.

(II) Soluble scFv screening:

- HB2151 bacterial strain (Nordic BioSite).
- M9/+Thi minimal plates, 2xYT-GA plates: see B.
- 2xYT medium, ampicillin stock solution, glucose stock solution: see A.
- Sterile 96-well PP round-bottom microplates, breathable sealing membrane, microplate replicator, 8-channel pipettes: see E-I.
- 1 M sucrose stock solution: 342.3 g/l dissolved in ultrapure water, filter-sterilized, stored at 4°C.

- Potassium phosphate buffer: 0.17 M KH_2PO_4, 0.72 M K_2HPO_4, adjusted to pH 7.0, filter-sterilized, stored at 4°C.
- Buffered 2xYT (pH 7.0): 900 ml 2xYT medium supplemented with 100 ml potassium phosphate buffer, filter-sterilized, stored at 4°C.
- Induction medium 2xYT-SAI: buffered 2xYT supplemented with 50 ml sucrose stock solution, 1 ml ampicillin stock solution, and 50 μl IPTG stock solution (IPTG concentration depending on phagemid vector used), prepared right before use.
- Primary scFv-tag-specific monoclonal antibody (e.g., anti-myc, anti-His): 5 μg/ml diluted in MPBS.
- Secondary anti-primary polyclonal antibody HRP conjugate (e.g., Jackson ImmunoResearch): 1:10,000 diluted in MPBS.

F. Identification of complete scFv fragments by colony PCR and sequencing

- LB high salt medium: 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, dissolved in ultrapure water, autoclaved, stored at RT.
- Taq PCR Core Kit, QIAquick PCR Purification Kit, QIAprep Spin Miniprep Kit, and QIAquick Gel Extraction Kit (optional): all from Qiagen.
- Colony PCR/sequencing primers flanking the scFv insert (most pHEN phagemids):

P1 (LMB3long, forward) 5′-CAGGAAACAGCTATGACCATGATTAC-3′; and

P2 (fdseqlong, reverse) 5′-GACGTTAGTAAATGAATTTTCTGTATGAGG-3′.

- 96-well PCR plate and sealing (e.g., BRAND).
- Sterile 96-well PP round-bottom microplates (e.g., Greiner Bio-One).
- 2xYT-glycerol: see E-I

G. Small-scale expression of soluble scFvs for functional characterization

- HB2151 bacterial strain: see E-II.
- M9/+Thi minimal plates, $MgSO_4$ stock solution: see B.
- 2xYT-GA, 2xYT-GK agar plates (100 mm), IPTG stock solution: see A.
- Induction medium 2xYT-GA with 0.1% glucose: 995 ml 2xYT medium, supplemented with 5 ml glucose stock solution and 1 ml ampicillin stock solution right before use.
- Periplasmic preparation buffer: 200 g/l sucrose, 30 mM Tris-HCl, 1 mM EDTA, diluted in ultrapure water, adjusted to pH 8.0, stored at 4°C.
- PBS: see C.
- Low protein-binding sterile syringe filters (0.22 μm): e.g., Millex-GV Filter (PVDF, 4 mm diameter) by EMD Millipore.

- Dialysis: D-Tube™ Dialyzer (MWCO 12–14 kDa) by EMD Millipore or dialysis membrane (e.g., Spectra/Por 4 dialysis membrane, MWCO 12–14 kDa, by Spectrum Laboratories).
- Lysozyme solution: 50 mg/ml lysozyme, in ultrapure water, prepared right before use.
- NPI-10 buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, dissolved in ultrapure water, adjusted pH to 8.0, filter-sterilized, stored at 4°C.
- Ni-NTA Spin Columns (Qiagen).

H. Functional antibody characterization by plaque reduction neutralization test

- Complete culture medium: e.g., Vero cells, 10% heat-inactivated fetal bovine serum (FBS) (optional: supplemented with penicillin/streptomycin solution).
- Sterile cell culture plates (6-well, 12-well, and 48-well): e.g., by Greiner Bio-One.
- Carboxymethyl cellulose (CMC) medium: 2/3 volume of sterile complete culture medium and 1/3 volume of CMC solution, stored at 4°C.
- CMC solution: 20 g/l carboxymethyl cellulose sodium salt dissolved in PBS (added slowly under stirring), autoclaved, stored at RT.
- Crystal violet solution: 200 mg/l crystal violet dissolved in 1 ml ethanol, filled up to 1 l with ultrapure water, stored at RT.
- 5% formaldehyde solution: 135 ml/l formaldehyde stock solution (37%), diluted in PBS, stored at RT.

I. Production of helper phage

- Top-agar: 2xYT supplemented with 7.5 g/l agar, autoclaved.
- 2xYT-agar plates (100 mm): 16 g/l tryptone, 10/l g yeast extract, 5/l g NaCl, and 15/l g agar dissolved in ultrapure water, autoclaved, poured as thin layers, stored at 4°C.
- 2xYT medium, helper phage VCSM13 or M13K07, kanamycin stock solution, PEG/NaCl solution: see A.
- TG1 bacteria strain, M9/+Thi minimal plates: see B.

4. Protocols

A. Preparation of scFv-phages

Cloned scFv antibody libraries are stored at −80°C as bacteria glycerol stocks and has to be packed into scFv-phages for selection using target antigen or virus stock (see Protocol C). The following protocol describes the superinfection of a log phase library bacterial culture with helper phage comprising kanamycin resistance (see Section I for the production of helper phages) and PEG purification of produced scFv-phages.

A1. Inoculate prewarmed 2xYT-GA to an initial OD_{600nm} of 0.1 with freshly thawed library glycerol stock (~1 ml). Typically, we inoculate 250 ml in a 1 L baffled Erlenmeyer flask for

libraries of large library size up to 10^9 independent bacteria clones. The inoculated volume depends on library diversity and might vary between 50 ml and > 2 l. For libraries cloned from virus-infected donors, an initial library size of 10^7 and inoculating 100 ml is sufficient (**Note 1**).

