Preface

When the first edition of Antibody Phage Display: Methods and Protocols was published, I and my co-editor Philippa O’Brien expressed the hope that the book would form a resource of real value for scientists who had followed the development of phage display technology through its first decade. The large number of citations to the book’s content and the success of its sales provided some assurance that we met that goal.

The aim of providing a resource that will guide readers in the design and execution of experiments based around antibody phage display holds true for this second edition; it goes alongside the hope that the methods will inspire investigators with the possibilities presented by this and related technologies. These include the huge range of methods for isolating recombinant antibodies from phage display libraries, how the targets recognised by antibodies of interest can be identified, the identification and exploitation of antibodies that can enter cells and bind to cytosolic targets, and novel approaches to the expression of recombinant antibodies.

This is a field of biology in which innovation is the norm: in assembling contributions, I have therefore tried to stimulate the reader’s imagination as much as guide their practice in the laboratory.

I am indebted to all the contributing authors for sharing their expertise with the wider scientific community. I also pay thanks to my graduate students past and present with whom I have enjoyed exploring an area of biology that is forever moving forward.

Glasgow, UK

Robert Aitken
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Chapter 1

Antibody Phage Display: Overview of a Powerful Technology that Has Quickly Translated to the Clinic

Beatrix Kotlan and Mark C. Glassy

Summary

Antibody-based immunologic reagents are useful for identifying, isolating, or eliminating cells with particular characteristics related to different diseases. Phage display is a highly valuable technique for antibody selection related to this purpose. In brief, a diverse group of antibody genes prepared from a patient or generated in vitro are inserted into a phagemid vector or the phage genome so that when the protein is expressed, it becomes anchored on the surface of the phage by fusion to a coat protein. A diverse library of recombinant antibodies is generated in this way and can then be exposed or panned on the antigen of interest, typically, this being a molecule associated with a particular pathological condition. Phage that carry proteins or peptides bind preferentially to the target and can thus be isolated from the library. The viruses that are recovered in this way also carry the gene for the binding moiety facilitating its over-expression or manipulation. Recent reviews highlight key milestones in the development of antibody libraries and their screening by phage display, and the impact of these technologies on drug discovery seems assured.

Key words: Antibody fragment, Phage display library, Target antigen, Specific binding

1. Introduction

Since its introduction almost 20 years ago (1–4), phage display technology has completely transformed approaches to the analysis of biomedical problems. Initial drawbacks and skepticism were quickly overcome by growing evidence of the technology’s strong and well-defined scientific process. The ability to rapidly select and analyze binding interfaces has been its most powerful application.
The use of various display vectors and in vitro selection technologies has influenced the process of generating antibody or peptide ligands and selecting for interaction with a target. The advent of this technology relies in the ability to design repertoires of ligands from scratch and the power of phage selection to select those ligands with the desired biological properties.

One of the most widely used phage library methodologies is based on the use of filamentous phage, a family of viruses that can infect and replicate within Escherichia coli. Exploiting the capacity of molecular biology to generate libraries containing more than several hundred million different peptides or proteins, the ease with which phage display can aid the isolation clones with binding properties has proved to be its most successful application. There has been significant progress in this rapidly developing field, including the use of large phage antibody libraries to discover novel therapeutic targets and methods for selection of biologically active ligands. This technology has strongly influenced the fields of immunology, cell biology, biotechnology, pharmacology, and drug discovery.

The power of phage display derives from the observation that DNA encoding millions of variants of a ligand can be batch-cloned into phagemid vectors or the viral genome to create fusions to genes that encode the phage coat proteins pIII, pVI, or pVIII. Expression of the fusion product and its incorporation into the phage coat presents the ligand on the viral surface, while the coding sequence for the fusion resides within the phage particle. Display enables virus bearing a relevant ligand to bind to an immobilized target of interest while non-adherent phage can be washed away. To amplify the small numbers of virus that may be recovered in this way, phage can be infected into bacteria and regrown for further rounds of selection and analysis of their binding characteristics. The overall process depends on the synthesis of large combinatorial repertoires on the phage, the success of ligand display on the phage, and the combination of selection and enrichment.

Interest in recombinant antibody technologies has rapidly increased because of the wide range of possible applications in therapy and diagnostics but interest has been especially high with respect to cancer biology. Several recombinant antibody libraries associated with different screening technologies have been generated in order to isolate human antibody molecules. To increase the chances of isolating antibody with virtually any specificity, innovative strategies have been developed to clone natural antibody repertoires and to increase library diversity beyond the scope of the immune system. The first phase of development entailed capture and cloning of the natural diversity of antibody repertoires from an individual or groups of donors; this has been succeeded by libraries that have been partially or completely
designed in silico and in vitro, based upon an understanding of the antibody–antigen interactions derived from structural data. Efficient display strategies are essential to maximize the availability of the library’s diversity. Phage display is one of the important developments and has provided innovative tools to efficiently display, screen, and extract the desired binding specificities from billions of different antibodies. Yeast display, ribosome display, and robotic platforms are other innovations in this respect. Phage display is the longest-standing platform among the molecular display technologies and has played a dominant role in the engineering of synthetic binding proteins of all sorts. Novel interfaces have been generated from libraries of antibody fragment frameworks and alternative scaffolds, as well as assisting combinatorial methods to further enhance diversity. The ability to rapidly select and analyze binding interfaces and the compatibility of phage display with high-throughput methods ensures its future in the engineering of synthetic binding proteins for very many applications (8).

Phage display is founded upon efficient procedures for construction of libraries of antibodies (or antibody fragments), selection, screening, and expression. Chapters in this volume present detailed methods for each of these processes. Phagemid vectors are most commonly used for phage display, libraries being constructed with antibody Fab fragments (see Chapter 2) or single chain Fv (scFv) proteins (see Chapters 3 and 6). The antibody coding sequence(s) is placed under the control of the lac promoter for ease of control, at this stage. Independent cloning of antibody light (L) and heavy (H) chains maximizes combinatorial diversity in the library. Although other display options exist, it is most common for the L (or H) chain coding region to be fused in-frame with the phage gene III. The phage gene III codes for a truncated version of the phage surface protein pIII, a protein present at the tip of the phage filament in five copies. To drive viral replication and assembly, bacteria carrying the phagemid construct are superinfected with a helper phage. On low-level induction of p lac (usually, relieving glucose repression is sufficient; induction with IPTG is usually reserved for high-level expression [see Chapters 10 and 16]), the cloned genes are expressed from the phagemid, the antibody fragments fold in the bacterial periplasm, and after packaging of single-stranded phagemid DNA into nascent virus, a mixture of native pIII (encoded by the helper phage) and antibody-pIII fusion proteins (encoded by the phagemid) complete the assembly of the viral filament. Phage released from the infected bacteria thus display antibody at their tip and carry the encoding sequences. To extract from a library those phage able to bind to the specific antigen, selection is carried out by several rounds of panning in antigen-coated microtiter wells (see Chapter 10), immunotubes (see Chapters 6 and 7) or magnetic beads (see Chapter 11). Phage that are extracted are
reinfected into *E. coli* to allow replication and repeated rounds of selection (9–11).

Although this volume focuses exclusively of antibody phage display, the original experiments demonstrated that both small peptides and folded proteins can be displayed on the surface of filamentous bacteriophage (1, 12). Hence, phage display allows the presentation of a very wide range of ligands on the surface of filamentous phage, and the selection of peptides and proteins, including antibodies, with high affinity and specificity to almost any target.

The principles of phage display are well established and selected papers from the older literature illustrate conveniently the general principles (1, 5, 13, 14). As the methods for antibody phage display evolved from their early beginnings (2, 9, 14), it became possible to isolate antibodies with sub-nanomolar affinities from very large libraries of scFv and Fab proteins. With the range of selection and screening techniques that are possible, the same non-immunized “single pot” library can be used to derive many high-affinity antibodies with different specificities (15, 16). When libraries of the requisite size and diversity are unavailable, custom-made resources from vaccinated, infected, or other individuals undergoing an immune response to an antigen of interest may be constructed and screened (see Chapters 4 and 5) (see Note 1).

1. To maximize diversity, immunoglobulin sequences are amplified from peripheral blood lymphocytes (PBL), or lymphoid tissue isolated from several donors (see Notes 2 and 3). The procedure begins with total RNA or messenger RNA (mRNA) extracted from these cells using appropriate kits and the protocols recommended by the manufacturers (see Note 4).

2. The concentration and quality of the isolated RNA is estimated by spectrophotometry at wavelengths of 260 and 280 nm. The optimal 260:280 ratio of these absorbance measurements lies between 1.7 and 1.9.

3. RNA is denatured (65°C, 10 min) and is checked by gel electrophoresis on 1% agarose as a further assessment of quality.

4. First strand complementary DNA (cDNA) is synthesized using a commercial cDNA synthesis kit. The reaction can be primed with random hexamers for convenience as, at a later stage, immunoglobulin sequence will be amplified with polymerase chain reaction (PCR) with specific primers. Random priming of first-strand synthesis may also overcome some of the
limitations of sub-optimal RNA quality. If the RNA is of a very high standard, oligo-dT or immunoglobulin-specific primers (see Chapter 6) can be used.

5. Immunoglobulin sequences are amplified from the cDNA pool (see Note 5). The design of the oligonucleotide primers will govern the range of immunoglobulin sequences that are recovered by the PCR. In order to form a functional library, it will be necessary to recover both heavy and light chain sequences from the cDNA pool in separate reactions. However, options exist within this broad goal. The reaction might bias recovery toward the class (e.g., IgM or IgG) or isotype (e.g., light chain κ or λ) of the coding sequences (see Chapter 4). Equally, the reaction may specifically recover variable (V) region sequences for the assembly of scFv antibodies, or, by using primers that anneal to sequences encoding the V region and constant domains, the foundation can be laid for the construction of Fab libraries. To assist, PCR primers can be designed with additional information on V region sequences from the V-BASE directory (MRC Centre for Protein Engineering; URL vbase.mrc-cpe.cam.ac.uk), a resource within which germline sequences for human immunoglobulin segments are compiled. The design of the PCR primers and the number of separate amplification reactions must maximize the diversity of the immunoglobulin sequences that are recovered. For example, in the early studies reported by Marks et al. (4), primers are described that hybridize to the 5′ (termed “back” primers) and 3′ ends (termed “forward” primers) of the rearranged VH or VL regions. In this example, the comprehensive recovery of immunoglobulin sequences helped to increase the overall size of the final library, which in turn increased the chance of isolating high-affinity antibodies (4).

6. Primers used for recovery and amplification of the immunoglobulin repertoires may include restriction sites so that the products can be cloned into the phage display vector. Sites must be placed with due regard to reading frames (see step 8).

7. The heavy and light chain sequences are amplified in separate PCRs. The PCR products are run on 1.5% low-melting agarose gel, and the correctly sized products are purified using gel extraction reagents.

8. If primers used for recovery of the immunoglobulin repertoires lack restriction sites for cloning, a secondary PCR can be performed with primers that place the sequences required into appropriate reading frames for successful translation, export, and assembly. Display vectors for the construction
of Fab libraries will typically carry leader sequences for heavy and light chain inserts, and the reading frame for the heavy chain component of the Fab must allow fusion to the phage coat protein (e.g., pIII) so that the translated and assembled Fab is incorporated into the viral particle. For a vector such as pComb3H, a derivative of pComb3 (14), the heavy chain products need to be fitted with *Xho*I and *Spe*I sites at the 5′ and 3′ termini, respectively. For the light chain amplicons, *Sac*I and *Xba*I sites are required.

Similar considerations apply to cloning into vectors for the construction of scFv libraries: PCR products must be cloned so as to form a contiguous reading frame spanning the vector-borne leader sequence, the VH insert, the linker, the VL insert, and the pIII coding sequence. For the construction of scFv libraries in the pHEN2 vector, a derivative of pHEN1 (9), an *Nco*I restriction site needs to be present at the 5′ terminus of VH products, with *Xho*I at the 3′ terminus. For *Vk* or *Vλ* PCR products, an *Apa*LI site is required at the 5′ terminus with a *Not*I site at the 3′ terminus.

9. scFv display vectors such as pHEN2 incorporate a linking sequence such as (Gly₄Ser)₃ that tether the VH and VL domains together. When this is absent, the linker can be incorporated with a further overlap PCR (see Chapter 5) (see Note 6). Initial sizes of the PCR products as revealed by agarose gel electrophoresis should be around 400 bp for VH, 380 bp for *Vk*, and 370 bp for *Vλ*. Once the overlap reaction has been conducted, the product should increased in size to around 800 bp.

10. Once amplified products are ready, they and the display vector of choice need to be digested with the relevant restriction enzymes. For example, overlap products for construction of a scFv display library in a vector that lacks a linker (e.g., pHEN1) need to be digested with *Neol* and *Not*I. In this instance, 1 µg of DNA is treated with *Not*I at 37°C for 2 h, followed by addition of *Neol* and digestion for a further 2 h. Where the buffering requirements for the first and second enzymes are very different, the DNA may have to be purified and exchanged into a second buffer system in order to ensure efficient digestion. For example, when cloning VL products into pHEN2, buffering requirements for *Apa*LI and *Not*I are sufficiently dissimilar that one or another enzyme is unlikely to operate with more than 50% efficiency in a single, commercially available buffer system. It may be prudent to dephosphorylate the digested vector with calf intestinal alkaline phosphatase to guard against vector religation.
11. Digested samples of vector and insert DNA are purified from a 1% low melting agarose gel in Tris acetate EDTA buffer, using a commercial purification kit (see Note 7).

12. For the ligation that will create the final library of DNA products, multiple ligations may need to be set up with appropriate molar ratios of vector and insert and long incubation times (16°C, 12 h). The products of the ligation reactions can be used immediately in transformation or kept frozen at −20°C to await the next step.

The library of DNA molecules is transferred into an *E. coli* host to amplify the size of the resource and to provide an environment within which expression and viral assembly can take place in order to create a display library. The choice of bacterial host is critical. Many phagemid display vectors carry an amber stop codon between the immunoglobulin-coding insert and the gene for the viral coat protein (e.g., pHEN2 and its derivatives; Fig. 1).

**2.2. Generation of a Library of Recombinant Antibodies in a Display Vector**

![Diagram](image)

Fig. 1. Schematic flow chart to illustrate the generation of an antibody phage display library. Features of phagemid: **VH–VL**, cloned antibody sequences in scFv format; **L**, bacterial leader sequence; **Tag**, sequences for purification (e.g., hexahistidine) and detection (e.g., c-myc epitope); **amber codon**, TAG stop codon that is suppressed in *E. coli* strains such as TG1; **gIII**, gene for minor phage coat protein pIII; **Amp**, ampicillin resistance marker for selection; and **M13**, viral sequence used in packaging of single-stranded phagemid DNA into nascent viral filaments. Diagram of scFv phage illustrates display of scFv fusion protein at the tip of the viral filament along with wild-type pIII (designated g3p), and packaging of phagemid DNA into the phage.
Depending on the bacterial host, this allows convenient expression of either a fusion protein (for phage display) or recombinant antibody in a soluble form (for evaluation of the properties of clones selected from the library). For production of a display library, it is therefore vital that a bacterial strain with a suppressor phenotype is chosen.

In addition, the vast majority of display vectors in common use are phagemids that require superinfection with a helper phage system to enable assembly of virus that will carry recombinant antibodies at their surface (Fig. 1). Infection of filamentous fd phage (M13 and its relatives) requires expression of the F pilus as a viral receptor. The *E. coli* strain TG1 is one of the most commonly used bacterial strains for amplification and assembly of phage display libraries because it carries a *supE* mutation that suppresses the TAG amber stop codon and it carries an F′ episome that cannot be transferred by conjugation to F− cells as a result of the *traD36* mutation. The F′ episome also bears genes that complement chromosomal mutations for proline biosynthesis, hence the presence of the episome can be confirmed by growth of the bacteria on minimal medium. Another strain well suited to these objectives is *E. coli* XL1-Blue; this is nominated in several chapters in this volume. One advantage of the strain is that the F′ episome carries a tetracycline-resistance marker.

Finally, a protocol is required that can transform the ligation products into the bacterial host with efficiency that is sufficiently high to ensure complete representation of the DNA library in the resulting phage displayed resource. Although chemical transformation methods can be effective for library construction, electroporation provides a combination of high efficiency and experimental simplicity. This method is proposed in several chapters. While electrocompeent bacteria can be purchased commercially, Chapter 2 (also Chapter 6) provides methods for their preparation in-house. The following overview is summarized in Fig. 1.

1. To create the phage display library, electrocompeent *E. coli* TG1 bacteria are transformed with the ligation products through electroporation. Multiple transformations may be required in order to use all the ligation products and hence capture all potential components in the DNA library.

2. The transformed bacteria are allowed to recover in rich medium containing glucose. The glucose serves to suppress expression from the *lac* promoter in the phagemid vector (where applicable), hence reducing the chances of loss of clones that might otherwise express recombinant antibodies inhibitory to growth.

3. For selection of transformants, the bacteria are plated onto large petri dishes containing rich medium, an antibiotic, and glucose, for the reasons stated above. They are grown
overnight at 30°C; again, this limits the potential toxicity of expression products.

4. The overnight cultures are resuspended in liquid medium and, for security, aliquots are frozen at −80°C with glycerol (15–20% [v/v]) as a cryopreservative. Other samples are diluted (typically 1:1,000) into fresh liquid medium and grown at 37°C with shaking into mid-log phase. This can be judged by spectrophotometry at 600 nm, an absorbance of 0.4–0.6 indicating that the required phase of growth has been reached. This should ensure successful expression of the F pilus to which helper phage can attach.

5. Several strains of helper phage are commercially available (e.g., VCSM13 or M13KO7). The literature describes other strains (e.g., KM13) that have been engineered to enhance their properties with respect to phage display. Phage should be added at a multiplicity of infection that ensures that all bacteria in the culture are likely to yield recombinant phage and will thereby contribute to the display library. As the F pilus is a relatively fragile structure, static incubation at 37°C is often used (30 min) to allow phage attachment and infection to take place. As many helper phage confer resistance to a second antibiotic, it is customary to spin down bacteria at the end of this incubation and resuspend in fresh medium supplemented with antibiotics before resuming shaken incubation at 30°C, overnight. Glucose is excluded or present at reduced concentrations in order to allow limited transcription from the lac promoter. During this phase, viral replication and assembly takes place, each virus bearing at its surface copies of the fusion between the recombinant antibodies, and carrying within the particle the phagemid vector with its selectable marker and the gene for the recombinant antibody (see Note 8).