A2. Grow bacteria at 250 rpm at 37°C until they reach log phase (OD_{600nm} of about 0.5). This typically takes about 2½ h (**Note 2**).

A3. Infect the log phase bacteria culture by adding freshly thawed helper phage VCSM13 or M13K07 (for preparation of helper phage: see Protocol I) with a multiplicity of infection of 20:1 (phage-to-cell-ratio). Infection is best performed by swirling the flask to distribute phages, followed by 30 min standing and 30 min shaking at 250 rpm and 37°C. Exclusively in the first round of selection, infection of the antibody library can be done by hyperphage M13 K07ΔpIII for oligomeric display of scFvs on the phage surface (**Note 3**).

A4. Superinfection of bacterial culture can be monitored by plating 1 μl of the infected bacteria (diluted in 100 μl 2xYT) onto 2xYT-GK agar plates. Successful infection should result in a bacteria lawn the next day.

A5. To induce expression of scFv-pIII fusion proteins, harvest bacteria by centrifugation (4000 × *g*, 10 min, 4°C) in PP tubes (either 50 or 250 ml) and resuspend bacteria in glucose-free induction medium 2×YT-AKI (**Note 4**).

A6. Incubate culture overnight shaking at ≤30°C (**Note 5**).

A7. On the next day, pellet bacteria (4000 × *g*, 10 min, 4°C) and transfer supernatant containing the antibody phages into fresh 50 ml PP tubes (40 ml per tube).

A8. Add 8 ml of prechilled PEG/NaCl to 40 ml supernatant (1/5 volume). Mix well and incubate for at least 1 h on ice (**Note 6**).

A9. Harvest phages by centrifugation (10,000 × *g*, 20 min, 4°C). Make sure to remove PEG/NaCl completely since remaining PEG leads to losing phages in the next step. Therefore, pour away the PEG solution and remove residuals with gauze or centrifuge again and aspirate remaining solution (**Note 7**).

A10. Pure phage preparation gives white pellets. Brownish pellets indicate contamination with bacteria debris. One (*Option 1*) or two (*Option 2*) precipitation steps may be performed. Especially in the first round of selection, we recommend to precipitate twice.

Option 1: Resuspend phage pellets in 1 ml of PBS per tube transfer in 2 ml tubes and centrifuge at high speed in a microcentrifuge (3 min, 4°C). Transfer the phage-containing supernatant into a fresh tube and determine the phage titer as colony-forming units (see Protocol B).

Option 2: Resuspend the phage pellets in 40 ml of ice cold PBS and pellet the bacteria by centrifugation (4000 × *g*, 10 min, 4°C). Save the supernatant and precipitate a second time (8 ml PEG/NaCl to 40 ml supernatant) for ≥1 h on ice or overnight. Proceed as described in *Option 1*.

A11. Store the phage at 4°C and proceed as soon as possible with scFv selection. *Optional*: filter supernatant through 0.45 μm filter. Filtered phages may be stored up to 2 weeks. To prevent proteolysis of the antibody fragments, proteolysis inhibitors might be added. Although not recommended for selection since displayed antibody fragment might be denatured,

long-term storage of packaged library can be done by adding sterile glycerol (15% final concentration) and freezing at −80°C (**Note 8**).

B. Determination of phage titer

Prior to selection, titer of produced scFv-phages should be determined. Phage titration can be done by different methods including counting plaque-forming units (pfu) on bacterial lawns or counting the total particle number. More easily, phage titer is determined as colony-forming units (cfu) by easy countable bacteria colonies on selection plates as described below.

B1. Streak out TG1 from frozen, uninfected bacteria stock onto minimal M9/+Thiamine plates (**Note 9**).

B2. Inoculate 5 ml of 2xYT with grown single colony of TG1 and grow overnight (250 rpm, 37°C).

B3. On the next day, inoculate 10 ml of 2xYT with 100 μl of overnight culture and grow culture until the log-phase is reached (OD_{600} of about 0.5). This will take about 2½ h. *Optional*: Check overnight TG1 cultures for infection by plating 100 μl/plate on 2xYT-GA agar and 2xYT-GK agar. No colonies should grow (**Note 10**).

B4. In parallel, prepare serial dilution for phage titration. Expect about 10^{13} to 10^{14} cfu/ml out of 1 l culture. To guarantee that all phages can infect bacteria, prepare serial dilutions of phage in PBS by diluting 10 μl into 990 μl (10^2, 10^4, and 10^6). Pipette 10 μl of the 10^6 dilution to 990 μl of the log-phase TG1 culture (10^8 dilution) and infect bacteria at 37°C (30 min standing, 30 min shaking) (**Note 11**).

B5. After infection, prepare up to five 1:10 serial dilutions (100–900 μl) of infected bacteria in 2xYT medium. Plate 100 μl of infected bacteria and dilutions onto 2xYT-GA plates. Additionally, plate 100 μl of TG1 culture as negative controls on 2xTY-GA and 2xYT-GK plates. Incubate plates overnight at 30°C. Alternatively, spot 10 μl/dilution in triplicates onto 2xYT-GA plates and dry before overnight incubation.

B6. The next day, count colonies on countable plates and calculate phage titer as colony-forming units (cfu/ml). If colonies are too small to count, increase temperature to 37°C. No colonies should be visible on control plates.

C. Selection of antigen-specific scFv-phages

The following protocol describes the selection of target-specific scFv-phages using recombinant viral protein or virions immobilized on immunotubes. Usually ≥3 selection rounds are performed to enrich specific binding scFv-phages.

C1. For the first round of selection, coat an immunotube with 10–50 μg protein dissolved in 1 ml PBS and incubate the parafilm-sealed tube overnight at 4°C. For subsequent rounds, decrease the protein concentration for more stringent conditions (1–5 μg/ml). If oligopeptides are used for the selection procedure, coat 2–5 μg/ml for all rounds. Alternatively, immunotubes might be coated with a virus stock if the target antigen is unknown (**Note 12**).

C2. On the same day, prepare uninfected TG1 overnight culture as described (see B1–B3).