6. Phage accumulate in the culture supernatant. After centrifugation to remove the bacterial cells, phage can be recovered from the supernatant by precipitation with a solution containing polyethylene glycol and sodium chloride. Buffer exchange returns the phage to a soluble state.

7. The yield of virus and hence the size of the library can be assessed by serial dilution of the phage stock and infection into a susceptible strain of *E. coli*. Plating onto agar containing antibiotic reveals the frequency of infection. This procedure is often termed “panning.” The following section describes the most straightforward approach to selection in which a surface is coated with the target of interest and interaction with the phage display library then takes place. A detailed protocol can be found in Chapter 7. Interaction can also take place in solution followed by capture of the complex formed between the
target and phage that have attached (see Chapter 11). Many other formats are possible (17–19), and the investigator can draw upon other chapters in this volume for ideas.

1. To pan an antibody phage display library on an immobilized target of interest, small petri dishes, special immunotubes or immunoassay microtiter plates are coated with the purified antigen overnight. The concentration of the purified target typically lies in the range of 1–1,000 μg/ml. Coating can take place in neutral, physiological buffers or slightly denaturing solutions (e.g., a bicarbonate buffer at pH 9.6) that promote interaction between the target and the plastic surface.

2. The surface must be washed several times and then saturated with a protein-rich solution to block nonspecific interactions at a later stage in the protocol. Two percent (w/v) skimmed milk in phosphate-buffered saline (PBS) is frequently used.

3. An aliquot of the phage display library is prepared in a small volume appropriate to the capacity of the selecting surface. At this stage, it is critical that the sample contains the full diversity contained in the library and that this diversity is present with sufficient frequency. It is recommended that sufficient phage are added to ensure at least 1,000-fold representation (i.e., if the library is estimated to contain $10^8$ different clones, an minimum of $10^{11}$ phage should be added to the selecting surface). To avoid the recovery of antibodies directed against protein(s) in the blocking material, the phage can be resuspended in blocking solution. This competitive inhibition prevents attachment to the selecting surface.

4. After incubation in the antigen-coated tube step, phage that have failed to attach to the surface and nonspecific bound virus are removed by extensive, repeated washing with PBS containing detergent (e.g., 0.1% Tween 20). Those phage that remain attached can be eluted. Brief exposure to 100 mM triethylamine has been widely used; eluates should be immediately neutralized in 1 M Tris-HCl, pH 7.4 (see Note 9). Other approaches include the use of denaturants, protease, competitive elution, etc. (see Chapter 7).

5. Depending upon the size of the library and the representation of clones able to bind to the target, modest numbers of phage may be recovered from the first round of selection. The population may also be made up of nonspecific binders, and phage with low, intermediate, and high affinities for the target. Amplification increases the numbers of virus without bias so that, in later rounds, selection can be directed toward virus with properties of interest. To amplify, the eluate is incubated with \textit{E. coli} TG1 that are in exponential growth, thereby allowing infection to take place. Titration of an aliquot of the infection
mix can be used to then assess the recovery of phage through conversion of the bacterial host to antibiotic resistance. The majority of the infection mix is then used to grow and harvest phage as described above. The panning procedure should be conducted several times to enrich for phage with the desired characteristics from the initially heterogeneous mix (round 1). By assessing input and recovery at each stage, the efficiency of each selective step can be monitored.

Progressive rises in phage recoveries during the course of selection should be observed, but only through immunoassay or other characterization can it be confirmed that target-specific phage are being recovered. Larger laboratories and organizations may have access to robotic systems for high-throughput screening, but even the smallest operation can make use of microtiter-based protocols to screen.

1. Taking the titration plates prepared for each round of selection, sterile toothpicks can be used to transfer individual bacterial colonies into microtiter plates prepared with liquid medium (100 µl/well) containing antibiotics and glucose (see Note 10).

2. The cultures are incubated overnight and then subcultured to allow the bacteria to grow into exponential phase (typically 2 h at 37°C). By adding a cryopreservative (e.g., glycerol), the original, overnight cultures can be frozen to provide a reference stock. When the subcultures have reached log phage, medium containing helper phage is added and infection takes place.

3. The microtiter plates are spun in a centrifuge fitted with appropriate carriers to pellet the bacteria. The medium is discarded and the pellets are resuspended in medium supplemented with antibiotics that select for the phagemid (e.g., ampicillin) and successful infection with helper phage (e.g., kanamycin). The medium should have a reduced concentration of glucose (e.g., 0.1% [w/v]) to allow low-level translation of the antibody-pIII fusion from the phagemid vector. Cultures are grown overnight at 30°C to allow the production of phage bearing the recombinant antibody.

4. After centrifugation, the supernatants are transferred from each well to ELISA plates that have been coated with the antigen of interest and blocked. If wells of the ELISA plates are preloaded with blocking solution (e.g., skimmed milk) at twice the concentration required for ELISA (e.g., 4% [w/v]), manipulation can be kept to a minimum, thereby easing the logistics of large-scale screening. Interaction between the phage and the target can now take place.
5. After washing, phage binding can be detected with commercially available antibody against M13 phage and an appropriate antibody conjugate when necessary.

6. After further washing, the reaction can be developed with a substrate solution appropriate to the reporter enzyme in use.

7. If selection is progressing as intended, target-specific clones detected in ELISA should increase as a proportion of those picked from each round for testing.

Phage display is a well-established approach for the identification of bioactive peptides and antibody fragments from large, highly diverse libraries. The specificity, selectivity, and potency of peptides and antibodies discovered in this way may have clinical advantages over traditional small molecule chemotherapeutics, and “targeted therapeutics” is a growing area (20–23).

The emphasis of this volume is the isolation of antibodies from large libraries using phage display, but the more free-thinking reader might like to reflect on the wider applications of phage display technology in drug discovery. When Smith and Scott first developed the concepts of phage display almost 20 years ago, they recognized that libraries of peptides could be used to identify regions of a protein recognized by an antibody by panning peptide libraries and mapping back the sequences carried by isolated phage to the sequence of the antigen (24). From this has emerged the idea of peptide mimics (mimotopes)—peptides that resemble an epitope in conformation but not in amino acid sequence. As illustrated by work with the envelope protein from hepatitis B virus (HbsAg), mimotopes can also be isolated by phage display (25). Phage libraries were helpful in defining peptide structures recognized by major histocompatibility molecules, and these peptide may serve for the design of MHC-specific antagonists. Phage display technology has also been used to analyze cell surface integrins, protein kinase SH2, peptides affecting protein-DNA interactions, and peptides binding to carbohydrates and to other chemical compounds. Phage display has been applied in enzymology to isolate novel enzymes (26), to develop a thrombin-specific inhibitor (27), and to develop DNA-binding proteins with novel specificities (28). Further, phage can be used to display cDNA libraries to identify allergens recognized by serum IgE from allergic individuals (29).

The last decade has shown rapid acceptance of the general principles of phage display and an accelerating pace of development in techniques and applications. This book aims to provide the investigator with a guide to a range of basic methods and an insight to more recent innovations. As a platform technology, phage display holds enormous promise for the development of new and improved diagnostic and therapeutic agents.
1. Although the general concept of antibody phage display process can be easily summarized, the construction of a good quality library is time consuming, and requires precision, well-developed laboratory skills, and some specialized equipment.

2. Cells used for RNA preparation can be characterized using specific markers and analyzed by immunofluorescence and flow cytometry. They can be sorted by cytometry or preselected to enrich for those bearing an antibody of particular specificity (see Chapter 3).

3. Cells can be directly used for RNA preparation or treated with an RNA protectant before freezing in liquid nitrogen, pending RNA isolation.

4. Successful extraction needs care if the integrity of the RNA is to be preserved. Work on ice, using gloves and dedicated reagents and plasticware certified to be RNase-free or treated so as to ensure this is the case.

5. Sterile, aerosol-resistant tips are essential to avoid cross contamination. Reactions are best conducted in an environment separate to that where analysis of the amplified products is to take place.

6. For the assembly reaction, equal amounts of the purified VH and VL amplicons have to be achieved so that all possible pairings have the chance of being formed. The yields of individual VH and VL products can be assessed by analysis on a 1% agarose gel and by comparison to markers, but it is best to quantify the yields by spectrophotometry.

7. Products have to be purified to remove DNA fragments that could interfere in the subsequent ligation reaction.

8. Handle phage preparation very carefully as phage contamination in the laboratory is extremely difficult to eradicate. Never discard supernatants that contain phage into the laboratory sink, even after decontamination. Solutions containing phage that are not required should be kept in capped containers as far as is practical and discarded after autoclaving.

9. Although the phage are robust, do not incubate phage with triethylamine longer than 10 min, as the alkaline solution may impair the infectivity of the virus.

10. To avoid any cross contamination during culture of bacteria in 96 well microtiter plates, an adhesive, gas-permeable sealing membrane can be applied over the surface of the plate. This seals individual wells more effectively than the lids often supplied with microtiter plates.
Acknowledgments

The work was supported by Fulbright No: 120610 and OTKA T048933 grants.

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Design and Construction of Synthetic Phage-Displayed Fab Libraries

Jenny Bostrom and Germaine Fuh

Summary

Diversity—the variability carried by the amino acid sequences of a synthetic antibody library—can be generated by synthetic degenerate oligonucleotides. One can experiment with different diversity designs in the variable domains of light and heavy chains (V<sub>H</sub> and V<sub>L</sub>) to generate antibody libraries with different properties. The ability to precisely define the final diversity of a library facilitates the process of isolating, characterizing, and optimizing an antibody lead. Here we describe detailed protocols for the design and construction of phage-displayed synthetic antibody libraries in which diversity is generated in the complementarity determining regions (CDRs) of the V<sub>H</sub> of a single humanized bivalent Fab scaffold. The example used in the protocol provides a general methodology for generation of libraries with engineered CDR diversity that can be applied to a template antibody sequence of choice.

Key words: Diversification, Synthetic, Scaffold, Random, Degenerate

1. Introduction

Synthetic antibody libraries have proven to be highly effective for the generation of functional, high-affinity antibodies against a wide variety of antigens (1–4). This chapter describes a method to design and construct such a synthetic antibody library in which diversity is created in the heavy chain variable domain. The method involves introducing diversity into an antibody scaffold by site-directed mutagenesis using synthetic oligonucleotides. In contrast to natural immune repertoires, the diversity of synthetic antibody libraries can generate well-defined repertoires.
There are numerous examples of different design strategies that have generated functional antibody libraries (2–5). The ability to precisely define the diversity of the final library facilitates the process of isolating, characterizing, and optimizing an antibody lead. Another advantage of synthetic libraries is that they allow control over parameters such as the subgroups of the light and heavy chain. The diversity of libraries generated in this protocol is restricted to 17 positions in the complementarity determining regions (CDRs) of the V_H. A single humanized bivalent Fab served as the scaffold. The libraries have proven to be efficient in generating antibodies with high affinity and specificity. This protocol will guide the reader through the process of defining the diversity to be achieved, using degenerate oligonucleotides to generate the library, and finally constructing the phage-displayed antibody library.

2. Materials

2.1. Generation of Library DNA

1. Competent *Escherichia coli* CJ236 (New England Biolabs).
2. Carbenicillin stock (5 mg/mL). Sterile filter and store at 2–8°C.
3. LB agar plates: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 20 g/L agar. Mix all components, heat to dissolve, autoclave, and allow to cool. Store at room temperature. Melt in a microwave when required, cool to 60°C, and, when required, supplement with carbenicillin to a final concentration of 50 μg/mL before pouring plates.
4. 2YT broth: 10 g/L yeast extract, 16 g/L tryptone, and 5 g/L NaCl in deionized water. Mix all components, autoclave, and cool down. Store at room temperature.
5. M13KO7 helper phage (New England Biolabs).
6. Kanamycin stock (5 mg/mL). Sterile filter and store at 2–8°C.
7. Phage precipitation solution: 20% (w/v) polyethylene glycol (PEG 8000) and 2.5 M NaCl in deionized water.
8. Phosphate-buffered saline (PBS).
9. QIAprep Spin M13 Kit (QIAGen).
10. Degenerate oligonucleotides diluted in water to 10 OD_260_/mL.
11. 10× Tris-Magnesium (TM) buffer: 0.5 M Tris-HCl pH 7.5, 0.1 M MgCl₂.
12. 100 mM adenosine 5'-triphosphate (ATP, New England Biolabs).
13. 100 mM dithiothreitol (DTT).
14. T4 polynucleotide kinase (10,000 U/mL, New England Biolabs).
15. Deionized water.
16. 25 mM deoxynucleotide solution mix (dNTPs, New England Biolabs).
17. T4 DNA ligase (400,000 U/mL, New England Biolabs).
18. T7 DNA polymerase (10,000 U/mL, New England Biolabs).
19. Agarose.
20. Tris acetate EDTA (TAE) buffer: prepare a 50× stock containing 242.2 g/L Tris base, 18.6 g/L of Na₂EDTA.2H₂O (EDTA disodium dihydrate), and 57.47 mL/L of 17.4 M glacial acetic acid. Dilute 50-fold with deionized water before use.
21. Ethidium bromide (EtBr).
22. QIAquick Gel Extraction kit (QIAGen).

2.2. Generation of Electrocompetent SS320 for Phage Production

1. *E. coli* SS320 (see Note 1).
2. 2YT top agar: 10 g/L yeast extract, 16 g/L tryptone, 5 g/L NaCl, and 7.5 g/L agar. Mix components and heat to dissolve. Autoclave. Store at 2–8°C. Melt in a microwave when required then hold at 44°C until required.
3. Tetracycline stock (5 mg/mL): dissolve initially in ethanol then add deionized water to 50% (v/v). Store at −10°C in brown bottles to protect from light.
4. LB agar plates supplemented with tetracycline to a final concentration of 10 μg/mL.
5. Super broth. Solution A: 12 g tryptone, 5 mL glycerol, 24 g yeast extract, and deionized water to 900 mL. Solution B: 125 g/L K₂HPO₄ and 38 g/L KH₂PO₄. Prepare solutions A and B and autoclave separately. Add 100 mL of Solution B to 900 mL of Solution A. Store at room temperature.
6. 1 mM HEPES, pH 7.2: 238 g HEPES (Cellgro) dissolved in 750 mL sterile deionized water. Adjust to pH 7.2 using 50% (w/v) NaOH. Add deionized water to 1 L. Store at room temperature. Dilute to 1 mM in sterile deionized water before use.
7. Glycerol.
8. Dry ice.
The performance of a synthetic library depends on the scaffold chosen as the foundation for the library, the design strategy for diversity, and the overall size of the final library. The first step in designing a synthetic phage-displayed antibody library is therefore to choose an appropriate scaffold for the library. Synthetic libraries have been reported that employ either single or multiple frameworks (1, 5–8). Libraries that are built on a single framework rely mainly on the CDR diversity that is introduced with synthetic degenerate oligonucleotides; while this is also true of libraries based upon multiple scaffolds, these resources also generate diversity through the provision of different frameworks.

A single framework facilitates the library construction and the downstream characterization and optimization of antibodies that are isolated from the library through their interaction with target. In the example described here, the library template is the Fab derived from the humanized antibody 4D5 (hu-4D5). The antibody binds to the extracellular domain of the human receptor tyrosine kinase ErbB2 with high affinity and is a validated therapeutic (9). This scaffold was chosen because it contains variable domain subgroups (V H 3 and V kappa 1) that are prevalent among naturally occurring human antibodies. It has been optimized for protein expression and it displays well on phage. The hu-4D5 antibody has been extensively characterized and structural information is available, which facilitated the strategy for library construction (9–11). These issues should be borne in mind when choosing a template for library production.

In the example described here, Fabs are engineered to assemble as dimers on the phage particle, thereby presenting the antibody in a bivalent format. Protein assembly results from fusion of a dimerizing leucine zipper motif to the Fab heavy chain as described below. This enables bivalent display (Fig. 1). The bivalent format mimics natural IgG in that avidity effects can allow the recovery of antibodies from the library that possess moderate affinity to the target. Antibodies recovered can then be readily reformatted into IgG.

A phagemid for displaying hu-4D5 as bivalent Fabs on phage particles is outlined in Fig. 2. The vector contains a bicistronic operon under the control of the alkaline phosphatase promoter. The first open-reading frame of the operon encodes a polypeptide consisting of the stII secretion signal (12), and the light chain variable (V L ) and light chain constant domains (C L ) of hu-4D5 followed by a gD epitope tag (sequence: MADPNRFRGKDLGG) (13). The second reading frame encodes the stII signal peptide followed by the heavy chain variable domain (V H ) and the first
constant domain (C\textsubscript{H1}) of hu-4D5 fused to the GCN4 leucine zipper (14). In turn, the leucine zipper is fused to the C-terminal domain of the M13 gene 3, which encodes the minor phage coat protein (cP3) (15). When expressed in \textit{E. coli}, the light chain and the heavy chain-cP3 fusion are secreted into the periplasm where they associate to form a functional Fab, which is anchored on the inner membrane via cP3 (Fig. 1). Infection of the \textit{E. coli} host with M13 helper phage results in the assembly of phage particles that display the Fabs on their surface as bivalent dimers. The M13KO7 helper phage used in this method renders infected cells resistant to kanamycin. Stop codons (TAA) were incorporated into CDR-H1, -H2, and -H3 of hu-4D5. The CDRs are repaired by Kunkel mutagenesis using a mixture of degenerate oligonucleotides that anneal to the TAA-containing CDRs of the template as described in \textbf{Subheading 3.6.2}. Unmodified template is thus blocked from forming a functional protein product.

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**Fig. 1.** The phage in the synthetic libraries display Fabs in a bivalent format. The leucine zipper enables the bivalent display of Fabs on the phage.