C3. The next day, wash tube three times with PBS by filling the tube with PBS using a wash bottle and removing the liquid immediately by pouring.

C4. Block the remaining binding sites by filling the empty immunotube completely with MPBS to the brim and incubate for about 2 h at RT.

C5. In parallel, preincubate packaged library (10^{12} to 10^{13} cfu) or phages from subsequent rounds (10^{11} to 10^{12} cfu) in MPBS (2–4% final milk concentration) for at least 1 h by slow overhead rotation in PP tubes (volume depends on volume used for coating immunotubes). Especially in the first round of selection, use 100-fold excess of scFv-phages compared with final library size to increase capturing promising binders (**Note 13**).

C6. Inoculate 20 ml of 2xYT with 200 μl of overnight culture and grow culture until the log-phase is reached (OD_{600} of about 0.5). This will take about 2½ h. Log phase TG1 can be stored on ice until infection with eluted phage. *Optional*: Check for infection of overnight TG1 culture by plating 100 μl on 2xYT-GA and 2xYT-GK plates.

C7. Empty the immunotube, add the preblocked phage solution, seal it with parafilm, and incubate for 90 min gently shaking followed by 30 min standing at the bench.

C8. In the last 30 min of incubation, start washing the immunotubes. In the first selection round, wash 10× (5× with PBST and 5× with PBS). In the following rounds, increase stringency of washing by adding more wash cycles (second round: 20× cycles, third round: 30× cycles, etc.).

C9. Elution of antigen-bound scFv-phages can be done by different methods. We prefer either acid elution (*Option 1*) or protease elution (*Option 2*).

Option 1 (*acid elution*): completely remove remaining buffer, add 1 ml acid elution buffer (pH 2.2) and incubate for about 8 min (longer incubation can destroy the phage). Rotate sealed tubes in an overhead rotator if using >1 ml for coating. Pipette eluted phage to about 100 μl neutralization buffer in a novel PP tube (**Note 14**).

Option 2 (*protease elution*): many common phagemid vectors possess a trypsin cleavage site between the scFv and pIII protein (e.g., all phagemids containing the myc or FLAG tag) that can be used for elution. Add 1 ml freshly prepared trypsin-PBS to the tubes and incubate for 10 min standing (1 ml coated tubes) or overhead rotation (>1 ml coated tubes) (**Note 15**).

C10. Per selection, inoculate 14 ml of log-phase TG1 culture with the eluted phage solution in fresh 50 ml tubes. Infect at 37°C by 30 min standing and 30 min shaking. *Optional*: fill the empty immunotube with 5 ml of log-phase TG1 and perform infection at 37°C as described above. After infection, combine both cultures for following steps.

C11. To monitor the success of an antibody selection, the eluted phage titer can be determined after each selection round. Therefore, make serial dilutions of the TG1 culture after infection (e.g., by plating 100 μl of undiluted culture, 10^2, 10^4, and 10^6 dilutions onto 2xYT-GA plates). Incubate plates at 30°C overnight and count colonies to determine the eluted phage titer after each round. Typically for successful enrichment of specific binders, the eluted phage titer should increase from about 10^4–10^6 in the first round up to the total number used for bacterial infection in subsequent rounds (~10^{10}).

C12. Centrifuge the remaining TG1 culture (3500 × *g*, 10 min, RT), resuspend pellets in about 1 ml 2xYT, and plate onto either one large square plate (245 × 245 mm) or 3 round 15 cm plates containing 2xYT-GA agar.

C13. Grow plates overnight at 30°C and harvest bacteria by adding sterile-filtered 15% glycerol in 2xYT (about 10 ml totally). Harvest cells with flamed glass spreader. Mix very well and freeze bacteria glycerol stock at −80°C.

C14. Perform subsequent selection rounds. Resolve 100–400 μl of the glycerol stock from the previous round in 100 ml 2xYT-GA to start OD_{600nm} of 0.1 and perform selection following the Protocols A, B, and C (**Note 16**).

D. Polyclonal phage ELISA (ppELISA)

After performing repeated rounds of selection, the scFv-phage preparations from the different rounds should be analyzed by ppELISA to identify if target-specific scFv-phage were successfully enriched.

D1. Coat a 96-well ELISA plate with the target antigen (or virus stock: usually 1×10^5–1×10^7 pfu/ml) and control proteins at 2–10 μg/ml (100 μl/well) in PBS, seal plate with parafilm, and incubate overnight at 4°C. In total, coat two wells of target and control proteins plus two wells as blank for each selection round (R0, R1, R2, R3, etc.).

D2. The next day, remove coating solution and block entire plate with MPBS (400 μl/well) for 2 h at RT.

D3. Dilute phages from the different selection rounds to 10^{12} cfu/ml in MPBS, pipette 100 μl/well, and incubate for 1 h at RT.

D4. Wash plate 3× with PBST and 3× with PBS. **Note 17**

D5. Add 100 μl/well of HRP-conjugated anti-M13 antibody diluted 1:5000 in MPBS and incubate for 1 h at RT.

D6. Wash plate 3× with PBST and 3× with PBS.

D7. Add 100 μl/well of TMB substrate solution and incubate until blue color has developed (up to 30 min). Stop reaction by adding 50 μl/well of stop solution. Read absorbance at 450 nm in a microplate reader (**Note 18**).

E. Screening for monoclonal binders

After successful antibody selection (see Protocols A–D), you will end up with an enriched pool of target-specific scFv-phages that must be screened for single binding antibodies ("monoclonals"). Screening can be done differently, using scFv-phages, scFv-pIII fusion proteins, or soluble scFv fragments. For beginners, we recommend screening as monoclonal phages by mpELISA (see Protocol E-I) since scFv-phages can be easily produced and detected by anti-phage HRP conjugates. Sometimes, screening as soluble fragments (see Protocol E-II) is preferred since screening as scFv-phages might result in false positive binders; meaning binding is dependent on the entire antibody-pIII fusions/phage. However, soluble screening requires switching the bacteria strain to a nonamber suppressor strain (e.g., HB2151).

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E-I. Monoclonal phage (mpELISA) screening

E1. Plate TG1 glycerol stocks from target-enriched selection rounds as confirmed in ppELISA (see Protocol D) on 2xYT-GA selection plates to obtain single colonies. Alternatively, use the plates that have been prepared for the determination of the phage titer (see Protocol B).

E2. Fill a sterile 96-well round-bottom plate with 100 μl/well of 2xYT-GA.

E3. Pick (single!) TG1 colonies from selection plates that are positively enriched rounds using sterile toothpicks or pipette tips and inoculate one well/colony. Keep wells H6 and H12 free as blank. Seal plate with breathable membrane (**Note 19**).

E4. Incubate plate at 37°C overnight while shaking. This will be your master plate (**Note 20**).

E5. On the next day, transfer an aliquot of the culture to a new plate containing 100 μl/well 2xYT-GA. Pipette either about 5 μl/well from the master plate using a multichannel pipette to the new induction plate or use a 96-well induction device with sterile metal pins for induction.

E6. Add 50 μl/well 2xYT containing 50% glycerol to the masterplate and freeze sealed plate at −20°C.

E7. Incubate induction plate for about 2½ h in the phage orbital shaker until bacteria reach log-phase (37°C, 200 rpm).

E8. Infect bacteria 1:20 with helper phage, i.e., 10 μl/well of a 10^{11} cfu/ml dilution (100 μl log-phase bacteria containing 5×10^7 bacteria and should be infected by 10^9 phages). Infect for 30 min standing and 30 min shaking at 37°C.

E9. Centrifuge plate (10 min, 3000 × *g*, 4°C), discard supernatant, and resuspend bacteria pellets in 180 μl/well of induction medium 2xYT-AKI. No glucose!

E10. Seal plate with a breathable membrane and incubate the induction plate overnight at 37°C.

E11. Coat two ELISA Maxisorb™ plates per induction plate by pipetting 100 μl/well of antigen/virus stock to the first half (columns 1–6) and control protein to the second-half (columns 7–12) of the plates. Use 2–10 μg/ml antigen/control protein diluted in PBS for coating. Seal plates and incubate overnight at 4°C.

E12. The next day, block ELISA plates with MPBS for 2 h at RT.

E13. Remove blocking solution and add 50 μl/well 4% MPBS.

E14. Centrifuge the induction plate for 10 min at 3000 × *g* and 4°C. The supernatant can be transferred to a new PP microplate and stored at 4°C.

E15. Pipette 50 μl/well of the supernatant from the induction plate to one antigen-coated column and one control protein-coated column (e.g., column 1 of the induction plate to columns 1 and 7 of the ELISA plate, column 2 to columns 2 and 8) and incubate for 1 h at RT.

E16. Wash plate 3× with PBST and 3× with PBS.

E17. Add 100 μl/well of HRP-conjugated anti-M13 antibody diluted 1:5000 in MPBS and incubate for 1 h at RT.

E18. Wash plate 3× with PBST and 3× with PBS.

E19. Add 100 μl/well of TMB substrate solution and incubate until blue color has developed (up to 30 min). Stop reaction by adding 50 μl/well of stop solution. Read absorbance at 450 nm in a microplate reader (**Note 21**).

E-II. Soluble scFv screening

E20. Grow bacteria strain HB2151 on M9/+Thi minimal plates and prepare log phase-HB2151 culture (OD_{600nm} of 0.5) as described before (see B1–B3).

E21. Take 10 μl of eluted phages from the different positively enriched selection rounds and infect 1 ml of log-phase HB2151 at 37°C (30 min standing, 30 min shaking).

E22. Prepare different dilutions of the phage-infected HB2151 culture in 2xYT (e.g., 10^2, 10^4, 10^6), plate 100 μl of the dilutions on 2xYT-GA plates, and grow overnight at 30°C. Alternatively, spot 10 μl/dilution on 2xYT-GA plates in triplicates.

E23. The next day, prepare a masterplate as described above (see E2–E4) by picking single HB2151 colonies.

E24. Inoculate an induction plate (see E5) and freeze the masterplate after adding 50% 2xYT/glycerol (see E6).

E25. Grow induction plate for around 3 h until OD_{600nm} of about 1 is reached.

E26. Centrifuge the induction plate (10 min, 3000 × *g*, 4°C) and completely remove the supernatant by carefully pipetting without disturbing the bacteria pellet.

E27. Resuspend the bacteria pellets in 180 μl/well of buffered 2xYT-SAI medium. No glucose! Seal the plate with breathable membrane and incubate the induction plate overnight at 30°C while shaking (**Note 22**).

E28. Perform ELISA as described in E11 to E19. However, soluble scFvs must be detected by a tag-specific antibody (e.g., anti-myc and anti-His) followed by washing and incubation with anti-primary HRP conjugated antibody. Alternatively, when using scFv libraries entirely based on the kappa light chain, detection can be performed with protein A-HRP or protein L-HRP using 3% BSA for blocking (lower backgrounds).

F. Identification of complete scFv fragments by colony PCR and sequencing

After screening for monoclonal binders (scFv-phages or soluble scFv fragments), hits should be analyzed for the presence of the full-length scFv insert (VH and VL) prior to sequencing. Selection of bacterial clones with incomplete inserts is a common issue observed with antibody phage display. Bacteria encoding for incomplete fragments with lower affinities often show growth advantages compared with those encoding for full-length scFvs and might be predominantly enriched during selection. To retrieve complete scFvs, we recommend analysis of colonies after screening by colony PCR as described below prior to sequencing. The following protocol allows PCR amplification of scFv inserts for size analysis and fast sequencing. Moreover, prepared plasmid DNA and glycerol stocks are useful for sequencing, cloning,

electroporation in HB2151, or direct expression of soluble scFv (Protocol G) that can be used for subsequent functional analysis (see Protocol H).

F1. Streak out the masterplate glycerol stocks from wells that have been identified as antigen-specific binders by screening (see Protocol E) on 2xYT-GA plates and grow overnight at 30°C.