**Fig. 2.** Phagemid expression construct for bivalent display of Fabs on phage.
As discussed previously, the productivity of a synthetic antibody library depends on the design strategy for introducing diversity and the final size of the repertoire. The size of phage libraries generated by the method described in this chapter is limited to $10^9$–$10^{10}$ by the efficiency with which library DNA can be electroporated into the *E. coli* host. Therefore, the design strategy for introducing diversity is key to a phage display resource that will be of general utility. One challenge is to select which residues to diversify (spatial diversity) and the range of amino acids (the degree of randomization) that will be incorporated at these positions (chemical diversity). Varying the length of the hypervariable loops present in the variable domain (length diversity) can further increase the diversity of the library. Different CDR-H3 lengths allow diverse structural conformations to form in this region.

The diversity of natural antibodies has been successfully used to guide the design of antibody libraries (1, 2, 16–19). In constructing a functional repertoire of high-affinity antibodies, it is important that the degeneracy of the DNA does not greatly exceed the actual library size. Naturally occurring antibodies provide guidance on how to limit synthetic diversity.

The diversity of the libraries described here (“V<sub>H</sub>-Fab” libraries) is designed to mimic the natural antibody repertoire (2). Heavy chain variable domains of natural human antibodies are more diverse than light chain variable domains, in both amino acid sequence and structural conformations. The heavy chain is the main direct contributor to interaction with antigen (mainly through CDR-H3) (20–23). Based on these observations, the diversity of the V<sub>H</sub>-Fab library is focused to the three CDRs of the V<sub>H</sub> domain of the Fab, while the V<sub>L</sub> domain is of fixed sequence.

Analysis of the sequences of natural human V<sub>H</sub> domains in the Kabat database (24, 25) reveals that the degree of amino acid variation at individual positions in the CDRs is itself variable. This kind of analysis identifies positions with the greatest natural variability. In the absence of structural information on the template chosen for library construction, one approach to diversification is to randomize amino acids at these naturally variable positions. In the case of hu-4D5, detailed structural information is available (11), and can be used to further guide library design by identifying solvent-exposed residues in the CDRs. These residues have the greatest potential to directly impact upon interaction with antigen. Overall, then, residues in the CDRs that are both highly variable in the natural V<sub>H</sub> repertoire and solvent exposed
in the hu-4D5 structure were selected for randomization (Fig. 3, Tables 1 and 2). These comprised residues 30–33 in CDR-H1 and 50, 52–54, 56, and 58 of CDR-H2. The framework residue at position 49 was also included, as this position has a degree of variation in the natural repertoire (Table 1). For CDR-H3, residues 95–101 were randomized. In contrast to CDR-H1 and CDR-H2, natural CDR-H3 loops are highly diverse in sequence and length. To mimic CDR-H3 length diversity, oligonucleotides were designed that insert residues between positions 98 and 100 (Table 2). The CDR-H3 length thus varies from 7 to 19 residues.
Table 1
Residues of hu-4D5 that are randomized in the libraries

| hu-4D5 residue | Diversity design | Codon | Encoded residues | Coverage (%) | Oligonucleotide | Pool | Target diversity |
|----------------|------------------|-------|-----------------|--------------|-----------------|------|-----------------|
| H1-28 N        |                  | ACC   | T               | 54           | H1 a, b         | H1   | T S             |
|                |                  |       |                 |              |                 |      | 54 36           |
| H2-30 K        |                  | AVT   | STN             | 90           | H1 a, b         |       | S T N R D G     |
|                |                  |       |                 |              |                 |      | 68 18 3 2 2     |
| H1-31 D        |                  | RRT   | SNGD            | 82           | H1 a, b         |       | S N G T D R A   |
| H1-32 T        |                  | WMY   | YSN T           | 80           | H1 a, b         |       | Y S N G F A     |
|                |                  |       |                 |              |                 |      | 64 9 7 4 3 3    |
| H1-33 Y        |                  | KMT   | AYSD            | 57           | H1 a            |       | A Y W G S D T N V |
|                |                  |       |                 |              |                 |      | 22 20 14 3 3 2 2 |
|                |                  | KGG   | WG              | 31           | H1 b            |       |                |
|                |                  |       |                 |              |                 |      |                |
| H2-49 A        |                  | GST   | GA              | 77           | H2 a-c          | H2   | G S A           |
|                |                  |       |                 |              |                 |      | 58 22 19        |
| H2-50 R        |                  | DGG   | RWG             | 35           | H2 a            |       | R Y W V G I E A S N L |
|                |                  |       |                 |              |                 |      | 17 10 9 9 8 8 6 6 6 4 |
|                |                  | DHT   | YVIASN          | 45           | H2 b            |       |                |
|                |                  |       |                 |              |                 |      |                |
|                |                  | GAA   | E               | 8            | H2 c            |       |                |
| H2-52 Y        |                  | DMT   | SYNDT A         | 74           | H2 a-c          |       | S Y N K I R D T |
|                |                  |       |                 |              |                 |      | 26 25 17 8 5 3 3 3 3 |
The target diversity was defined as the amino acids that cover 90% of the ~3,500 human amino acid sequences that were analyzed in the Kabat database (24). The occurrence of each amino acid is listed as a percentage. The diversity design depicts the amino acids that are encoded by the degenerate codons listed. The codons were designed to maximize the coverage (shown as percentages) while minimizing the occurrence of residues that are not part of the target diversity (shown in **bold**). Several oligonucleotides were mixed in the pools shown here in the Kunkel mutagenesis reaction, which is further described in the text.

| H2-53 T | DMT | SDYNT | A | 66 | H2 | a–c | S | D | Y | G | H | N | I | T | W |
|---------|-----|-------|---|----|----|-----|---|---|---|---|---|---|---|---|---|
|         |     |       |   |    |    |     | 24| 20| 11| 10|  9|  8|  5|  3|  2|
| H2-54 N | RRC | GSDN  |   | 81 | H2 | a–c | G | S | D | N | K | F | T |   |   |
|         |     |       |   |    |    |     | 37| 26| 11|  7|  6|  5|  4|   |   |
| H2-56 Y | DMT | STNDYA|   | 81 | H2 | a–c | S | T | N | D | Y | E | G | A |   |
|         |     |       |   |    |    |     | 28| 16| 15| 10| 10|  5|  5|  2|   |
| H2-58 R | DAC | YND   |   | 69 | H2 | a–c | Y | N | D | R | S | I | T | H |   |
|         |     |       |   |    |    |     | 32| 25| 12|  7|  4|  4|  3|  2|   |
### Table 2
**Designed diversity of CDR-H3**

| Oligonucleotide | Length | 93 | 94 | 95 | 96 | 97 | 101 | 102 Pools |
|-----------------|--------|----|----|----|----|----|-----|------------|
| H3-7a           | 7      | A  | R/K|     |    |    | F   | D          | Y          |
| H3-7b           | 7      | A  | R/K|     |    |    | F   | D/A        | Y          |
| H3-7c           | 7      | A  | R   | H/S/N|   |    | F/L | G/V/ Y     | H3 1       |
| H3-8a           | 8      | A  | R/K|     |    |    | F   | D          | Y          |
| H3-8b           | 8      | A  | R   | H/S/N|   |    | F/L | G/V/ D/A   | Y          |
| H3-8c           | 8      | A  | R   | H/S/N|   |    | F/L | D/A        | Y          |
| H3-9a           | 9      | A  |     | NNS| NNS| NNS| Y   | A/G/V      | M          |
| H3-9b           | 9      | A  |     | NNS| NNS| NNS| W/S/A/G  | M          |
| H3-9c           | 9      | A  | R/K|     |    |    | F   | D          | Y          |
| H3-9d           | 9      | A  | R/K|     |    |    | F   | D/A        | Y          |
| H3-9e           | 9      | A  | R/K|     |    |    | M   | D          | Y          |
| H3-9f           | 9      | A  | R/K|     |    |    | M   | D          | Y          |
| H3-10a          | 10     | A  |     | NNS| NNS| NNS| Y   | A/G/V      | M          |
| H3-10b          | 10     | A  |     | NNS| NNS| NNS| W/S/A/G  | M          |
| H3-10c          | 10     | A  | R/K|     |    |    | F   | D          | Y          |
| H3-10d          | 10     | A  | R/K|     |    |    | F   | D          | Y          |
| Oligonucleotide | Length | Pools |
|-----------------|--------|-------|
| H3-10e          | 10     | A/R/K |
| H3-11a          | 11     | A     |
| H3-11b          | 11     | A     |
| H3-11c          | 11     | A/R/K |
| H3-11d          | 11     | A     |
| H3-11e          | 11     | A/R/H/S/N |
| H3-12a          | 12     | A     |
| H3-12b          | 12     | A     |
| H3-12c          | 12     | A     |
| H3-12d          | 12     | A/R/K |
| H3-12e          | 12     | A/R/H/S/N |
| H3-12f          | 12     | A/R/H/S/N |
| H3-13a          | 13     | A     |
| H3-13b          | 13     | A     |
| H3-13c          | 13     | A     |
| H3-13d          | 13     | A/R/K |

(continued)
| Oligonucleotide | Length | CDR-H3 positions | 101  | 102 Pools |
|-----------------|--------|------------------|------|-----------|
| H3-13c          | 13     | A R/K            | XYZ  | M D Y     |
| H3-13f          | 13     | A R/K            | XYZ  | M D Y     |
| H3-13g          | 13     | A R/K            | XYZ  | M D Y     |
| H3-14a          | 14     | A R/K            | XYZ  | M D Y H3 7|
| H3-14b          | 14     | A R/K            | XYZ  | M D Y     |
| H3-15a          | 15     | A R/K            | XYZ  | M D Y H3 8|
| H3-15b          | 15     | A R/K            | XYZ  | M D Y     |
| H3-16a          | 16     | A R              | XYZ  | M D Y H3 9|
| H3-16b          | 16     | A R              | XYZ  | M D Y     |
| H3-17a          | 17     | A R              | XYZ  | M D Y H3 10|
| H3-17b          | 17     | A R              | XYZ  | M D Y     |
| H3-18a          | 18     | A R              | XYZ  | M D Y H3 11|
| H3-18b          | 18     | A R              | XYZ  | M D Y     |
| H3-19           | 19     | A R              | XYZ  | M D Y H3 12|

The table shows the oligonucleotide design encoding different diversity and length of CDR-H3. The designed diversity is shown as degenerate codons (bold italics) or amino acids (plain text). The oligonucleotides were mixed in different pools as indicated and used in the Kunkel mutagenesis reaction as described in the text. For specifications of the XYZ codon, see Note 3.
Analysis of natural human antibody sequences enables the calculation of amino acid occurrence at each variable position. This often reveals a bias toward certain residues. By aligning thousands of sequences (~3,500) in the Kabat database, the frequency with which each amino acid occurs at each position can be determined (Table 1). Where variation is observed, a limited number of residues may occur in more than 90% of all sequences in the database. For example, at position 49, glycine is the most common amino acid (58% of sequences in the database), followed by serine (22%) and alanine (19%). Together, these three amino acids account for the diversity at position 49 in 99% of natural sequences. These three amino acids are therefore taken forward into the strategy for diversification at position 49.

This analysis works well for CDR-H1 and CDR-H2 but is more difficult for CDR-H3 because this region is highly variable in naturally occurring antibodies. Due to the substantial diversity in the length and sequence of CDR-H3, the occurrences of amino acids at positions 94 to the position preceding 101 are totaled and analyzed as a group to determine the target diversity for all positions in this CDR (Fig. 4).

Having assessed where diversity is best generated in the template and the degree/length of diversification that should be introduced, the goal moves to designing degenerate oligonucleotides with the capacity to encode the desired range of amino acids.

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**Fig. 4.** The amino acid composition of natural human CDR-H3 sequences are calculated for position 94–101. The graphs depict the frequency at which each amino acid occurs in this region. The results are compared with the amino acid composition encoded by the degenerate codons NNS and XYZ. The latter was designed to closely mimic the natural CDR-H3 diversity. A standard single-letter amino acid code is used here and in all other figures. The text gives a detailed description of the XYZ codon.
for each selected position. Degenerate codons were designed to prioritize the most frequently observed amino acid residues, while encoding a minimum number of nontarget residues and excluding stop codons and cysteines (Tables 1 and 2, Fig. 4). DNA degeneracies are denoted by the IUB code (see Note 2). In creating these sequences, the goal should be to use an equimolar mix of the required nucleotides during synthesis. The codon XYZ (Table 2) is designed to represent the amino acid diversity of CDR-H3 sequences observed in natural antibodies. This is created by synthesizing the XYZ codon from different proportions of the nucleotides at each of the three positions in the codon (see Note 3). In some cases it is difficult to generate the diversity required using a single nucleotide mix. When this arises, several oligonucleotides are included that carry different codons at a single position. This is the case for positions 31, 33, and 50 of CDR-H1 and CDR-H2 (Tables 1 and 2).

3.6. Generation of Library DNA

3.6.1. Generation of Single-Stranded DNA Carrying the Antibody Library for Library Construction

1. Take phagemid vector DNA carrying the antibody sequence to be diversified and transformed into competent E. coli CJ236. Recall that, in our methods, stop codons are incorporated into the CDRs in a preliminary stage (Subheading 3.3).

2. Plate onto LB agar containing an antibiotic suitable for selection of the vector (carbenicillin in the case described here) and grow overnight at 37°C.

3. Pick a single colony of E. coli CJ236 harboring the phagemid vector and transfer to 5 mL of 2YT broth supplemented with 50 µg/mL carbenicillin. Grow at 37°C until log-phase (an OD 600 in the range 0.3–0.6). Infect the cells with M13KO7 helper phage (4 × 10^10 pfu/mL) for 60 min at 37°C.

4. Add 25 mL 2YT supplemented with 50 µg/mL carbenicillin and 50 µg/mL kanamycin and grow at 37°C for 20 h.

5. Centrifuge the culture at 24,000 × g for 10 min. Harvest the supernatant and add 1/5 volume of phage precipitation solution. Incubate on ice 5 min.

6. Centrifuge at 24,000 × g for 10 min. Decant the supernatant and resuspend the phage pellet in 5 mL of PBS. Centrifuge again at 24,000 × g for 5 min to remove any remaining bacterial debris.

7. Transfer the supernatant to a new tube and purify single-stranded DNA from the phage particles using two spin columns from a QIAprep Spin M13 kit. Elute the DNA from the columns with deionized water.
8. Determine the yield of single-stranded DNA by spectrophotometry (1.0 \( A_{260} \) is equivalent to 33 ng/\( \mu \)L of single-stranded DNA).

Mutations are introduced simultaneously in all heavy chain CDRs to repair the engineered stop codons and introduce sequence diversity using the tailored degenerate codons. Oligonucleotide pools of defined composition are generated (Table 1).

For CDR-H1, oligonucleotides H1a and H1b are mixed 2:1 (Pool H1).

For CDR-H2, oligonucleotides H2a, H2b, and H2c are mixed 1:2:0.1 (Pool H2).

For CDR-H3, the oligonucleotides encoding sequences of the same length (Table 2) are each pooled at equimolar ratios, except for the two shortest lengths that are pooled together. This generates 12 separate pools (Pools H3 1–12) (see Note 4). A separate library is constructed for each length of CDR-H3 that is created from these pools (Libraries 1–12). This entails 12 mutagenesis reactions in which pools H3 1, H3 2, H3 3, etc. are individually mixed with Pool H1 and Pool H2 at a 1:1:1 ratio as detailed in Table 3.

### Table 3

| Library | Oligonucleotide pools | Molar ratio |
|---------|-----------------------|-------------|
| 1       | H1 H2 H3 1            | 1:1:1       |
| 2       | H1 H2 H3 2            | 1:1:1       |
| 3       | H1 H2 H3 3            | 1:1:1       |
| 4       | H1 H2 H3 4            | 1:1:1       |
| 5       | H1 H2 H3 5            | 1:1:1       |
| 6       | H1 H2 H3 6            | 1:1:1       |
| 7       | H1 H2 H3 7            | 1:1:1       |
| 8       | H1 H2 H3 8            | 1:1:1       |
| 9       | H1 H2 H3 9            | 1:1:1       |
| 10      | H1 H2 H3 10           | 1:1:1       |
| 11      | H1 H2 H3 11           | 1:1:1       |
| 12      | H1 H2 H3 12           | 1:1:1       |

The oligonucleotide pools H1, H2, and H3 1–12 are mixed according to the table and used for library DNA generation by Kunkel mutagenesis.
Standard Kunkel mutagenesis (26) is used to generate each synthetic antibody library (Libraries 1–12).

1. A kinase reaction is set up, preparatory to mutagenesis. Mix 1.5 μL aliquots from pool H3 1, H3 2, H3 3, etc. with 1.5 μL from pool H1 and 1.5 μL from pool H2 (1:1:1 molar ratio).

2. Add 4 μL of 10x TM buffer, 0.5 μL of 100 mM ATP, 2 μL of 100 mM DTT, and 2 μL T4 polynucleotide kinase (10 U/μL) to each mixture. Add deionized water to a final volume of 40 μL. Mix and incubate at 37°C for 45 min. Place on ice.

3. In the next step, an “annealing reaction,” the oligonucleotides are allowed to anneal to the single-stranded library template (Subheading 3.6.1, step 7). Add 20 μg single-stranded template to the kinase reaction mixture, and 25 μL of 10x TM buffer. Add deionized water up to 250 μL. Incubate at 95°C for 2 min, then at 50°C for 5 min. Place the samples on ice.

4. The last step is a “filling-in reaction” that uses T7 polymerase and T4 ligase. To the 250 μL annealing reaction, add 1 μL of 100 mM ATP, 15 μL of 100 mM DTT, 10 μL of 25 mM dNTPs, 6 μL T4 DNA ligase (400 U/μL), and 3 μL T7 DNA polymerase. Mix and incubate at room temperature for at least 3 h.

5. Prepare a 1% agarose gel in 1x TAE buffer. Add EtBr to a final concentration of 0.5 μg/mL. Take 0.5μL aliquots of the mutagenesis products and load onto the agarose gel adjacent to the single-stranded template DNA. Run at 110 V for 20 min. Visualize the DNA bands under ultraviolet illumination. The mutagenesis products should appear higher on the gel than the single-stranded template and, after the reaction, three bands are normally visible on the gel, with the bottom band representing the correct, mutated product. The two upper bands are undesired products of the Kunkel mutagenesis reaction, which may lead to the presence of un-mutated template sequences in the final library.

6. Excise the mutagenesis product and purify using a QIAquick Gel Extraction kit using two spin columns per mutagenesis reaction. To elute the purified DNA from the spin columns, add 40 μL deionized water and incubate the spin columns for 30 min at room temperature before centrifuging. Collect the eluate and measure the $A_{260}$. The total recovery should be at least 20 μg.

1. Plate out E. coli SS320, pick a single colony, and grow into exponential phase (Subheading 3.6.1, step 3).

2. Set up infections of the bacteria with M13KO7 starting at $6 \times 10^8$ pfu/mL (see Note 5) and, after allowing the infection to take place, mix the infected bacteria with just-molten 2YT top agar and pour to a dry LB agar plate supplemented with
10 μg/mL tetracycline. Shake to distribute the top layer then incubate overnight for growth at 37°C.

3. Pick a single plaque and inoculate into 1 mL 2YT supplemented with 5 μg/mL tetracycline and 25 μg/mL kanamycin at 37°C for 4 h. Transfer to 50 mL of medium and grow overnight at 37°C.

4. Sample 5 mL of the overnight culture and inoculate into two flasks, each containing 900 mL Super broth with 5 μg/mL tetracycline and 25 μg/mL kanamycin. Incubate at 37°C.

5. When the OD<sub>600</sub> reaches approximately 0.6, centrifuge at 700 × g at 4°C. Discard the supernatant.

6. All further steps require the cells to be kept on ice. Wash salt from the cells by resuspending the pellet in cold 1 mM HEPES, pH 7.5.

7. Pellet the cells as described previously and repeat the wash step with more cold 1 mM HEPES, pH 7.5.

8. Pellet the cells as described previously and resuspend in cold 1 mM HEPES, pH 7.5, containing 10% (v/v) glycerol.

9. Finally, resuspend the cells in 1 mL 10% (v/v) cold glycerol, aliquot, and snap freeze on dry ice. Store the cells at −80°C.

3.8. Phage Production and Purification

1. The 12 mutagenesis reactions are electroporated individually into the electrocompetent <i>E. coli</i> SS320 cells harboring M13KO7 helper phage (I2). Each reaction should use 10 μg of the mutated DNA template and approximately 10<sup>11</sup> electrocompetent <i>E. coli</i> cells. Approximately 1–5 × 10<sup>9</sup> transformed clones should be expected from each transformation.

2. Recover all transformed cells from each reaction and grow overnight at 30°C in 2YT broth supplemented with 50 μg/mL carbenicillin and 50 μg/mL kanamycin.

3. Centrifuge the cultures for 10 min at 24,000 × g and transfer the supernatants to fresh tubes.

4. Harvest phage from the culture supernatants by the addition of 1/5 volume of phage precipitation solution followed incubation on ice for 5 min.

5. Pellet the precipitated phage by centrifugation at 24,000 × g and resuspend the pellets in 1/20 volume of PBS.

6. Remove insoluble matter by centrifugation at 24,000 × g and repeat twice to ensure purity.

7. The yields of phage can be estimated by measuring the optical density at 268 nm. 1 OD/mL corresponds to a phage concentration of approximately 0.5 × 10<sup>12</sup> particles/mL.

8. Add glycerol to a final concentration of 50% (v/v). The phage libraries should be stored at −20°C until they are used for selection (see Note 6 and 7).
1. For high-efficiency DNA transformation, the *E. coli* strain SS320 was constructed. The strain is generated by mating MC1061 (Sigma-Aldrich) and XL1-blue (Stratagene) and selection on agar containing tetracycline and streptomycin. *E. coli* SS320 retains the high-efficiency transformation qualities of MC1061, while acquiring the F' episome from XL1-blue that is required for bacteriophage infection and propagation (12).

2. IUB code for DNA degeneracy:

| N = A/T/G/C | D = A/G/T | V = A/C/G | B = C/G/T |
|-------------|-----------|-----------|-----------|
| H = A/C/T   | K = G/T   | M = A/C   | R = A/G   |
| S = G/C     | W = A/T   | Y = C/T   |

3. For the XYZ codon, the proportion of nucleotides at each codon triplet is as follows:

- X: 38% G, 19% A, 26% T, 17% C
- Y: 31% G, 34% A, 17% T, 18% C
- Z: 24% G, 76% C (2).

4. The generation of pools facilitates the precise mixing of the different oligonucleotides at specified ratios.

5. The generation of competent *E. coli* SS320 cells harboring M13KO7 helper phage facilitates library production, as there is no need for helper phage superinfection after the electroporation of DNA. In this way, each cell is guaranteed to harbor M13KO7 helper phage.

6. After purification and storage, the phage-displayed Fab libraries are stable at −20°C for at least a year.

7. Selection can use the 12 libraries individually or as a pooled stock according to preference.

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Chapter 3

Affinity Isolation of Antigen-Specific Circulating B Cells for Generation of Phage Display-Derived Human Monoclonal Antibodies

Henrik J. Ditzel

Summary

A method is described for affinity isolation of antigen-specific circulating B cells of interest for subsequent generation of immune antibody phage display libraries. This approach should overcome the problem of low yields of monoclonal antibodies of interest in the libraries generated from peripheral blood lymphocytes caused by the low abundance of antigen-specific B cells in the circulation. The preselection of B cells is based on the specificity of the surface Ig receptor and is accomplished using the antigen of interest conjugated to magnetic beads. This method should significantly increase the frequency of antibody phage particles of interest in the library and allow for efficient isolation monoclonal antibodies with the predefined specificity.

Key words: Phage display, Antibody libraries, Human monoclonal antibodies, B cells, Affinity selection, Preselection, Memory B cells, Ig surface expression, Autoantibodies, Antiviral antibodies

1. Introduction

Human bone marrow is a major repository for maturated antibody-secreting plasma cells, which produce the majority of the antibodies found in serum (1–3). This makes bone marrow an attractive source of RNA for generating human immune antibody libraries for isolation of monoclonal antibodies with similar specificity as that found in the donor serum (4–7). Unfortunately, bone marrow is not always readily available, because health and ethical concerns often prohibit bone marrow aspiration and leave
peripheral blood mononuclear cells (PBMCs) as the only readily available source. Although human immune libraries can be generated from circulating B cells, the low frequency of antigen-specific B cells in the circulation may limit representation of monoclonal antibodies of interest in the resulting library.

In this chapter, we describe a preselection strategy to enrich for antigen-specific B cells prior to antibody library construction (see Fig. 1). We have applied this approach to isolate a panel of human anti-HIV-1 gp120 monoclonal antibodies derived from circulating B cells of HIV-1-seropositive individuals (8). The strategy should be generally applicable, enabling the retrieval of antibodies against other viral antigens and infectious antigens, autoantigens, alloantigens, etc. The preselection of B cells is based on the specificity of the surface Ig receptor and is accomplished using antigen-conjugated magnetic beads (see Fig. 1); in our model system, recombinant HIV-1 gp120 is conjugated to magnetic beads.

Preselection as an enrichment procedure before EBV transformation of B cells for the generation of human monoclonal antibodies has been described (9–10). These preselection strategies utilized erythrocyte rosetting for either identification of Ig-expressing B cells with specificity for blood group antigens or haptens coupled to erythrocytes, and were shown to increase the number of antigen-specific human monoclonal antibodies (Abs) generated. However, the availability of magnetic beads with different surfaces suitable for antigen binding makes such an

Fig. 1. Affinity isolation of antigen-specific circulating B cells using antigen-conjugated magnetic beads for subsequent generation of phage display libraries.
approach far more efficacious (11). The benefit of preselection is clear from a comparison of the number of antigen-specific antibodies isolated from an anti-gp120-preselected B cell library and from unselected B cells (8), even though the preselected libraries may contain fewer clones and exclude rare plasma cells that do not express Ig on the surface.

In our recent study, IgG antibody libraries were generated from affinity-selected circulating B cells, predominantly of the memory/activated B cell phenotype, to obtain high-affinity antibodies (8). However, antibody phage display libraries of other isotypes (IgM, IgA, or IgE) may also be generated (12). Analysis of the anti-gp120 antibodies derived from the circulating B cells of HIV-1 donors generally resemble those from bone marrow plasma cells with respect to epitope specificity, affinity, and neutralization ability (8).

2. Materials

1. Magnetic streptavidin beads (Dynabeads M-280 streptavidin, Dynal, Invitrogen).
2. Biotinylated antigen.
3. Phosphate-buffered saline (PBS).
4. Bovine serum albumen (BSA).
5. Magnetic particle concentrator (Dynal, Invitrogen).
6. Blood sample containing anticoagulant (EDTA, heparin, acid citrate dextrose).
7. Lymphoprep, sodium diatrizoate 9.1% (w/v) and polysaccharide 5.7% (w/v) (Axis-Shield).
8. Heparin.
9. RPMI tissue culture medium.
10. Total RNA isolation kit (Stratagene).
11. Absolute ethanol.
12. Water treated with diethylpyrocarbonate (DEPC) or otherwise rendered RNase-free (Ambion).

3. Methods

3.1. Preparation of Antigen-Coated Magnetic Beads

1. Coat magnetic streptavidin beads with the antigen of interest (a recombinant protein, purified protein, etc.) by incubating $8 \times 10^7$ beads (133.33 μL) with 12 μg biotinylated antigen
in a total volume of 100 μL PBS for 1 h at room temperature (see Note 1).

2. Remove unbound antigen by washing the beads twice in PBS with 0.1% BSA using the magnetic concentrator (see Note 2).

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### 3.2. Lymphocyte Isolation

1. Dilute blood samples 1:2 in RPMI with heparin.
2. Carefully layer 6 mL of diluted blood over 3 mL of Lympho-prep in a 12 to 15 mm centrifuge tube and centrifuge at 800 \( \times g \) for 30 min at 20°C.
3. Collect PBMCs from the interface.
4. Wash the cells twice with RPMI and centrifuge 250 \( \times g \) for 15 min at 20°C.
5. Discard the supernatant and resuspend the cell pellet in 2 mL RPMI.

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### 3.3. Preselection of Antigen-Specific B Lymphocytes with Antigen-Coated Magnetic Beads and Isolation of RNA

1. Incubate coated Dynabeads \( (2 \times 10^7, 25 \text{ mL}) \) with \( 5 \times 10^6 \) PBMCs resuspended in 975 mL RPMI for 1 h at 4°C with constant gentle mixing.
2. Wash the magnetic beads four times in RPMI using the magnetic concentrator.
3. Add bound B lymphocytes in the presence of the magnetic beads to 10 mL of the denaturant, guanidinium isothiocyanate (supplied as solution D in the recommended RNA isolation kit) (see Note 3), in a thick-walled 50 mL polypropylene centrifuge tube and resuspend the pellet (see Note 4).
4. Add 1 mL of 2 M sodium acetate, pH 4 (supplied in the recommended kit). Mix the contents of the tube thoroughly by repeatedly inverting the tube.
5. Add 10 mL of phenol (pH 5.3–5.7) saturated with water (supplied in the recommended kit). Mix the contents thoroughly by inversion.
6. Add 2 mL of chloroform-isoamyl alcohol mixture (supplied in the recommended kit) to the sample. Mix by shaking the tube vigorously for 10 s.
7. Incubate the tube on ice for 15 min. If two layers do not form, add another volume of chloroform–isoamyl alcohol mixture and repeat.
8. Spin the tube in a centrifuge at 10,000 \( \times g \) for 20 min at 4°C (see Note 5).
9. Transfer the upper, aqueous phase to a fresh tube (see Note 6).
10. Add an equal volume of isopropanol to the tube and mix the contents by inversion.
11. Add 2 μL glycogen and incubate the tube for ≥1 h at −20°C to precipitate the RNA.

12. Spin the tube in a centrifuge at 10,000 × g for 20 min at 4°C to pellet RNA.

13. Dissolve the pellet in 3 mL of solution D (supplied in the recommended kit). Gently pipette the pellet to resuspend, if necessary.

14. Add 3 mL of isopropanol (supplied in the recommended kit) to the tube and mix the contents well.

15. Incubate the tube for 1 h at −20°C.

16. Remove an aliquot (e.g., 1 mL) of RNA into a microcentrifuge tube and pellet by centrifugation at 10,000 × g for 10 min at 4°C. Remove and discard the supernatant from the tube.

17. Wash the pellet with 1 mL of 75% (v/v) ethanol in RNase-free water (e.g., treated with DEPC), pellet by centrifugation at 10,000 × g for 10 min at 4°C, and remove and discard the supernatant.

18. Dry the pellet under vacuum for 2–5 min at room temperature (see Note 7).

19. Resuspend the RNA in 50 μL RNase-free water. Remove an aliquot (2–5 μL) to determine the absorbance at 260 and 280 nm to determine the RNA purity and concentration. The A_{260}/A_{280} ratio should be ≥2.0. Lower values indicate protein and/or phenol contamination. RNA concentration (in micrograms per milliliter) = absorbance at 260 nm × dilution factor × 40. For long-term storage, resuspend the RNA in RNase-free water, add 2 M sodium acetate to a final concentration of 0.25 M, and then add 2.5 volumes of 100% (v/v) ethanol. Store the RNA at −80°C.

3.4. Antibody Library Construction

Reverse transcribe RNA to complementary DNA (cDNA) and construct IgG κ/λ libraries using the pCombH phage display vector or similar system, as described in the literature (4–5, 13–14) and elsewhere in this volume.

4. Notes

1. In some cases the antigen of interest can be obtained commercially in an already biotinylated form. If this is not the case, the antigen of interest can be biotinylated using standard biotinylation kits. A panel of different easy-to-use
biotinylation kits are provided by Pierce, including a micro biotinylation kit that allows biotinylation of very small amounts (50–200 μg) of protein with recoveries generally >80%.

2. To assure that the antigen of interest is still in its native conformation and that it has not been denatured during the biotinylation process, perform an enzyme-linked immunosorbent assay (ELISA) using appropriate antibodies against the antigen of interest.

3. Prepare solution D by adding 100 μL of β-mercaptoethanol to 14 mL of denaturing solution, warmed to room temperature. Solution D may be stored at room temperature for up to 1 month.

4. Total RNA may also be isolated using Trizol (Invitrogen) or messenger RNA (mRNA) may be isolated with an mRNA isolation kit using magnetic beads coated with poly dT₁₆ (Dynal).

5. After centrifugation, two phases should be clearly visible. The upper, aqueous phase contains the RNA. The lower, phenol phase and interface contain DNA and proteins.

6. Taking care to avoid the interface and the lower phase, transfer the upper, aqueous phase, which contains the RNA, to a fresh centrifuge tube. Discard the lower phenol phase, which contains proteins and DNA.

7. Do not over-dry the sample or the pellet will be difficult to redissolve.

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Construction of Phage Antibody Repertoires from the Blood of West Nile Virus-Infected Donors

Mark Throsby and John de Kruif

Summary

A method for the construction of West Nile virus immune donor antibody repertoires is described. B cells are harvested from a suitable donor and the antibody variable genes are amplified using polymerase chain reaction (PCR). The PCR fragments are cloned in a phage display vector to construct a repertoire that can be used in panning procedures to identify many unique monoclonal antibodies.

Key words: Immune library, Phage display, Antibody, scFv, Immune donor

1. Introduction

Antibody phage display has been used for almost two decades for the isolation of antibodies directed against numerous antigens. Synthetic and naïve libraries have been used successfully but these need to be of very large size and high quality to yield diverse repertoires of high-affinity antibodies; typically, these libraries are not available to the general public. The likelihood of obtaining antibodies with optimal characteristics from smaller repertoires is much higher when the peripheral B cell pools are used from donors that have been immunized or infected with a target pathogen. These antibody libraries are highly enriched for high affinity antibodies because the donor repertoires have been shaped by affinity maturation in vivo. Further, the donors used for library construction can be selected a priori for favorable characteristics such as the presence of high-affinity virus-neutralizing antibodies in the serum.
This protocol describes the construction of an antibody phage display library using the antibody genes harvested from the blood of West Nile virus (WNV)-infected donors in order to obtain potent virus-neutralizing antibodies. Selected phage antibodies were reformatted and tested for their capacity to neutralize (1). The protocol can be used for the isolation of antibodies against other pathogens, depending on the donors who are used.

Briefly, donors were recruited and their sera tested for WNV binding and neutralization activity (1). The donors with highest antibody titers were identified and RNA extracted from samples of lymphocytes. The RNA was used for complementary DNA (cDNA) synthesis followed by polymerase chain reaction (PCR) amplification of the antibody genes. The PCR fragments were then cloned into a phage display vector. Quality of the resulting library was verified by estimation of insert and open reading frame frequencies. The libraries were rescued and used in selection procedures as described elsewhere (1, 2).

2. Materials

2.1. RNA Isolation and cDNA Synthesis

1. Isolated peripheral blood mononuclear cells from donors of the appropriate medical and immunological history.
2. RNase Blaster solution (Clontech).
3. TRIzol RNA reagent (Invitrogen).
4. Chloroform.
5. Isopropanol.
6. 70% (v/v) ethanol in DEPC-treated water.
7. DEPC-treated water.
8. 10 mM Tris–HCl, pH 8.5.
9. Random oligonucleotide hexamers (Promega).
10. 5× FS-buffer (Invitrogen).
11. 10 mM dNTPs.
12. 100 mM dithiothreitol.
13. RNAse inhibitor (40 U/μL) RNasin Plus (Promega).
14. MMLV reverse transcriptase, SuperScript III (200 U/μL) (Invitrogen).