F2. Fill sterile 96-well plate with 100 μl/well LB medium.

F3. Prepare PCR master mix using Taq PCR Core Kit according to manufacturer's recommendations preparing 50 μl/reaction and using scFv insert flanking primers, e.g., P1 (LMB3long) and P2 (fdseqlong) if using pHEN derivatives.

F4. Distribute 50 μl/well of PCR master mix into a 96-well PCR plate.

F5. Use sterile pipette tips to pick single colonies and inoculate one well of the PCR plate and afterward the corresponding well of the medium-filled masterplate.

F6. Run PCR according to manufacturer's recommendations using an annealing temperature of 53°C and 30 cycles.

F7. Analyze 25 μl/sample on a 1% agarose gel containing ethidium bromide. Complete scFv inserts run at about 1 kb when using pHEN phagemids and primer P1 and P2.

F8. Purify remaining 25 μl PCR product of full-length scFvs by QIAquick PCR Purification Kit according to manufacturer's recommendations by eluting DNA in ultrapure water (**Note 23**).

F9. Sequence inserts using a primer designed to either bind downstream or upstream of the forward (P1) or reverse primer (P2) used for colony PCR. Although colony PCR primer (or shorter versions thereof) might be used as well, sequencing primer binding within the amplified scFv insert might increase sequence quality.

F10. Use 100 μl of infected medium from positive binders for inoculation of 6 ml LB high-salt medium supplemented with 100 μg/ml ampicillin and grow culture overnight at 37°C.

F11. The next day, use 1 ml of grown culture to prepare bacterial stock by adding 0.5 ml 50% 2xYT/glycerol and freeze at −20°C. Use remaining culture to prepare plasmid DNA using QIAprep Spin Miniprep Kit according to manufacturer's recommendations by eluting DNA in ultrapure water (**Note 24**).

F12. Analyze obtained scFv sequences for sequence inaccuracies using molecular cloning software (e.g., SnapGene). VH and VL genes can be analyzed by the Fab Analysis online tool by aligning the scFv sequences to the VBASE2 database [26] to identify closest antibody germline sequences as well as to identify the CDR regions of the scFvs.

Preliminary notes prior to antibody fragment production and functional characterization

Antigen/virus-specific scFvs needs to be further characterized for virus neutralization. This step is challenging since the neutralization capacity of antibodies often depends on the antibody valency as observed for HSV, varicella-zoster virus, HIV, and rabies [27, 28]. In these

cases, bivalent antibodies (IgG) or antibody fragments ($F(ab)_2$) neutralized the virus with an exceptional efficiency, while monovalent Fab or scFv fragments did not or only when used at very high concentrations (e.g., 300 times higher than IgG). Although the production of soluble scFvs from phagemid libraries for functional characterization is a simple procedure (see Protocol G), it allows only the production of monovalent fragments that might be insufficient for virus neutralization in some cases. Therefore, the cloning of the VH and VL genes of virus-specific scFvs into other bivalent antibody formats (e.g., scFv-Fc, diabody, minibody) using a high-throughput, recombinant protein expression systems should be considered to increase the likelihood of isolating potent antiviral antibodies (e.g., transient HEK 293 expression platform from National Research Council Canada). The screening of virus-specific antibodies for virus neutralization can be performed either with bivalent or monovalent antibodies. The choice of the method (e.g., reporter virus and cell line) strongly depends on the virus itself. You need the suitable cell culture system to test neutralizing effects of antibodies on the viral replication. In this chapter, we demonstrate the identification of an HSV-neutralizing scFvs as an example for the isolation of neutralizing antibodies from phage display libraries. As mentioned above, this procedure can be modified for the screening of bivalent antibodies and for other viruses.

G. Small-scale expression of soluble scFvs for functional characterization

After the identification of scFvs with a unique sequence, soluble scFv can be produced in small scale to analyze their biological function, e.g., for their neutralizing capacity in case of antiviral antibodies. The protocol below describes the soluble expression of scFvs in the periplasm of nonamber suppressor strain HB2151 that allows the correct formation of disulfide bonds. ScFvs are recovered by periplasmic extraction followed by one-step purification using Immobilized metal ion affinity chromatography (IMAC) for His-tagged scFvs. Please adapt the protocol when using other tags for purification or upscale if higher quantities of scFvs are needed.

G1. Streak out HB2151 glycerol stock of unique scFv clones on 2xYT-GA agar plates to obtain single colonies (**Note 25**).

G2. Inoculate 5 ml 2xYT-GA with single picked colony and grow overnight at 37°C while shaking.

G3. The next day, inoculate 200 ml 2xYT-GA containing reduced concentration of 0.1% glucose with 2 ml of overnight culture and grow culture at 37°C until OD_{600nm} of 1 is reached.

G4. Add 0.2 ml IPTG stock solution (i.e., 1 mM final concentration) to the cultures and grow culture overnight at RT (**Note 26**).

G5. Harvest bacteria by centrifugation (6000 × *g*, 15 min, 4°C) and resuspend the pellets in 5 ml periplasmic preparation buffer supplemented with 1 ml/l of freshly prepared lysozyme stock solution.

G6. Incubate preparation on ice for 30 min and stabilize spheroblasts by adding 50 μl 1 M $MgSO_4$ (i.e., 10 mM final concentration).

G7. Centrifuge preparations (12,000 × *g*, 30 min, 4°C) to clarify periplasmic fractions.

G8. Dialyze scFvs using D-Tube™ Dialyzer (MWCO 12–14 kDa) overnight at 4°C against NPI-10 buffer (**Note 27**).

G9. Purify scFvs by IMAC employing Ni-NTA spin columns following the manufacturer's protocol and loading columns several times with the scFv preparations.

G10. Analyze samples by reduced SDS-PAGE and Coomassie stain (and/or Western blot) to check for purity.

G11. Dialyze scFvs against PBS buffer, filter-sterilize using low-protein-binding filter (0.22 μm) and measure protein concentration (e.g., by Nanodrop using calculated extinction coefficient and molecular mass).