2.2. PCR, DNA Purification, and Transformation

1. Taq polymerase (5 U/μL), 10× PCR buffer, 50 mM MgCl₂ (Invitrogen).
2. DMSO.
3. dNTPs (25 mM).
4. Human κ chain constant region primer (antisense direction):

| Primer          | Sequence  |
|-----------------|-----------|
| HuCVκ           | 5′ ACA CTC TCC CCT GTT GAA GCT CTT 3′ |

5. Human Vκ primers (sense direction):

| Primer | Sequence  |
|--------|-----------|
| HuVκ1B | 5′ GAC ATC CAG WTG ACC CAG TCT CC 3′ |
| HuVκ2  | 5′ GAT GTT GTG ATG ACT CAG TCT CC 3′ |
| HuVκ3  | 5′ GAA ATT GTG WTG ACR CAG TCT CC 3′ |
| HuVκ4  | 5′ GAT ATT GTG ATG ACC CAC ACT CC 3′ |
| HuVκ5  | 5′ GAA ACG ACA CTC ACG CAG TCT CC 3′ |
| HuVκ6  | 5′ GAA ATT GTG CTG ACT CAG TCT CC 3′ |

Nucleotide codes: A = adenosine, C = cytidine, G = guanosine, T = thymidine, K = G or T, B = C, G or T not A, R = A or G, M = A or C, D = A, G or T not C, Y = C or T, S = G or C, H = A, C or T not G, N = A, C, G or T, W = A or T, V = A, C or G not T. See Note 1 with respect to primer sequences.

6. Human λ chain constant region primers (antisense direction):

| Primer | Sequence  |
|--------|-----------|
| HuCL2  | 5′ TGA ACA TTC TGT AGG GGC CAC TG 3′ |
| HuCL7  | 5′ AGA GCA TTC TGC AGG GGC CAC TG 3′ |

7. Human Vλ primers (sense direction):

| Primer | Sequence  |
|--------|-----------|
| HuVλ1A | 5′ CAG TCT GTG CTG ACT CAG CCA CC 3′ |
| HuVλ1B | 5′ CAG TCT GTG YTG ACG CAG CCG CC 3′ |
| HuVλ1C | 5′ CAG TCT GTG CTG ACT CAG CCG CC 3′ |
| HuVλ2  | 5′ CAR TCT GCC CTG ACT CAG CCT 3′ |
| HuVλ3A | 5′ TCC TAT GWG CTG ACT CAG CCA CC 3′ |
| HuVλ3B | 5′ TCC TCT GAG CTG ACT CAG GAC CC 3′ |
| HuVλ4  | 5′ CAC GTT ATA CTG ACTCAA CCG CC 3′ |
| HuVλ5  | 5′ AAT TTT ATG CTG ACT CAG CCC CA 3′ |
| HuVλ7/8| 5′ CAG RCT GTG GTG ACY CAG GAG CC 3′ |
| HuVλ9  | 5′ CWG CCT GTG CTG ACT CAG CCM CC 3′ |

8. 10× gel loading buffer for agarose gel electrophoresis.
9. QIAquick Gel Extraction Kit (Qiagen).
10. Human Vk primers containing *SalI* sites:

| Primer          | Sequence                                                                 |
|-----------------|--------------------------------------------------------------------------|
| **HuV\(k\)1B-SAL** | 5’ TGA GCA CAC AGG TCG ACG ACT CAG WTG ACC CAG TCT CC 3’ |
| **HuV\(k\)2-SAL** | 5’ TGA GCA CAC AGG TCG ACG GAT GTT GTG ATG ACT CAG TCT CC 3’ |
| **HuV\(k\)3B-SAL** | 5’ TGA GCA CAC AGG TCG ACG GAA ATT GTG WTG ACR CAG TCT CC 3’ |
| **HuV\(k\)4B-SAL** | 5’ TGA GCA CAC AGG TCG ACG GAT ATT GTG ATG ACC CAC ACT CC 3’ |
| **HuV\(k\)5-SAL**  | 5’ TGA GCA CAC AGG TCG ACG GAA ACG ACA CTC ACG CAG TCT CC 3’ |
| **HuV\(k\)6-SAL**  | 5’ TGA GCA CAC AGG TCG ACG GAA ATT GTG CTG ACT CAG TCT CC 3’ |

11. Human J\(k\) primers containing *NotI* sites:

| Primer          | Sequence                                                                 |
|-----------------|--------------------------------------------------------------------------|
| **HuJ\(k\)1-NOT** | 5’ GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT TTC CAC CTT GGT CCC 3’ |
| **HuJ\(k\)2-NOT** | 5’ GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAG CTT GGT CCC 3’ |
| **HuJ\(k\)3-NOT** | 5’ GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT ATC CAC TTT GGT CCC 3’ |
| **HuJ\(k\)4-NOT** | 5’ GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT ATC CAC CTG GGT CCC 3’ |
| **HuJ\(k\)5-NOT** | 5’ GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT AAT CTC CAG TCG TGT CCC 3’ |

12. Human V\(\lambda\) primers containing *SalI* sites:

| Primer          | Sequence                                                                 |
|-----------------|--------------------------------------------------------------------------|
| **HuV\(\lambda\)1A-SAL** | 5’ TGA GCA CAC AGG TCG ACG ACT CAG TCT GTG CTG ACT CAG CCA CC 3’ |
| **HuV\(\lambda\)1B-SAL** | 5’ TGA GCA CAC AGG TCG ACG ACT CAG TCT GTG YTG ACT CAG CCG CC 3’ |
| **HuV\(\lambda\)1C-SAL** | 5’ TGA GCA CAC AGG TCG ACG ACT CAG TCT GTG ACG ACT CAG CCG CC 3’ |
| **HuV\(\lambda\)2-SAL**  | 5’ TGA GCA CAC AGG TCG ACG CAR ACT GTG AC CCA CCG CC 3’ |
| **HuV\(\lambda\)3A-SAL** | 5’ TGA GCA CAC AGG TCG ACG TCC TAT GWG CTG ACT CAG CCA CC 3’ |
| **HuV\(\lambda\)3B-SAL** | 5’ TGA GCA CAC AGG TCG ACG TCT ACT GTG ACT CAG GAC CC 3’ |
| **HuV\(\lambda\)4-SAL**  | 5’ TGA GCA CAC AGG TCG ACG ACT CAA CCA CCG CC 3’ |
### Construction of Phage Antibody Repertoires from the Blood of West Nile

|      | 5’ TGA GCA CAC AGG TCG ACG CAG GCT GTG CTG ACT CAG CCG TC 3’ |
|------|-------------------------------------------------------------|
| HuVλ.5-SAL |                                              |
| HuVλ.6-SAL | 5’ TGA GCA CAC AGG TCG ACG AAT TTT ATG CTG ACT CAG CCC CA 3’ |
| HuVλ.7/8-SAL | 5’ TGA GCA CAC AGG TCG ACG CAG RCT GTG GTG ACY CAG GAG CC 3’ |
| HuVλ.9-SAL | 5’ TGA GCA CAC AGG TCG ACG CGW CCT GTG CTG ACT CAG CCM CC 3’ |

13. Human Jλ primers containing NotI sites:

|      | 5’ GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT GAC CTT GGT CCC 3’ |
|------|---------------------------------------------------------------------|
| HuJλ.1-NOT |                                                      |
| HuJλ.2/3-NOT | 5’ GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT CAG CTT GGT CCC 3’ |
| HuJλ.4/5-NOT | 5’ GAG TCA TTC TCG ACT TGC GGC CGC ACY TAA AAC GGT GAG CTG GGT CCC 3’ |

14. QIAquick PCR Purification Kit (Qiagen).
15. Restriction enzymes, 10x buffers, supplements, and ligase (New England Biolabs): Sall (20 U/μL), NotI (10 U/μL), BSA (10 mg/mL), T4 DNA ligase (400 U/μL), AseI (5 U/μL), XhoI (20 U/μL), SfiI (10 U/μL).
16. Phenol/chloroform/isoamyl alcohol (25:24:1).
17. Pellet paint (Novagen).
18. 3 M Sodium acetate, pH 5.2.
19. 0.1 cm electroporation cuvettes.
20. SOC medium containing 5% (w/v) glucose (Invitrogen).
21. Electrocompetent Escherichia coli TG1 bacteria (Stratagene).
22. 2TY agar containing ampicillin (100 μg/mL) and glucose (1% [w/v]).
23. Sterile 245 × 245-mm plastic dishes and conventional 94-mm diameter petri dishes.
24. 2TY liquid medium containing ampicillin (50 μg/mL) and glucose (5% [w/v]).
25. QIAGen Plasmid Maxi Kit (Qiagen).
26. Human IgG constant region primer (antisense direction):

|      | 5’ GTC CAC CTT GGT GTT GCT GGG CTT 3’ |
|------|--------------------------------------|
| HuCIgG |                                      |
27. Human VH primers (sense direction):

| Primer | Sequence |
|--------|----------|
| HuVH1B/7A | 5' CAG RTG CAG CTG GTG CAR TCT GG 3' |
| HuVH1C | 5' SAG GTC CAG CTG GTR CAG TCT GG 3' |
| HuVH2B | 5' CAG RTC ACC TTG AAG GAG TCT GG 3' |
| HuVH3B | 5' SAG GTG CAG CTG GTG GAG TCT GG 3' |
| HuVH3C | 5' GAG GTG CAG CTG GTG GAG WCY GG 3' |
| HuVH4B | 5' CAG GTG CAG CTG GAG TCS GG 3' |
| HuVH4C | 5' CAG GGA CAG CTG GTG GAG TCT GG 3' |

28. Human JH primers containing \textit{XhoI} sites (antisense direction):

| Primer | Sequence |
|--------|----------|
| HuJH1/2-\textit{XhoI} | 5' GAG TCA TTC TCG ACT CGA GAC GGT GAC CAG GCC 3' |
| HuJH3-\textit{XhoI} | 5' GAG TCA TTC TCG ACT CGA GAC GGT GAC CAT TGT CCC 3' |
| HuJH4/5-\textit{XhoI} | 5' GAG TCA TTC TCG ACT CGA GAC GGT GAC CAG TCC 3' |
| HuJH6-\textit{XhoI} | 5' GAG TCA TTC TCG ACT CGA GAC GGT GAC CGT GCC 3' |

29. Human VH primers containing \textit{SfiI}/\textit{NcoI} sites (sense direction):

| Primer | Sequence |
|--------|----------|
| HuVH1B/7A-\textit{NcoI} | 5' GTC CTC GCA ACT CGG GCC CAG CCG GCC ATG GCC CAG RTG CAG CTG GTG CAR TCT GG 3' |
| HuVH1C-\textit{NcoI} | 5' GTC CTC GCA ACT CGG GCC CAG CCG GCC ATG GCC SAG GTC CAG CTG GTR CAG TCT GG 3' |
| HuVH2B-\textit{NcoI} | 5' GTC CTC GCA ACT CGG GCC CAG CCG GCC ATG GCC CAG RTG CAG CTG GTG CAR TCT GG 3' |
| HuVH3B-\textit{NcoI} | 5' GTC CTC GCA ACT CGG GCC CAG CCG GCC ATG GCC SAG GTG CAG CTG GAG TCT GG 3' |
| HuVH3C-\textit{NcoI} | 5' GTC CTC GCA ACT CGG GCC CAG CCG GCC ATG GCC GAG GTG CAG CTG GTG GAG WCY GG 3' |
| HuVH4B-\textit{NcoI} | 5' GTC CTC GCA ACT CGG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTA CAG CAG TGG GG 3' |
In our published work, RNA from WNV convalescent patients was pooled to generate a single immune library that was used for selection procedures (1). However, in subsequent libraries we have chosen to keep donor materials separate throughout the procedure. The rationale for this approach is that the chances of combining cognate heavy and light chains are enhanced. A generic procedure using this approach is described below.

1. Prepare a sample of peripheral blood cells via one of the routine procedures such as Ficoll density gradient centrifugation.
2. Pellet a maximum of $1 \times 10^7$ cells by centrifugation at $500 \times g$ for 2 min at room temperature. Add 1 mL of TRIzol RNA extraction reagent to the pellet.
3. Lyse the cells by repetitive pipetting through a 1,000 μL filter tip. Incubate for 5 min at room temperature to ensure complete lysis.
4. Extract RNA from the lysate by adding 200 μL chloroform and gently mixing for 20 s.
5. Separate the organic phase by centrifuging for 15 min at $13,000 \times g$, at 4°C. Transfer the aqueous phase containing total RNA to a fresh tube.
6. Extract once more by adding 500 μL chloroform. Mix and centrifuge for 5 min at $13,000 \times g$, 4°C. Transfer the aqueous phase to a new tube.
7. Precipitate the RNA by adding an equal volume of isopropanol and incubating for 10 min at room temperature.
8. Centrifuge for 15 min at $13,000 \times g$, 4°C. Discard the supernatant and wash the pellet with 1 mL of 70% ethanol. Centrifuge for 10 min at $7,500 \times g$ at 4°C.
9. Remove the ethanol carefully and air-dry the pellet for 5 min at room temperature.
10. Dissolve the pellet in 50 μL DEPC-treated water. Make a dilution of 1:25 in 10 mM Tris–HCl, pH 8.5, and determine
the RNA concentration by measuring the absorbance at 260 nm. The concentration of RNA in micrograms per microliter will be \( \frac{A_{260} \times 40 \times \text{dilution}}{1,000} \).

11. Dilute the sample to 0.1 \( \mu \text{g/\muL} \) in DEPC-treated water and store the sample at −80°C.

### 3.2. cDNA Synthesis

1. Mix the following components: 10 \( \mu \text{L} \) RNA (0.1 \( \mu \text{g/\muL} \)), 1 \( \mu \text{L} \) random hexamers (500 ng/\( \mu \text{L} \)), and 13 \( \mu \text{L} \) DEPC-treated water.

2. Heat the mixture at 65°C for 5 min, then cool immediately on ice for 1 min.

3. Add in the following order: 8 \( \mu \text{L} \) of 5× FS-buffer, 2 \( \mu \text{L} \) dNTPs (10 mM), 2 \( \mu \text{L} \) DTT (0.1 M), 2 \( \mu \text{L} \) RNase inhibitor (40 U/\( \mu \text{L} \)), and 2 \( \mu \text{L} \) SuperScript III (200 U/\( \mu \text{L} \)). Mix after addition of each component. Incubate for 5 min at room temperature, then for 1 h at 50°C. Inactivate the enzyme by heating at 75°C for 15 min.

4. Add 160 \( \mu \text{L} \) DEPC-treated water. Make a dilution of 1:25 in 10 mM Tris-HCl, pH 8.5, to determine the cDNA concentration. Use a spectrophotometer to measure the absorbance at 260 nm. The formula for calculating the concentration in micrograms per microliter is \( \frac{A_{260} \times 50 \times \text{dilution}}{1,000} \).

5. The cDNA can be used as a template for PCR amplification directly or stored at −20°C.

### 3.3. Primary PCR Amplification of Light Chains

1. PCR is performed to amplify the light chain variable regions. Prepare and keep all components on ice. Make a PCR master mix of all components except the template and the primers. Add Taq polymerase to the master mix just before use.

2. The master mix comprises 5 \( \mu \text{L} \) of 10× PCR buffer, 1.5 \( \mu \text{L} \) of 50 mM MgCl\(_2\), 2.5 \( \mu \text{L} \) of DMSO, 0.5 \( \mu \text{L} \) of dNTPs (25 mM), 30.25 \( \mu \text{L} \) water, and 0.25 \( \mu \text{L} \) Taq polymerase. These volumes are for a single reaction; scale appropriately for multiple reactions.

3. Set up six 0.2 mL reaction tubes, a separate reaction for each of the antisense primers. To each tube, add 5 \( \mu \text{L} \) cDNA template, 2.5 \( \mu \text{L} \) of the single antisense primer (10 \( \mu \text{M} \)), and 2.5 \( \mu \text{L} \) of each of the antisense primers (10 \( \mu \text{M} \)) in turn.

4. To each tube, add 40 \( \mu \text{L} \) of the PCR master mix.

5. Preheat a PCR machine to 96°C so that the reaction gets a hot start. For this primary amplification, use the following PCR program: 2 min at 96°C, 30 cycles of 30 s at 96°C, 30 s at 55°C, 1 min at 72°C, a 10 min incubation at 72°C, and hold at 4°C.
6. For the primary amplifications of the V\(\lambda\) light chain variable regions, follow the same procedure as above with minor modifications: the three sense primers for the V\(\lambda\)1 family (HuV\(\lambda\)1A, B, and C) can be mixed in a 1:1:1 equimolar ratio. The antisense primers HuC\(\lambda\)2 and HuC\(\lambda\)7 can also be mixed in equimolar proportions. In total, nine reactions will be required for recovery of the V\(\lambda\) light chain repertoire.

3.4. Analysis and Purification of the PCR Reactions

1. Add 5.5 \(\mu\)L of 10× loading buffer to each PCR reaction and mix.

2. Load each entire reaction to the enlarged sample wells in a 1% agarose gel. Separate the products by electrophoresis using DNA markers to confirm the size of the amplicons.

3. After running, cut the bands of the correct size (~650 bp) from the gel and place each gel slice in a 1.5 mL microfuge tube. Purify the fragments using gel extraction reagents.

3.5. Secondary Amplification of Light Chain Variable Regions

1. These reactions introduce restriction sites for cloning the products into the phagemid vector and thereby constructing a scFv display library. For the V\(\kappa\) light chain products, follow the same procedure as in the primary amplification, with the following modifications.

2. As PCR template, use 5 \(\mu\)L of each purified primary PCR product. For each reaction set, select the appropriate V\(\kappa\) sense primer carrying a SalI site and generate a series of reactions with each of the five J\(\kappa\) primers containing a NotI site. For example, if the primary PCR product was generated with primers HuV\(\kappa\)1B and HuCV\(\kappa\), the sense primer for secondary amplification will be HuV\(\kappa\)1B-SAL and all five J\(\kappa\) antisense primers containing NotI sites (HuJ\(\kappa\)1-NOT, etc.) will be used. This results in five reactions from a single primary product.

3. In total this procedure will generate 30 reactions. The products should be of approximately 400 bp. Cycle using the following PCR program: 2 min at 96°C, 30 cycles of 30 s at 96°C, 30 s at 60°C, 1 min at 72°C, a 10 min incubation at 72°C, and hold at 4°C.

4. For the V\(\lambda\) light chains, follow the same procedure with the following modifications. Use the human V\(\lambda\) primers containing SalI sites and the human J\(\lambda\) primers containing NotI sites. As before, primers for the V\(\lambda\)1 family (HuV\(\lambda\)1A, B, and C-SAL) can be pooled in a 1:1:1 equimolar ratio. Reactions should be set up by choosing the HuV\(\lambda\)-SAL primer (or pool) appropriate for the primary reaction and each of the 3 J\(\lambda\) primers containing NotI sites. Since primary amplification involved nine reactions, this means that a total of 27 PCRs need to be conducted.
3.6. Analysis and Pooling of the Light Chain PCR Reactions

1. Sample 6 μL from each secondary PCR reaction and mix with 6 μL of 2× loading buffer.

2. Load to a 1% agarose gel and separate by electrophoresis. Use a set of DNA markers to check that the sizes of the products are approximately 400 bp and to estimate the concentration of the DNA.

3. Pool the secondary amplification products from Vk reactions as follows (see Note 3). First, mix products that were amplified with common J primers. Then, guided by the Jk primers that were used (e.g., HuJk1-NOT), combine products in the ratio of 25% Jk1, 25% Jk2, 25% Jk4, 12.5% Jk3, and 12.5% Jk5.

4. Following similar principles, pool secondary amplification products from Vl reactions. Guided by the JL primers that were used, combine products in the ratio 30% JL1, 60% JL2, and 10% JL3.

5. Analyze the resulting pools on a 1% agarose gel, again estimating the DNA concentrations.

6. The pools are then combined into a single light chain pool according to Vk and Vl families, as identified by the names of the primers. Combine in the ratio: 30% Vk1, 4% Vk2, 1% Vk3, 19% Vk4, 1% Vk5, 5% Vk6, 14% Vl1, 10% Vl2, 10% Vl3, and 1% Vl4–9.

3.7. Digestion of Light Chain PCR Products

1. Purify the DNA from the pool using a kit for purification of PCR products. Elute the DNA in two aliquots of 52 μL using the EB buffer provided in the kit.

2. Prepare a restriction digestion with the following ingredients. One hundred microliters of DNA containing approximately 3 μg of purified PCR product, 20 μL of 10× buffer (e.g., NEB 3), 2.5 μL of SalI, 5 μL of NotI, and 72.5 μL of water. Digest overnight at 37°C.

3. Add 20 μL of 10× gel loading buffer to the digestion mix and load to a 1.5% agarose gel, cast with an enlarged loading well. Separate by electrophoresis.

4. Cut out the band of approximately 350 bp and recover the DNA using a gel extraction kit.

5. Determine the concentration of the purified DNA by measuring the absorbance at 260 nm on a spectrophotometer.

3.8. Preparation of the Phage Display Vector and Light Chain Ligation

1. Add the following reagents to a microfuge tube (see Note 4). One hundred microliters of DNA containing approximately 100 μg of phagemid vector (e.g., pDV-C06), 100 μL of 10× buffer (e.g., NEB 3), 25 μL SalI, 10 μL BSA (10 mg/mL), and 725 μL of water. Digest overnight at 37°C.

2. Add 100 μL of 10× loading buffer to the digested DNA and run on a 0.8% agarose gel cast with an enlarged loading well.
Cut the vector band from the gel and purify the DNA using gel extraction reagents. Elute the DNA in a total volume of 825 µL.

3. Perform a second digestion by adding the following reagents to the purified DNA: 100 µL of 10× buffer (e.g., NEB 3), 25 µL SalI, and 50 µL NotI. Digest overnight at 37°C.

4. Add 100 µL of 10× loading buffer to the digested DNA and run on a 0.8% agarose gel. Cut the vector band from the gel and purify the DNA using gel extraction reagents. Elute in 500 µL and measure the concentration of the DNA.

5. Ligations are carried out with different vector-to-insert ratios to determine empirically the most efficient ligation conditions and to assess the number of colonies generated per nanogram of ligated DNA. With a vector of approximately 5 kb and an insert of 350 bp, mixing 500 ng of vector DNA with 35 ng insert results in a molar ratio of 1:1. Prepare 50 µL ligations, each containing 5 µL of 10× ligase buffer, 2.5 µL of ligase and vector: insert ratios of 1:1, 1:2, and 1:4. Include a control with all components except the insert, and a control with vector but no ligase to control for the presence of uncut vector DNA. Incubate the ligations in a 16°C waterbath overnight.

6. Add 50 µL of 10 mM Tris-HCl, pH 8.5, to the ligation mix to double the volume. Then add 100 µL phenol/chloroform/isoamyl alcohol (25:24:1). Mix well and centrifuge for 1 min at top speed in a microcentrifuge at room temperature. Transfer the upper phase to a new tube.

7. Add 100 µL chloroform to the upper phase. Mix well and centrifuge for 1 min at top speed in a microcentrifuge at room temperature. Transfer the upper phase to a new tube.

8. Make a large-scale precipitation mixture with 1 µL Pellet paint, 10 µL sodium acetate, and 100 µL isopropanol for each ligation reaction, plus extra in case of shortfall. Add 111 µL of the mixture to each extracted ligation and incubate for 30 min at −20°C. Centrifuge 15 min at top speed in a microcentrifuge, cooled to 4°C.

9. Remove the supernatant and wash the pellet with 500 µL of 70% ethanol. Centrifuge for 15 min at top speed in a microcentrifuge, cooled to 4°C.

10. Remove the supernatant, air-dry the pellet, and dissolve in 50 µL of 10 mM Tris-HCl, pH 8.5. Store at −20°C until transformation.

3.9 Transformation of Light Chain Ligations

1. Chill 0.1 cm electroporation cuvettes and 1.5 mL microcentrifuge tubes on ice. Prewarm SOC medium containing glucose to 37°C.

2. Thaw electrocompetent E. coli TG1 cells on ice for approximately 5 min.
3. Gently mix 40 μL aliquots of bacteria with 2 μL ligation mix in a chilled microcentrifuge tube. Transfer the suspension to the prechilled 0.1 cm electroporation cuvette. Tap the cuvette on the bench to make sure the mixture settles evenly to the bottom and slide the cuvette into the electroporation chamber until the cuvette connects with the electrical contacts.

4. The following settings are recommended for use with a Bio-Rad Gene-Pulser II: field strength 17 kV/cm, capacitance 25 μF, and resistance 200 Ω. Pulse the sample once.

5. Quickly remove the cuvette from the apparatus and immediately add 750 μL of prewarmed SOC glucose to resuspend the cells.

6. Transfer the cell suspension to a sterile 15 mL tube and add another 750 μL SOC glucose medium to the tube. Shake in a 37°C incubator at 225 rpm for exactly 1 h.

7. Plate dilutions of the transformations onto 2TY agar with ampicillin and glucose. Incubate overnight at 37°C.

8. Count the colonies and determine the optimal vector-to-insert ratio for the final ligations.

9. For a large scale experiment (e.g., full library construction), perform multiple transformations as described above, each with 2 μL aliquots of an optimized ligation mix. Aim for a recovery of approximately 10^7 colonies, as predicted from the efficiency of the test transformations.

10. Plate the transformed bacteria on 2TY agar with ampicillin and glucose, poured to 245 × 245 mm plates in volumes predicted to yield approximately 10^5 colonies per plate. In addition, plate several dilutions onto conventional petri dishes for counting and to pick colonies. Count the colonies on conventional plates to estimate the size of the final library.

11. Perform PCR amplification and sequencing on 96 colonies of the intermediate light chain library to check for inserts of the expected length and the presence of open reading frames (see Note 5). Standard procedures can be used, which are outside the scope of this chapter.

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1. Gently scrape the bacteria from the 245 × 245 mm plates with a glass spreader, and, for each plate, resuspend the harvested bacteria in 15 mL 2TY medium containing ampicillin (50 μg/mL) and glucose (5%).

2. Pool the bacteria into a sterile bottle and mix until all bacteria are in suspension. Make a 1:50 dilution in 2TY medium containing ampicillin (50 μg/mL) and glucose (5%) and determine the absorbance at 600 nm using a spectrophotometer.
3. Prepare a series of 50 mL tubes, and add to each a volume of bacteria suspension equivalent to 500 U of absorbance. Pellet the bacteria by centrifugation for 10 min at 4,000 \( \times g \).

4. Discard the supernatant and use the bacterial pellets for DNA maxipreps using Qiagen reagents or equivalent. Quantify the yields of DNA by spectrophotometry.

5. Prepare digests of the light chain phage display library. To a 1.5 mL microfuge tube, add a volume of solution containing 100 \( \mu \)g DNA, 100 \( \mu \)L of 10x buffer (e.g., NEB 2), 25 \( \mu \)L of \( XhoI \), and water to a final volume of 1 mL. Incubate at 37\(^\circ\)C overnight.

6. Add 100 \( \mu \)L of 10x gel loading buffer to the digested DNA and run on a 0.8% agarose gel. Cut out the vector band (~5.3 kb for a vector such as pDV-C06) and purify from the agarose using gel extraction reagents. Elute the DNA in a total volume of 875 \( \mu \)L.

7. Continue the digestion by adding the following components to the purified DNA: 100 \( \mu \)L of 10x buffer (e.g., NEB 2) and 25 \( \mu \)L of \( SfiI \). Incubate at 50\(^\circ\)C overnight (see Note 6). Purify the DNA by agarose gel electrophoresis as described above but eluting in a volume of 500 \( \mu \)L. Estimate the concentration of DNA by spectrophotometry.

3.11. Primary PCR Amplification of Heavy Chains and Analysis

1. PCR is performed to amplify the repertoire of heavy chain variable regions from the donor cDNA.

2. Follow a similar procedure to that described for the light chain amplifications. Set up the reactions using the single antisense primer, HuCIgG, and the human VH sense primers (HuVH1B/7A, etc.). The primers HuVH1B/7A and HuVH1C can be combined in a 1:1 molar ratio (see Note 7). This results in a total of seven reactions.

3. Products of approximately 600 bp should result. Purify the PCR products as described for the light chain reactions.

3.12. Secondary Amplification of Heavy Chain Variable Regions

1. Follow a similar procedure as described for the light chains with the following modifications.

2. As the template for this PCR, use 5 \( \mu \)L of the purified primary heavy chain PCR product. The antisense primers are the four human JH primers containing \( XhoI \) sites. The sense primers for the reaction are the human VH primers containing \( NcoI \) and \( SfiI \) sites. As in the previous section, the HuVH1B/7A-\( NcoI \) and HuVH1C-\( NcoI \) primers can be combined in a 1:1 molar ratio.

3. In setting up, each of the VH sense primers or primer mixes should be combined with each of the four JH primers, resulting in a total of 28 reactions. Because the reactions recover sequences coding for the variable domain only, products of approximately 400 bp should be obtained.
3.13. Analysis and Pooling of the Heavy Chain PCR Reactions

1. Add 6 μL of 2× loading buffer to 6 μL samples from each of the secondary PCR reactions and load onto a 1% agarose gel. Use a marker in the first lane to check that the size of the products is as expected (~400 bp) and to determine the approximate concentration of DNA in each reaction.

2. Pool the secondary amplification products as follows (see Note 3). First, mix the PCR products according to the sense primer that was used, in the following ratio: 10% HuJH1/2-XhoI, 10% HuJH3-XhoI, 60% HuJH4/5-XhoI, and 20% HuJH6-XhoI. Then, combine the PCR products based upon VH family: 25% HuVH1B/7A-NcoI/HuVH1C-NcoI, 2% HuVH2B-NcoI, 25% HuVH3B-NcoI, 25% HuVH3C-NcoI, 2% HuVH4B-NcoI, 20% HuVH4C-NcoI, and 1% HuVH6A-NcoI. This results in one pool of heavy chain products.

3.14. Digestion of Heavy Chain PCR Products, Purification, and Ligation

1. Purify the DNA from the pool using PCR purification reagents and elute in two aliquots of 52 μL of buffer EB supplied with the kit.

2. Prepare a digestion mix with the following ingredients: 3 μg DNA, 20 μL of 10× buffer (e.g., NEB 2), 2.5 μL XhoI, and water to a final volume of 200 μL. Digest overnight at 37°C.

3. Add 2.5 μL SfiI to the mix and digest for a further 6 h at 50°C.

4. Purify the digested products as described above for the light chain inserts.

5. Set up ligations with the cut light chain library prepared in Subheading 3.10 using the methods described earlier. Test ligations and controls should be used to guide the final large-scale ligation.

6. Purify the ligation products and electroporate into E. coli TG1.

7. Select on 2TY agar containing ampicillin and glucose. Titrations should be used to establish the size of the final library containing heavy and light chain inserts.

8. A PCR amplification and sequencing reaction on 96 colonies of the library is performed to check for inserts of the expected length and the presence of open reading frames. Because this entails a standard procedure, a detailed described is out of the scope of this chapter.

3.15. Storage of the Library (see Note 8)

1. Gently scrape bacteria from the 245 × 245 mm plates with a glass spreader and resuspend bacteria from each plate in 15 mL of 2TY medium containing ampicillin and glucose.

2. Collect all bacteria from the library into a sterile container, and mix until all bacteria are in suspension.
3. Pipette 30 mL of the bacterial suspension into a 50 mL tube, add 15 mL sterile 50% (v/v) glycerol, and mix well.
4. Dispense the bacteria to multiple cryotubes, snap freeze on dry ice, and store at −80°C.

### 4. Notes

1. The oligonucleotides are optimized versions of previously described primer sets (3).
2. Keep the cells on ice during isolation to avoid degradation of RNA. All materials used should be free of RNase. Spray surfaces and pipettes with RNase Blaster solution and rinse with RNase-free water before beginning the procedure. Wear gloves during RNA isolation. In treating solutions with DEPC, note that DEPC is a suspected carcinogen and should be handled with care; always wear gloves and work in a fume hood.
3. To maintain natural distribution of the different J segments, and light chain and heavy chain families within the library and to ensure families are not over- or under-represented, the PCR products are combined in defined ratios into single pools and subsequently ligated into the phage display vector. The proportions are based on data in the literature (4).
4. In our laboratory, the pDV-C06 vector is used. This is a close derivative of vector pHEN1 (5).
5. More than 80% of clones that are analyzed should contain an insert and intact open reading frame. Lower insert frequencies may be caused by insufficient digestion of the cloning vector. Frame shifts are often caused by synthesis/purification of primers of insufficient quality for this PCR application.
6. An extra digestion step can be included when the VH-stuffer fragment present in the phage display vector contains a unique restriction site. This will reduce the background vector molecules that lack an insert after ligation and transformation. In the pDV-C06 vector, a unique AscI site is present in the VH stuffer.
7. No primers specific for the VH5 family are used in this protocol because the VH5 gene 5.51 will be readily amplified by other primers in the panel.
8. Using these procedures, libraries with a size of approximately $10^7$ clones per donor can be made. In our hands, these libraries perform well and yield many antibodies of interest (1, 2).
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Chapter 5

Anti-$\beta_2$ GP-I and Anti-prothrombin Antibodies Generated by Phage Display

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Summary

This chapter describes the construction and screening of a library of single-chain variable fragments (scFv) derived from patients with autoimmune disease. The methods cover the isolation of mononuclear cells from peripheral blood, preparation of RNA, and recovery of immunoglobulin-coding sequences by polymerase chain reaction (PCR). Cloning into a phage display vector and screening of the scFv display library by a simple panning procedure are described. These methods are applicable to library construction from any patient group or (with alternative primer sets) any mammalian species.

Key words: Peripheral blood mononuclear cells, cDNA synthesis, scFv, Panning

1. Introduction

Phage display is gaining acceptance as a powerful research tool in immunology. It has been used, for instance, to generate human monoclonal antibodies from patients exposed to microbial pathogens or suffering from autoimmune diseases and to elucidate the structure and specificity of autoimmune antibodies (1–3). The technology can accommodate large fragments (Fab), smaller variable fragments (Fv), and recombinant single-chain variable fragments (scFv). In the latter format, the $V_H$ and $V_L$ domains are artificially joined by a peptide linker, usually 15 amino acids in length, and are hence expressed as a single polypeptide chain of 25–30 kDa. These scFv chains are usually monomeric (i.e., they contain only one paratope), but, by reducing the length of the peptide linker or abolishing it altogether, the scFv may be expressed as dimeric, trimeric, or tetrameric molecules (see elsewhere in this volume).
To construct phage display scFv libraries, $V_H$ and $V_L$ genes are prepared by reverse transcription of messenger RNA (mRNA) obtained from naïve or immune B lymphocytes. The $V_H$ and $V_L$ sequences are then amplified and assembled into a single gene by way of a nucleotide linker fragment. This construct is subsequently inserted into a phagemid vector that is later introduced into competent *Escherichia coli* by transformation or electroporation. Phagemid-containing bacterial cells are grown and infected with a helper phage to yield recombinant phage that display scFv antibody fragments. Antibody libraries are then screened and enriched for antigen-specific clones by a technique known as biopanning. In this protocol, phage displaying the scFv are bound to the immobilized antigen of interest and eluted under appropriate buffer conditions. To date, ten human anti-phospholipid antibodies (five against $\beta_2$-glycoprotein-I [anti-$\beta_2$ GP-I] and five against prothrombin [aPT]) have been generated in scFv format via phage display (2, 4, 5). Complementary DNA (cDNA) sequence analysis has shown that all but two aPT antibodies (4) have variable domains that are the products of non-antigen-driven B cell clonal maturation. Their $V_H$ and $J_H$ genes are highly homologous to their nearest germ line genes (2, 5). Most anti-phospholipid antibodies characterized in this way are monospecific, but, interestingly, two prothrombin-selected scFv fragments cross-react with $\beta_2$ GP-I (2–5).

The pathogenic activity of anti-$\beta_2$ GP-I scFvs was studied by Blank et al. (6) who took four monoclonal anti-$\beta_2$ GP-I antibodies known to be pathogenic in mice, and converted the antibodies into the scFv format by a combination of phage display and bacterial expression to produce soluble scFv anti-$\beta_2$ GP-I antibodies (6). Blank et al. found that the scFvs showed a reduction in antigen-binding properties compared with the original, full-length monoclonals, but that normal mice immunized with any of the three anti-$\beta_2$ GP-I scFvs went on to develop anti-phospholipid syndrome. Control animals immunized with scFvs carrying the heavy chain sequence derived from a non-pathogenic antibody had no such activity (6).