G12. Store scFvs at 4°C and proceed as soon as possible to characterization. ScFv stability can differ dramatically depending on the sequence from few days to several years. Do not freeze scFvs since many tend to aggregate after thawing. Oligomeric state of scFv can be analyzed by gel filtration chromatography.

H. Functional antibody characterization by plaque reduction neutralization test

In the case of screening antiviral antibodies, scFvs can be screened in neutralization and/or protection assays that detect antiviral activities of antibodies *in vitro* or *in vivo* [29]. As the "gold standard" assay for screening and neutralizing capacity of antibodies, plaque reduction neutralization test (PRNT) can be performed where the $PRNT_{50}$ value is used to describe the neutralization activity. The protocol below describes a PRNT-based assay to screen for best neutralizing scFvs and was optimized for HSV-specific and neutralizing scFvs.

H1. Cultivate cell line suitable to form plaques when infected with virus of interest (e.g., Vero cells for HSV, dengue virus) according to supply recommendations. Most virus sustainable cell lines are recommended to be propagated and stored in low passage levels to guarantee susceptibility for plaque formation.

H2. Seed cells in 6-well cell culture plates with 5×10^5 cells/well and grow in 5 ml/well complete culture medium in a humidified cell incubator at 37°C and 5% CO_2 for one to three days and until a confluency of at least 90% is reached (**Note 28**).

H3. To screen for the best neutralizing antibody, dilute all scFvs to a constant concentration (e.g., 4 μM) in appropriate culture medium supplemented with penicillin/streptomycin solution. For some viruses, reduction of the FBS concentration is recommended. If the $PRNT_{50}$ should be determined, prepare twofold serial dilution of scFvs (at least five steps) that is the amount of antibody required to neutralize 50% of the infectious virus particles (**Note 29**).

H4. Freshly thaw cryostock with a known titer of plaque-forming units (pfu) at RT and dilute to 600 pfu/ml (same medium as in H3).

H5. Pipette 300 μl of diluted scFvs (or PBS as negative control) to 300 μl of the virus preparation in a sterile plate and incubate the scFv-virus mixtures at 37°C for 1 h.

H6. Carefully aspirate old medium from cell plates and wash with 2 ml PBS. Always pipette to the wall of the wells to not destroy the cell monolayer.

H7. Add 500 μl/well of the scFv-virus mixtures to each well and tilt plates as described above. Include virus only to every plate. ScFvs should be tested in at least duplicates.

H8. Incubate plates at 37°C for about 1 h (time can vary between 30 and 90 min depending on the cell/virus). During incubation, carefully tilt all the plates 10–15 min.

H9. Remove inoculum and wash cells with 3 ml/well PBS.

H10. Add 3 ml/well CMC medium and incubate plates in the humidified incubator (37°C, 5% CO_2) for three days.

H11. Fixate the cells with formaldehyde solution (3 ml/well for 5 min) and stain with crystal violet solution (800 μl/well) for 2 min.

H12. Wash wells once with PBS and twice with ultrapure water (2 ml/well).

H13. Count plaques and calculate percentage of neutralization as follows:

% of neutralization = 100 − [(no. of plaques: virus + antibody)/no. of plaques: virus only) × 100].

When analyzing serial dilutions of scFvs, percentage of neutralization can be plotted against scFvs concentration to determine the $PRNT_{50}$ values.

After functional characterization, produced scFvs should be characterized for specificity and affinity in binding to recombinant antigen and, more importantly, to intact virions. The specificity of scFvs to recombinant proteins can be easily analyzed by ELISA using recombinant virus antigens (or virus lysates). While scFv affinities has to be determined by kinetic measurements (e.g., by surface plasmon resonance or biolayer interferometry), estimation of apparent scFv affinities can be quickly performed by ELISA. Therefore, recombinant antigen is coated on ELISA plates and 1:2 serial dilutions of purified scFvs (e.g., 1 μM start concentration) are incubated in triplicates followed by detection via tag-specific IgGs and anti-IgG HRP conjugate. The half-maximal effective concentration (EC_{50}) of saturated binding corresponds to the KD value and can be used for affinity ranking of scFvs. However, immobilization of antigens/virions on plastic can alter conformation of coated proteins leading to antibodies recognizing epitopes that are not found on intact virions. Alternatively, binding affinity of antibody fragments can also be estimated by flow cytometry using antigen overexpressing cell lines [30], e.g., by using Vero cell being infected with HSV [18, 28].

In conclusion, broadly neutralizing human monoclonal antibodies represent an excellent opportunity for the prevention and therapy of viral infections and are a potent tool to identify neutralizing epitopes on viral proteins for vaccine approaches. Phage display technology became a potent tool to isolate human neutralizing antibodies and should be considered as a validated technique for future approaches.

I. Production of helper phage

Larger preparation of helper phage (VCSM13 or M13K07) being used for superinfection to prepare scFv-phages for selection (Protocol A) and screening (Protocol E-I) can be obtained following the protocol below. Hyperphage cannot be produced without recombinant *E. coli* strain and must be purchased.

I1. Thaw primary phage stock and prepare 10^2 dilutions (100 μl) in 2xYT medium.

I2. Mix helper phage dilution with equal volume of log-phase TG1 culture prepared as described above (see B1–B3) to infect bacteria at 37°C (30 min standing and 30 min shaking).

I3. Melt top agar in a microwave, aliquot in glass tubes while hot, and cool down tubes to 42°C in a prewarmed water bath.

I4. Pipette infected bacteria to top agar tubes, mix quickly, and immediately cast top agar onto prewarmed (37°C) 2xYT plates. Grow plates overnight at 30°C (**Note 30).**

I5. The next day, pick a single small plaque from grown bacteria lawns and transfer into 3 ml 2xYT that was inoculated with 100 μl of TG1 overnight culture right before.

I6. Incubate culture for 3 h at 37°C while shaking and use grown culture to inoculate 500 ml of 2xYT.