In this chapter, the construction of an scFv antibody library and the isolation of monoclonal antibodies against $\beta_2$ GP-I is described. The advantages of this approach are that phage display is capable of producing libraries with as many as $10^{10}$ different specificities, and the linkage between phenotype and genotype enables multiple rounds of selection on immobilized pure antigens to identify specific binding antibodies from these resources. The linkage of $V_H$ and $V_L$ domains into scFvs yields proteins that are functionally, structurally, and pathogenically similar to monoclonal antibodies isolated by hybridoma technology. Further, scFv libraries are usually stable if kept under appropriate conditions, allowing screens to be conducted against different antigens as project goals evolve. A relative disadvantage is the unavoidable need for highly purified antigen(s) of interest.
2. Materials

1. EDTA anticoagulant (2% w/v).
2. Phosphate-buffered saline (PBS).
3. Lymphoprep density gradient medium (Axis-Shield).
4. TRIzol RNA isolation reagent (Invitrogen) or equivalent.
5. SuperScript first-strand cDNA synthesis system for reverse transcriptase (RT)-PCR (Invitrogen).
6. Reagents for PCR including dNTPs (Promega) and LongAmp Taq polymerase (New England Biolabs).
7. Phage display system (e.g., pCANTAB 5b [Amersham Bioscience], or pSyn2) and an existing library in the vector system to provide template for linker recovery.
8. Reagents for gel electrophoresis including modified TAE buffer (40 mM Tris-acetate, pH 8.0, and 0.1 mM EDTA) containing 0.5 µg/mL ethidium bromide.
9. Reagents for purification of DNA from agarose.
10. Restriction enzymes, buffers, and ligase: NotI and SfiI with associated 10× buffers (New England Biolabs); T4 DNA ligase (concentrated stock; New England Biolabs).
11. Electrocompetent E. coli XL1-Blue strain (Stratagene).
12. Electroporation cuvettes (e.g., YorBio) and apparatus (e.g., Bio-Rad).
13. LB medium: 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl.
14. M13K07 helper phage (Amersham Bioscience).
15. PEG/NaCl: 20% (w/v) polyethylene glycol 8000 in 2.5 M NaCl.
16. TBS: 50 mM Tris-HCl and 150 mM NaCl, pH 7.5.
17. Purified human β2GP-I (25 µg/mL), or human prothrombin (Enzyme Research Laboratories) as a target antigen.
18. 12 × 75 mm polystyrene test tubes (Falcon).
19. Blocking buffer: 2% (w/v) powdered milk in PBS containing 0.01% (v/v) Tween.
20. Washing buffer: 0.05% (v/v) Tween 20 in PBS.
21. Elution buffer: 0.2 M glycine–HCl (pH 2.2) and 1 M Trizma base (pH 9.1).
22. 96 well culture plates (Nunc).
23. 2× TY culture medium (Difco) supplemented with 2% (w/v) glucose, ampicillin, and tetracycline.
24. Enzyme-linked immunosorbent assay (ELISA) microtiter plates (Nunc Immunoplate II, Nunc).
25. Peroxidase-conjugated anti-M13 antibody (Amersham Bioscience).
26. ELISA substrate: 1 mg/mL o-phenylenediamine and 0.01% (v/v) H₂O₂ in citrate buffer (pH 5.0).
27. Stop buffer: 5 M H₂SO₄.

3. Methods (see Note 1)

3.1. Recovery of the Antibody Repertoire (see Note 2)

1. Collect 60 mL of peripheral blood into tubes containing EDTA anticoagulant (2 mL/tube).
2. Dilute 20 mL of blood by addition of an equal volume of PBS, pH 7.2.
3. Carefully layer 20 mL of diluted blood over 10 mL of Lymphoprep.
4. Centrifuge at 800 × g for 30 min at room temperature in a swing out rotor.
5. Remove mononuclear cells from a distinct band at the sample/medium interface.
6. Wash the harvested fraction twice by suspension with 10 mL PBS, pH 7.2, and centrifugation at 800 × g for 10 min.
7. Purify total RNA from the pellet with TRIzol reagents or other specialist reagents according to the manufacturer’s recommendations.
8. Synthesize cDNA with SuperScript first-strand synthesis reagents for RT-PCR or other specialist materials according to the manufacturer’s recommendations.
9. Using the primers shown in Table 1, amplify the \( V_\text{H} \), \( V_\kappa \), and \( V_\lambda \) repertoires by PCR according to the following protocol. Set up 20 μL PCR reactions containing reaction buffer for the LongAmp Taq DNA polymerase at a final concentration of 1×, nucleotides at the concentration recommended by the polymerase supplier, and 10 pM per reaction of \( V_\text{H} \) or \( V_\kappa \) (κ or λ)-specific primers (see Table 1). Finally, add 0.8 U of LongAmp Taq polymerase. Sufficient reactions should be set up to ensure all combinations of forward and back primers for the \( V_\text{H} \), \( V_\kappa \), and \( V_\lambda \) reactions. This recovers the heavy and light chain repertoires from the cDNA pool.
10. Cycle with the following conditions: 4 min at 94°C, 25 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C, and one cycle of 10 min at 72°C.
### Table 1

**Oligonucleotides used to amplify antibody heavy and light chains**

| Chain | Family | Sequence (5’ to 3’) |
|-------|--------|---------------------|
| V<sub>H</sub> | VH1a | CAG GTG CAG CTG GTG CAG TCT GG |
| | VH2a | CAG GTC AAC TTA AGG GAG TCT GG |
| | VH3a | GAG GTG CAG CTG GTG GAG TCT GG |
| | VH4a | CAG GTG CAG CTG GAG TCG GG |
| | VH5a | GAG GTG CAG CGT TTG CAG TCT GC |
| | VH6a | CAG GTA CAG CTG CAG TCA GG |
| | JH1–2 | TGA GGA GAC GGT GAC CAG GGT GCC |
| | JH3 | TGA AAG GAC GGT GAC CAT TGT CC |
| | JH4–5 | TGA GGA GAC GGT GAC CAT GCC |
| | JH6 | TGA GGA GAC GGT GAC CAT TGT CCC |
| V<sub>k</sub> | VK1a | GAC ATC CAG ATG ACC CAG TCT CC |
| | VK2a | GAT GTT GTG ATG ACT BCAG TCT CC |
| | VK3a | GAA ATT GTG TTG ACG CAG TCT CC |
| | VK4a | GAC ATC GTG ATG ACC CAG TCT CC |
| | VK5a | GAA ACG ACA CTC ACG CAG TCT CC |
| | VK6a | GAA ATT GTG CTG ACT CAG TCT CC |
| | JK1 | ACG TTT GAT TTC CAC CTT GGT CCC |
| | JK2 | ACG TTT GAT TTC CAC CTT GGT CCC |
| | JK3 | ACG TTT GAT ATC CAC CTT GGT CCC |
| | JK4 | ACG TTT GAT TTC CAC CTT GGT CCC |
| | JK5 | ACG TTT AAT CTC CAG TCG TGT CCC |
| V<sub>λ</sub> | VL1a | CAG TCT GTG TTG ACG CAG GCG CC |
| | VL2a | CAG TCT GCC CTG ACT CAG CCT GC |
| | VL3a | TCC TAT GTG CTG ACT CAG CCA CC |
| | VL13a | TCT TCT GAG CTG ACT CAA CCG CC |
| | VL4a | CAC GTT ATA CTG ACT CAA CCG CC |
| | VL5a | CAG GCT GTG CTC ACT CAG CCC CA |
| | VL6a | AAT TTT ATG CTG ACT CAG CCC CA |
| | JL1 | ACC TAG GAC GGT GAC CTT GGT CCC |
| | JL2–3 | ACC TAG GAC GGT GAC CTT GGT CCC |
| | JL4–5 | ACC TAA AAC GGT GAG CTT GGT CCC |
Table 2
Oligonucleotides used to amplify the linker

| Sequence (5’ to 3’) |
|---------------------|
| **V\_1Fv** | GGC GGC TGC GTC AAC ACA GAC TGC GAT CCG CCA CCG CCA GAG |
| **V\_2Fv** | GCA GGC TGA TGA AGA GCA GAC TGC GAT CCG CCA CCG CCA GAG |
| **V\_3aFv** | GGT GGC TGA AGC ACA TAG GAC GAT CCG CCA CCG CCA GAG |
| **V\_3bFv** | GGG TCC TGA AGC TCA GAA GAC GAT CCG CCA CCG CCA GAG |
| **V\_4Fv** | GGC GGT TGA GTC ACG ATG TCC GAT CCG CCA GAG |
| **V\_5Fv** | GAC GGC TGA AGC ACA GAC TGC GAT CCG CCA CCG CCA GAG |
| **V\_6Fv** | TGG GGC TGA AGC ACA ATA AAA TTC GAT CCG CCA CCG CCA GAG |
| **Vk\_1a** | GGA GAC TGG GTC ATC TGG ATG TCC GAT CCG CC |
| **Vk\_2a** | GGA GAC TGA GTC ATC ACA ACA TCC GAT CCG CC |
| **Vk\_3a** | GGA GAC TGC GTC AAC ACA ATT TCC GAT CCG CC |
| **Vk\_4a** | GGA GAC TGC GTC ATC ACG ATG TCC GAT CCG CC |
| **Vk\_5a** | GGA GAC TGC GTG AGT GTC GTT TCC GAT CCG CC |
| **Vk\_6a** | GGA GAC TGA GTC AGC ACA ATT TCC GAT CCG CC |
| **JH1–2** | GCA CCC TGG TCA CCG TCT CCT CAG GTG G |
| **JH3** | GGA CAA TGG TCA CCG TCT CTT CAG GTG G |
| **JH4–5** | GAA CCC TGG TCA CCG TCT CTT CAG GTG G |
| **JH6** | GGA CCA CGG TCA CCG TCT CCT CAG GTG G |

11. Amplify the linker sequence from a display vector such as pCANTAB 5b using the reaction protocol described in step 9 and the primers in Table 2. DNA from an existing library should be used as template with all combinations of JH and V\_\k/V\_\l primers. The cycling conditions are: 4 min at 94°C, 25 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C, and one cycle of 10 min at 72°C.

12. Analyze the PCR reactions by electrophoresis on 1.5% agarose in TAE buffer with ethidium bromide.

13. Excise the products from the gel and purify using a gel extraction system.

14. The heavy chain and light chain products are now assembled into V\_\text{H}-linker-V\_\text{L} constructs by overlap PCR to generate scFv sequences. To do this, mix 175 ng samples of the V\_\text{H} products with 60 ng of the linker products and 150 ng of the V\_\k or V\_\l products in a series of 25 μL reactions. Amplify by
PCR as described above using combinations of \(V_H\) and \(V_L\) or \(V_L\) primers that will anneal to the termini of the overlap construct (Table 1; for example, VH1a and JK1). The conditions for cycling are: 4 min at 94°C, 25 cycles of 1 min at 94°C, 1 min at 64°C, and 1 min at 72°C, and one cycle of 10 min at 72°C. Consider carefully the inclusion of appropriate control reactions that lack components of the template mixture and/or primers to confirm the specificity of the reactions.

15. Purify the assembled scFv products from agarose gels as described earlier.

3.2. Library Construction (see Note 3)

1. In order to clone the scFv products into a phage display vector, restriction sites need to be created. For the vectors systems used in our laboratory, \(NotI\) and \(SfiI\) sites are required; these are introduced to the scFv products using PCR with primers shown in Table 3. The choice of primer combinations should be guided by those used in overlap assembly (e.g., if VH1a and JK1 were used in scFv assembly, VH1a \(SfiI\) and JK1 \(NotI\) will be required at this point). The primer sequences could be adapted to create other sites for cloning; attention must be paid to reading frames.

2. Set up 25 \(\mu\)L reactions containing reaction buffer at 1x, dNTPs, primers (Table 3) at 10 pM, 200 ng of the purified scFv products, and 0.8 U of LongAmp \(Taq\) polymerase. Carry out the reaction using the following cycling conditions: 4 min at 94°C, 25 cycles of 1 min at 94°C, 1 min at 64°C, and 1 min at 72°C, and one cycle of 10 min at 72°C.

3. Purify the products as described above, and digest first with \(NotI\) according to the manufacturer’s recommendations.

4. Purify the treated DNA by agarose gel electrophoresis, and then digest with \(SfiI\) according to the manufacturer’s recommendations.

5. Gel-purify the digested scFv construct.

6. Prepare 100 ng of the display vector (e.g., pCANTAB 5b or pSyn2) with the endonucleases \(NotI\) and \(SfiI\), as above.

7. Ligate the scFv sequence with the vector. To do this, mix 150 ng of digested scFv with 250 ng of digested vector in a 40 \(\mu\)L reaction containing 1x T4 DNA ligase buffer, and 100 U T4 DNA ligase. Incubate for 4 h at room temperature.

8. Purify the ligation products from an agarose gel using water to elute the DNA.

9. Transform electrocompetent \(E. coli\) XL1-Blue bacteria. Place 50 \(\mu\)L of the bacterial suspension and 3 \(\mu\)L of scFv ligation product into an ice-cold 1.0 mm electroporation cuvette. Carry out electroporation under conditions appropriate for the apparatus
being used. For example, with the Bio-Rad Gene Pulser Xcell, conditions of 25 μF, 200 Ω, 1.8 kV, and 3 ms work well.

10. Grow the transformed E. coli cells in LB medium for 12–24 h at room temperature with shaking.

11. Once the culture has reached an absorbance at 600 nm of between 0.3 and 0.6, infect with 10^{13} pfu/mL of helper phage M13KO7.

12. Grow the culture in LB medium overnight at 30°C.

13. Centrifuge the culture at 2,000 × g at 20°C and recover the supernatant that now contains phage displaying the scFv.

### Table 3

Oligonucleotides used to introduce endonucleases restriction sites

| Sequence (5’ to 3’) | VH1a SfiI | VH2a SfiI | VH3a SfiI | VH4a SfiI | VH5a SfiI | VH6a SfiI | Jk1 NotI | Jk2 NotI | Jk3 NotI | Jk4 NotI | Jk5 NotI | Jk1.1 NotI | Jk2–3 NotI | Jk4–5 NotI |
|---------------------|-----------|-----------|-----------|-----------|-----------|-----------|----------|----------|----------|----------|----------|-----------|-----------|----------|
|                     | GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG GTG CAG TCT GG | GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTC AAC TTA AGG GAG TCT GG | GTC CTC GCA ACT GCG GCC CAG CCG GCC ATC GCC GAG GTG CAG CTG GTG GAG TCT GG | GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG CAG GAG TCG GG | GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG TTG CAG TCT GC | GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG CAG CAG TCA GG | GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT TTC CAC CTT GGT CCC | GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAG CTT GGT CCC | GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT ATC CAC TTT GGT CCC | GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAC CTT GGT CCC | GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT AAT CTC CAG TCG TGT CCC | GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT GAC CTT GGT CCC | GAG TCA TTC TCG ACT TGC GGC CGC ACC TAA AAC GGT GAG CTG GGT CCC | GAG TCA TTC TCG ACT TGC GGC CGC ACC TAA AAC GGT GAG CTG GGT CCC |
14. Isolate the phage from the culture supernatant by two rounds of precipitation with PEG/NaCl.
15. Resuspend the pellet of phage in TBS and titer by serial dilution and infection of *E. coli* XL1-Blue.

**3.3. Selection (see Note 4)**

1. To select anti-β₂-glycoprotein-I or aPT scFvs from the library, dissolve purified human β₂GP-I (7) or human prothrombin at 25 μg/mL in PBS.
2. Incubate aliquots of human β₂GP-I or human prothrombin in 17 × 100 mm polystyrene test tubes overnight at room temperature to coat.
3. Block the surface of the tubes with blocking buffer for 2 h at room temperature.
4. Add 10¹³ particles of phage displaying the scFvs to each tube and incubate for 1 h at 37°C.
5. Rinse the tubes three times with washing buffer.
6. Recover the binding phage with elution buffer.
7. Titrate the recoveries by serial dilution and infection to *E. coli* XL1-Blue and infect bacteria with the majority of the eluate.
8. Grow the bacteria, infect with helper phage, and repeat the selection four times, at each stage assessing the phage input and recovery.
9. At each round of selection, reduce the concentration of the target coated to the selecting tube by half (last round, 1.6 μg/mL) to drive the recovery of phage with high affinity toward the target.
10. After the fourth round of selection, pick single colonies from titration plates and grow the clones in 96 well culture plates containing 2× TY culture medium supplemented with glucose, ampicillin (200 μg/mL), and tetracycline (12.5 μg/mL).
11. When cultures reach an absorbance at 600 nm of 0.7, infect the bacteria in each well with M13K07 helper phage. Spin in a centrifuge fitted with a plate carrier to recover the supernatant and thereby obtain monoclonal stocks of phage displaying scFv antibodies.
12. Analyze the culture supernatants by monoclonal phage ELISA.

**3.4. Monoclonal Phage ELISA**

1. To determine phage specificities, coat ELISA plates with β₂GP-I or PT (both at 10 μg/mL) according to previously published methods (8).
2. Wash with PBS and block with blocking buffer.
3. Add 1 × 10⁹ monoclonal phage as prepared above and incubate for 1 h at 37°C.
4. Wash the plates three times with PBS.
5. To detect phage binding, add a peroxidase-conjugated anti-M13 antibody and incubate at room temperature for 1 h at 37°C.
6. Wash three times with PBS.
7. Add ELISA substrate and allow the reaction to proceed at 37°C. Check regularly.
8. Stop the reaction with acid stop buffer and read the absorbance at 490 nm in an ELISA reader.

4. Notes

1. Further guidance on the execution of methods described herein can be gained from cross-referencing other chapters in this volume.
2. For additional insights to the recovery of the antibody repertoire (Subheading 3.1), refer to Chapters 3, 4, and 6.
3. Further guidance on library construction (Subheading 3.2) is also available from these chapters along with material presented in Chapter 2.
4. Chapter 7 provides complementary material on the topic of selection (Subheading 3.3).