I7. After one further hour of growing, add 200 μl kanamycin stock solution and grow overnight at 37°C while shaking.

I8. The next day, pellet bacteria (4000 × *g*, 10 min, 4°C), transfer 40 ml of supernatant into 50 ml PP tubes and precipitate phage as described in Protocols A8–A10 (**Note 31**).

I9. To inactivate the remaining bacteria, either heat helper phage preparation for 15 min at 65°C (recommended) or filter through low protein binding 0.45 μm filter (**Note 32**).

I10. Aliquot the phage preparation into 2 ml PP tubes and snap freeze the tubes in liquid nitrogen prior to storage at ≤−20°C.

I11. Determine phage titer as plaque-forming unit (pfu/ml) as described above (see I1–I4) using 1:10 serial phage dilutions from 10^8 to 10^{13} for infecting 100 μl log phage TG1 and counting plaques on countable plates (**Note 33).**

5. Notes

Note 1: To minimize loss of diversity, very large libraries (>10^9 independent clones) should always be stored as sublibraries that can be separately packed and combined prior to selection. Antibody selection should be only performed with freshly packaged (sub-)libraries that have been kept at 4°C for short as possible. Due to loss of diversity, we do not recommend selection with frozen phage preparations or phage antibodies that have been packaged from secondary library stocks. Importantly, minimize freeze and thaw steps of your primary library and keep it frozen at −80°C until needed.

Note 2: Correct growing temperature is crucial for phage display. Too low a temperature (<34°C) might result in ineffective formation of pili that are necessary for successful infection by phage.

Note 3: Oligomeric display of scFvs in the first round of selection by infection with hyperphage can greatly improve selection efficacy and can reduce loss of interesting binders during the initial selection step improving the average display from 0.01 up to 5 antibody fragments

per phage. Please note that hyperphage infection results in scFv-phages without wild-type pIII proteins that are necessary for successful infection. To restore the wild-type infectivity, hyperphage-packed libraries should be eluted by a protease cutting between the antibody fragment and the pIII protein (e.g., trypsin works well for pSEX and most pHEN derivatives). To check for suitable proteases for your phagemid, you might analyze your vector on PeptideCutter (ExPASy database) and perform Western blot analysis of digested antibody phages using an anti-pIII antibody for detection (see Note 8).

Note 4: Expression of scFv-pIII fusion proteins with *lac* promoter-based phagemid vectors can be performed by using glucose-free media without or a low concentration of IPTG (about 5 μM to 500 μM final concentration depending on used phagemid). However, strong induction of the *lac* promotor by too high concentration of IPTG might reduce the expression of complete scFv-pIII fusion proteins. Induction conditions should be optimized and antibody presentation on phages can be analyzed by SDS PAGE and Western blot using primary anti-pIII detection system (see Note 8).

Note 5: Reducing temperature to 30°C or lower guarantees better expression and folding of complete antibody fusions that otherwise might be overgrown by incomplete fusions. Moreover, lower temperature helps to reduce degradation/cleavage of antibody fragments on the phage surface.

Note 6: Longer incubation on ice or 4°C might result in better precipitation of antibody phage and higher yields.

Note 7: To save time, we perform phage pelletation in Beckman centrifuges, equipped with fixed angel rotors such as JA 16.250 that allows higher speed centrifugation in 250 ml reusable or 50 ml disposable PP tubes if using adapters. If not available, centrifugation with swinging buckets and lower speed can be performed, but centrifugation time should be increased accordingly.

Note 8: Helper phage/hyperphage packaged libraries can be analyzed by SDS-PAGE and Western blot. Run reduced phage samples on 10% SDS PAGE and incubate membrane with primary anti-pIII monoclonal antibody and secondary anti-mouse polyclonal serum HRP conjugate, using ECL substrate for detection. Although wild-type pIII has a calculated molecular weight of about 45 kDa, it runs at about 60 kDa in SDS-PAGE. Accordingly, complete scFv-pIII constructs can be detected at about 90 kDa.

Note 9. TG1 is an amber suppressor *E. coli* strain most widely used for antibody phage display. Growing TG1 on proline-deficient M9 minimal plates guarantees maintenance of the F′ episome important for production of pili necessary for phage infection. For growing of TG1, M9 plates must be additionally supplemented with thiamine due to a chromosomal mutation in the thiamine biosynthesis.

Note 10: OD_{600} of culture is critical. Do not overgrow or grow below 34°C. Bacteria can be kept on ice for a while (30 min up to a few hours), but cells might start losing pili after longer incubations.

Note 11: One ml of log phase TG1 (OD_{600nm} of 0.5) corresponds to about 5×10^8 bacteria. Always infect with at least 1:10 phage-to-cell ratio for titration, i.e., do not use more than 5×10^7 phages for 1 ml log-phase TG1.

Note 12: If enough antigen is available, increase volume of coating solution up to 5 ml, especially in the first round of selection. Only use highly pure (>90%), freshly prepared protein from sources you can trust. Do not use proteins that have been stored at 4°C for prolonged periods. If using oligopeptides or virions for selection, immobilization condition should be optimized due to lower coating efficacies and/or reduced accessibility of epitopes. Test different buffer/pH/additives for immobilization on MaxiSorp™ plates (e.g., 50 mM carbonate buffer pH 9.6) using tag-specific antibody enzyme conjugates for detection in ELISA. Alternatively, antigens might be biotinylated and immobilized on streptavidin-coated tubes/plates or be captured in solution using streptavidin dynabeads [31].

Note 13: If using proteins with large tags/fusions (e.g., Fc region, GST tag) for antibody selection, supplement the preincubation solution with respective proteins to reduce enrichment of binders against those parts. If using biotinylated oligopeptides immobilized on streptavidin-coated immunotubes, preincubate phages with streptavidin to deplete streptavidin-specific binders.

Note 14: To check pH of final solution, perform microtitration using pH indicator strips to determine volume of neutralization buffer required to get a final pH of 7.2–7.4.

Note 15: Always elute hyperphage-packaged libraries by protease elution to guarantee highest infectivity of eluted phages.

Note 16: We usually perform three rounds of selection, but this might be increased up to five or more. However, performing additional rounds might lead to loss of diversity and enrichment of binders having growth advantages that might occur (e.g., truncated scFvs).

Note 17: Washing can be done by hand using a small plastic box filled with buffer. Remove all liquid by inverting the plates and hitting it onto paper towels. Change buffer and towels frequently. For better reproducibility and when screening larger amounts of plates, wash plates using a microplate washer (e.g., BioTek Instruments, Tecan).

Note 18: Successful enrichment of specific binding scFv-phages should be seen as increasing of target-specific signals compared to control antigens. For a typical result see [18], Figure 4A.

Note 19: Try to pick single colonies. Sometimes picking of double colonies or a spillover from well to well might occur. Secondary screen of positive well (hit-picking) can be performed. If performing high-throughput screening on a regular basis, automation of the picking process by colony pickers (e.g., Molecular Devices) might be considered.

Note 20: Most universal orbital shakers for bacterial culture possess an orbit diameter of about one inch that works very well for Erlenmeyer flasks (25 ml up to 2 l) and shaking at about 200 to 250 rpm. However, growing bacterial culture in microtiter plates can be more challenging due to evaporation, contamination, and oxygen transfer. To prevent contamination, shaking

at a lower speed (about 180 rpm) can be performed although oxygen supply is reduced as well. Alternatively, PP deep well plates can be used for screening. To prevent evaporation, plates can be tape-fixed in an additional plastic bag or use PBS-filled plates at the bottom and top when using microplate holders. The highest yield of soluble scFvs can be obtained with special temperature-controlled shakers with a small orbital diameter (0.12 inch) and shaking at high speed (1000 rpm).

Note 21: Usually, antigen-specific binding is defined as signals at least five times higher than the background. For a typical result, see [18], Figure 4B and C.

Note 22: The 2% glucose guarantees repression of antibody expression within the first 3 h of growing. To induce antibody expression, medium must be replaced by induction medium without glucose and optimized concentration of IPTG. Many scFvs show very low yield in the supernatant after expression in *E. coli* and periplasmic extraction is highly recommend prior to ELISA, e.g., by resuspending bacteria pellets in 180 µl/well periplasmic preparation buffer (see Protocol G) for 30 min on ice. However, as shown by Hust et al. [32], buffered 2xYT-SAI and growing cultures overnight at 30°C can improve production in *E. coli* for some scFvs even without performing periplasmic extraction.

Note 23. Adjusting PCR conditions to obtain single scFvs bands on agarose gel is important for successful sequencing of PCR fragments, e.g., by increasing annealing temperature. Alternatively, scFv bands can be recovered by QIAquick Gel Extraction Kit or sequencing can be done using plasmid DNA (see F10).

Note 24: Plasmid DNA can be used for electroporation into self-made electrocompetent HB2151 if scFv-phage screening with TG1 bacteria was performed beforehand. If already in HB2151 after soluble screening, the glycerol stock can be used to obtain single colonies on 2xYT-GA plates for small-scale production of soluble scFvs (see Protocol G). Alternatively, plasmid DNA can be used for subcloning into bacterial expression vectors without pIII gene.

Note 25. For time reasons, we recommend soluble expression of scFvs in nonamber suppressor strain such as HB2151 when using phagemid with the amber stop codon between the scFv and the pIII protein. Otherwise, scFvs can also be subcloned into expression vector without the pIII gene. If clones are still in TG1 after phage screening, phagemid DNA can be transformed into self-made competent HB2151. Use standard protocol for generation of chemically or electrocompetent HB2151. Alternatively use commercially available competent nonamber suppressor strains for soluble production (e.g., SS320 by Lucigen, or Express I^q by NEB).

Note 26. Reducing the temperature for soluble scFv expression is important for proper folding and stability of produced scFvs.

Note 27: As a cheaper option for dialysis in small scale, we recommend using 2 ml PP tubes without lids, filled with scFv preparations, and sealed with square cut dialysis membrane fixed with rubber band and parafilm. If using other tags for purification, dialyze in recommended buffer prior purification.

Note 28: Determine best number/growing conditions for your cell line. Gently tilt plate about five times horizontally after seeding to guarantee that uniform monolayers of cells are

formed. PRNT assay can be adapted to plates with higher well number (e.g., 12–24 well/plates) by reducing pfu and volumes used. Presented protocol was optimized for PRNT with HSV and Vero cells. Cell line and optimal conditions are different for each virus and should be optimized for best results (e.g., scFv-virus incubation time, absorption time on cells, percentage of CMC for overlay, working virus dilution and volume, incubation time after infection).

Note 29: Since performing of the PRNT is laborious and time-consuming, using a single concentration of scFvs for neutralization usually allows a good head-to-head comparison to identify best neutralizing scFvs. In a second step, most promising scFvs can be cloned into other (bivalent) Ab formats (e.g., scFv-Fc) and tested in more detail by analyzing $PRNT_{50}$ (or more precisely even $PRNT_{70}$ or $PRNT_{90}$).

Note 30: The temperature of melted top agar should be exactly at 42°C before casting. Higher temperatures might kill *E. coli* bacteria, while at lower temperatures, the top agar might solidify too fast before pouring nice layers on plates.

Note 31: Although helper phage supernatant can be directly used after heat treatment, we recommend purifying and concentrating by one or two steps of PEG/NaCl precipitation.

Note 32: Using 0.22 μm filter for phage filtration is not recommended due to loss of filamentous phages. If clogging of 0.45 μm filter occurs, centrifuge phage preparation at high g-force at 4°C prior to filtration.

Note 33: Using 10^8 phage dilution guarantees an at least tenfold excess of helper phage to bacteria for infection. Avoid additional freeze-and-thaw cycles of helper phage preparations. Titer of frozen helper phage might drop over time during storage and titration should be repeated after several months. Sterile glycerol up to a final concentration of 50% prior to snap freezing can be added to prolong storage time. Review Ref. [33] for more information about correct storage of phages.

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