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Chapter 6

The Generation and Selection of Single-Domain, V Region Libraries from Nurse Sharks

Martin F. Flajnik and Helen Dooley

Summary

The cartilaginous fish (sharks, skates, and rays) are the oldest phylogenetic group in which a human-type adaptive immune system and immunoglobulins (Igs) have been found. In addition to their conventional (heavy-light chain heterodimeric) isotypes, IgM and IgW, sharks produce the novel isotype, IgNAR, a heavy chain homodimer that does not associate with light chains. Instead, its variable (V) regions act as independent, soluble units in order to bind antigen. In this chapter, we detail our immunization protocol in order to raise a humoral IgNAR response in the nurse shark (Ginglymostoma cirratum) and the subsequent cloning of the single-domain V regions from this isotype in order to select antigen-specific binders by phage display.

Key words: Cartilaginous fish, Shark, Immunoglobulin, IgNAR, Variable region, Single domain

1. Introduction

The cartilaginous fish (sharks, skates, and rays) are the oldest phylogenetic group in which a human-type adaptive immune system and immunoglobulins (Igs) have been found. In addition to their conventional (heavy-light chain heterodimeric) isotypes, IgM and IgW, sharks produce the novel isotype, IgNAR, a heavy chain homodimer that does not associate with light chains. Instead, its variable (V) regions act as independent, soluble units in order to bind antigen (1–3). In the nurse shark Ginglymostoma cirratum, IgNAR V regions can be classified into one of two highly similar groups, termed type I and II, based upon the number and location of their non-canonical cysteines (cys). Type I V regions generally
have an even number of cys (usually two) in complementarity determining region (CDR) 3 and one each in framework regions (FR) 2 and 4. Type II V regions have two non-canonical cys, one in CDR1 and one in CDR3 (1, 2). Crystal structures obtained for representatives of the two types revealed that the differential placement of these non-canonical cys induces very different CDR3 conformations, and, consequently, remarkably different binding site topologies in the two types. In type I V regions, the CDR3 is, on average, much longer than those found in type II (4) and is seen to “drape” over the outside of the FR, where it is fixed by two disulfide bonds. This generates a deep groove between CDR1 and the tips of the CDR3 loop where antigen can be bound. In type II V regions, the shorter CDR3 is tethered to CDR1 by a disulfide bond and protrudes from the top of the V region, where it can bind antigen (5–7).

The IgNAR V region primary repertoire is entirely CDR3 based. However, the use of three D regions, along with their four rearrangement events and unusually extensive N additions, provide the primary repertoire with unprecedented variability in CDR3 length and amino acid composition. Additional diversity is then introduced through extremely high levels of antigen-driven somatic hypermutation (8, 9). Our work has shown that immunization of adult nurse sharks results in the production of antigen-specific IgNAR as an integral part of the shark humoral response (10). We have also shown that it is possible to use polymerase chain reaction (PCR) to amplify IgNAR V regions from the immunized animals with V region-specific primers, clone the V repertoire into a phagemid vector, and subsequently use standard phage display selection methods to enrich for antigen-specific V regions. The V regions thus isolated have been shown to be highly antigen specific, generally of high affinity (nanomolar), small in size (~12 kDa), and extremely stable at nonphysiological temperatures (3, 11).

In this chapter, we detail our immunization protocol in order to raise an antigen-specific, humoral IgNAR response in nurse sharks and the subsequent isolation of peripheral blood lymphocytes (PBLs) from these animals. We also detail our methods to enable cloning of the single-domain V regions of this isotype and for the subsequent selection of antigen-specific Vs by phage display.

2. Materials

2.1. Shark Immunization and Isolation of PBLs

1. Tricaine methanesulfonate (MS222) (Argent Laboratories). Warning: potentially carcinogenic. Wear gloves and appropriate protective clothing.

2. Porcine heparin.
3. Shark phosphate-buffered saline (PBS): 100 mL of 10× mouse PBS (1.38 M sodium chloride, 0.027 M potassium chloride, and 0.1 M phosphate, pH 7.4), 100 mL of 3.5 M urea, 10 mL of 5 M NaCl, and water to 1 L.

2.2. Preparation of Total RNA from PBLs

1. TRIzol (Invitrogen). Warning: contains phenol—take appropriate precautions.
2. Chloroform (molecular biology grade).
3. Isopropanol (molecular biology grade).
4. Diethylpyrocarbonate (DEPC)-treated water (Quality Biologicals). Alternatively, add 0.1% (v/v) DEPC to water and incubate overnight at 37°C. Autoclave twice at 121°C and 15 psi to inactivate any residual DEPC.
5. DEPC–ethanol (75% [v/v]): mix 37.5 mL molecular biology grade absolute ethanol with 12.5 mL DEPC-treated water in a 50 mL tube.

2.3. cDNA Production and Amplification of IgNAR V Regions

1. Ready-to-go reverse transcriptase (RT)-PCR beads (GE Healthcare).
2. PCR primers:
   - NAR F1 Rev: 5′-ATA ATA AGG AAT TCC ATG GCT CGA GTG GAC CAA ACA CCG-3′.
   - NAR F4 For1: 5′-ATA ATC AAG CTT GCG GCC GCA TTC ACA GTC ACG GCA TTC ACA GTC ACG ACA GTG CCA CCT C-3′.
   - NAR F4 For2: 5′-ATA ATC AAG CTT GCG GCC GCA TTC ACA GTC ACG ACA GTG CCA CCT C-3′.

   Figure 1 illustrates how the NAR F4 For and NAR F1 Rev primers were designed. Changes should be made to the restrictions sites and/or handling if cloning into a plasmid other than pHEN2.
3. Taq DNA polymerase.
4. Reagents and apparatus for agarose gel electrophoresis.
5. QIAquick reagents for isolation of DNA from agarose gels (QIAden).
6. Restriction enzymes NeoI and NcoI.

2.4. Library Construction

1. Phage display vector pHEN2.
2. T4 DNA ligase.
3. Phenol:chloroform for extraction and absolute ethanol for DNA precipitation.
4. Escherichia coli TG1 supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM) 5 (rK− mK−) [F′ traD36 proAB lacIq ZΔM15] (Stratagene).
5. 2× TY media: 16 g/L tryptone, 10 g/L yeast extract, and 5 g/L sodium chloride. Autoclaved.
6. 2× TY/G1%: Sterile 2× TY medium supplemented with sterile glucose to 1% (w/v).

7. TYE agar: 10 g/L tryptone, 5 g/L yeast extract, 8 g/L sodium chloride, 20 g/L agar. Autoclaved.

8. TYE/A100/G2%: Sterile TYE agar supplemented with ampicillin to 100 mg/mL and sterile glucose to 2% (w/v).

9. Primers:
   - LMB3: 5’-CAG GAA ACA GCT ATG AC-3’.
   - pHEN Seq: 5’-CTA TGC GGC CCC ATT CA-3’.

10. 2× TY/A100/G2%: Sterile 2× TY medium supplemented with ampicillin to 100 µg/mL and sterile glucose to 2% (w/v).

11. Sterile glycerol.

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2.5. Phage Display and Selection

1. M13KO7 helper phage (GE Healthcare). Store at 4°C.

2. 2× TY/A100/K50/G0.2%: Sterile 2× TY medium supplemented with ampicillin to 100 µg/mL, kanamycin to 50 µg/mL, and sterile glucose to 0.2% (w/v).

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Fig. 1. (a) Germline translations for type I and type I V regions from the nurse shark. Fr1 and Fr4 sequences used for primer design are shown in bold. Stars indicate CDR3; (b) illustrates how the NAR F4 For and NAR F1 Rev primers were designed. Primer anchor sequences are shown in bold. Restriction sites engineered into the handles to allow cloning into pHEN2 are underlined. Direction of amplification is shown by the arrowheads.
3. Methods

3.1. Shark Immunization and Isolation of PBLs

1. Prior to immunization or bleeding, remove the animals from their tank and place them into a smaller container containing artificial seawater, in which MS-222 has been dissolved, in order to anesthetize them (see Note 1). Place the animals in the solution until the desired level of narcosis is attained then remove them for immunization or bleeding.

2. Our current standard protocol is to immunize adult sharks (longer than 3 ft in length) initially with one shot of antigen (~250 μg) emulsified in complete Freund’s adjuvant in the lateral fin. Deliver the immunization just under the skin with a 20-gauge needle, spreading the antigen across the fin with the fingers so it lies evenly under the skin.

3. Boost at 4-week intervals intravenously into the caudal vein (on the underside of tail) as soluble antigen (~250 μg) in PBS (sample filtered through a 0.45-μm porosity filter system) with a 20-gauge needle. Using this method, we usually see an IgNAR response after three to four immunizations.

4. Harvest blood samples from the caudal vein with an 18-gauge needle fitted to a 30 mL syringe containing ~200 μL of a filtered stock solution of porcine heparin (1,000 U/mL in shark PBS).

5. After treatment, return the sharks to their original tank and resuscitate by flushing the gills with seawater until they are able to swim unaided.

6. Spin blood samples at 300 × g for 10 min to separate the buffy coat, which can then be carefully removed with a 1 mL pipette.
If the sample is excessively contaminated with red cells, re-spin the samples in a microcentrifuge at low speed for 5 min and repeat the isolation of the buffy coat.

3.2. Preparation of Total RNA from PBLs

1. Place the isolated buffy coat into a clean 50 mL conical-bottom tube, add 5 mL of TRIzol, and homogenize by pipetting up and down or using a power homogenizer. Incubate the sample at room temperature for 5 min to permit complete dissociation of nucleoprotein complexes.

2. Add 1 mL of chloroform, cap the samples securely, and shake the tubes vigorously by hand for 15 s. Incubate at room temperature for a further 2–3 min prior to spinning at 12,000 × g for 50 min at 4°C.

3. After centrifugation, remove the upper (colorless) layer, being careful not to interrupt the interface, and transfer to a fresh tube.

4. Precipitate the RNA from the sample by mixing with 2.5 mL isopropanol that has been warmed to room temperature, and incubate at room temperature for 10 min. Spin out the RNA by centrifugation at 12,000 × g for 30 min.

5. Remove the supernatant and wash the gel-like pellet once with 1 mL of ice-cold 75% DEPC–ethanol. Centrifuge at 7,500 × g for 5 min at 4°C.

6. Aspirate the ethanol and briefly air-dry the pellet (5–10 min at room temperature). Be careful to avoid over-drying the sample.

7. Once dry, resuspend the pellet in DEPC-treated water (see Note 2), then incubate at 55–60°C for 10 min. Measure the absorbance of the sample at 260 and 280 nm to assess yield and purity, and store at −80°C until ready for use (see Note 3).

3.3. cDNA Synthesis and Amplification of IgNAR V Regions

For first-round cDNA synthesis, we favor the use of RT-PCR beads that contain the reverse transcriptase, nucleotides, thermostable DNA polymerase, and buffer components for combined reverse transcription and amplification (see Note 4):

1. Reconstitute beads in 45 μL of DEPC-treated water and incubate on ice until the bead is completely dissolved.

2. To each tube, add 1 μg RNA and 1 μL NAR F4 For1 and F4 For2 primer mix (see Note 5) at 25 μM. Gently flick tubes to mix contents, and incubate on a prewarmed PCR block at 45°C for 30 min.

3. After first-strand cDNA synthesis, incubate tubes at 95°C for 7 min to inactivate the reverse transcriptase and denature the template.

4. To enable amplification, add 1 μL of the common primer NAR F1 Rev (see Note 6) at 25 μM. This primer contains an
Neol site in its handle to enable cloning of the amplified products into pHEN2, our favored phage display vector. Preheat the reactions to 95°C and add an additional 1 μL of Taq DNA polymerase at 1 U/μL before cycling 32 times at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 s.

5. After amplification, run the amplification reactions on a 1.5% agarose gel. Excise the 400-bp V region product and recover the DNA using QIAquick reagents. Similar purification systems may be used.

6. Incubate the isolated DNA with the restriction enzymes Neol and NotI to cut at the sites included in handles incorporated into the primers used for amplification. Digest overnight at 37°C then repeat the agarose gel purification as above.

3.4. Library Construction

1. Prepare the phagemid vector pHEN2 by digestion of purified vector DNA with Neol and NotI. Gel-purify the vector as described in Subheading 3.3, step 5 (pHEN2 is derived from pHEN1 (12); see Notes 7 and 8). To quantify vector and insert prior to ligation, run 2 μL of each DNA sample on a 1% agarose gel with a suitable DNA marker. Band intensities are evaluated by eye to judge relative amounts of DNA present.

2. Perform ligations with equal amounts of vector and insert DNA in the presence of 2.5 μL of 10× ligase buffer and 1 μL of T4 ligase. Make up the final volume to 25 μL with sterile water and incubate overnight at 15°C. For library construction, 30–40 such ligations are generally performed.

3. After incubation, pool the ligation reactions, extract with phenol:chloroform, and precipitate the DNA with ethanol according to standard methods. Reconstitute the resultant DNA pellet in approximately 100 μL of molecular biology-grade water, prior to transformation as 10 μL aliquots into electrocompetent E. coli TG1 cells (see Note 9).

4. After electroporation, resuspend the cells in 100 μL ice-cold 2× TY/G1%. Sample aliquots from each transformation and prepare serial 100-fold dilutions in 2× TY/G1%. Plate dilutions of 10⁻², 10⁻⁴, and 10⁻⁶ from each transformation on TYE/A100/G2%. Plate the remaining bacterial suspensions onto 140 mm petri dishes containing TYE/A100/G2%. Incubate all plates overnight at 30°C.

5. After incubation, count colonies from the dilution plates to estimate the final size of the library.

6. Colonies can be PCR screened to estimate the percentage insert and/or sequenced to assess diversity using the pHEN2 vector-specific primers LMB3 and pHEN seq (see Note 10).

7. To store the library, scrape colonies from the agar plates using a sterile spreader into a final volume of 10 mL of 2× TY/A100/G2% medium. Combine the cells suspensions with sterile
glycerol to a final concentration of 20% (v/v), and, after thorough mixing, aliquot in 500 μL unit shots for flash-freezing and storage at −80°C.

### 3.5. Phage Display and Selection

1. Thaw a single aliquot of library stock on ice. Add the stock to 200 mL of prewarmed 2× TY/A100/G2%. Grow at 37°C with shaking at 250 rpm until log phase (OD₆₀₀ 0.4–0.8) is reached (typically 1–3 h).

2. Take 50 mL of the culture to a fresh flask and add a total of approximately 1 × 10¹⁵ M13KO7 helper phage. Incubate at 37°C for 30 min without shaking to allow viral infection to take place.

3. After incubation, spin the culture at 2,000 × g at 4°C for 10 min and resuspend the cell pellet in 100 mL of 2× TY/A100/K50/G0.2%. Incubate overnight at 30°C with shaking at 250 rpm to allow library expression and rescue.

4. Spin the overnight culture at 2,000 × g at 4°C for 20 min and recover 80 mL of the supernatant. Add to 20 mL of PEG/NaCl, mix well, and incubate on ice for at least 1 h to precipitate phage displaying the IgNAR V regions. Pellet the phage by centrifugation at 2,000 × g at 4°C for 20 min. The supernatant can be discarded; resuspend the phage pellet in 2 mL PBS. Spin the phage suspension at top speed in a microfuge for 10 min to remove any remaining bacterial debris. Retain the supernatant. The phage stock can be stored short term at 4°C or, after the addition of sterile glycerol to 20% (v/v), at −20°C for the longer term.

5. To prepare for selection, coat Maxisorp Immunotubes with the antigen(s) of interest (see Note 11) in 4 mL PBS for 2 h at room temperature. Wash the tubes twice with PBS before blocking with MPBS overnight at 4°C (see Note 12). Selection is conducted by incubating each coated, washed, and blocked immunotube with 1 mL of phage stock in 3 mL of 2% MPBS. Seal and mix for 1 h at room temperature using an over-and-under tumbler. Incubate for a further hour without tumbling before discarding the supernatant containing unbound phage.

6. Wash the immunotubes with PBST (see Note 11), drain off excess liquid, and elute the bound phage by adding 1 mL of fresh 100 mM TEA. Return the tube to the tumbler for a maximum of 10 min then recover the phage solution and neutralize by mixing with 500 μL of 1 M Tris–HCl. Add a further 200 μL of Tris–HCl to the immunotube, rotate to coat the immunotube, and then pool with the rest of the recovered phage.

7. Grow *E. coli* TG1 into log phase and add 15 mL of the bacterial culture to 750 μL of the TEA-eluted phage and incubate at 37°C without shaking for 30 min.
8. Prepare serial 100-fold dilutions from an aliquot of the infected culture in 2× TY and plate on TYE/A100/G2% plates to assess the number of phage recovered from the first round of selection.

9. Centrifuge the remaining infected culture for 10 min at 2,000 × \( g \), resuspend in 100 μL of 2× TY, and plate on a 140 mm petri dish containing TYE/A100/G2%. Incubate the plates overnight at 30°C.

10. After overnight growth, scrape colonies from the large petri dishes into 2 mL of 2× TY medium with a sterile scraper and mix the suspension thoroughly. Use 50 μL to inoculate 50 mL of 2× TY/A100/G2%. Mix the remaining bacterial suspension with glycerol to a final concentration of 15% (v/v) and store at −80°C as a stock.

11. Incubate the 50 mL culture at 37°C with shaking at 250 rpm until the \( \text{OD}_{600} \) reaches 0.4. Remove 10 mL, add approximately 1 × 10^{10} helper phage, and incubate without shaking for 30 min at 37°C. Spin at 2,000 × \( g \) for 10 min and resuspend the resultant cell pellet in 50 mL of 2× TY/A100/K50/G0.2%. Incubate overnight at 30°C with shaking (250 rpm).

12. Centrifuge the overnight culture at 2,000 × \( g \) for 10 min and recover 40 mL of the supernatant. Add this to 10 mL of PEG/NaCl and mix well prior to incubation on ice for at least 1 h to allow precipitation of the phage. Recover the virus by centrifugation and, again, resuspend in 2 mL of PBS. Spin as before to remove any remaining bacterial debris. Store the phage at 4°C for the short term.

Further rounds of selection are carried out on antigen-coated immunotubes with phage rescued from the previous round of selection, as above. Once completed, the results of selection can be analyzed by polyclonal and monoclonal phage enzyme-linked immunosorbent assay (ELISA) using an HRP-conjugated anti-M13 monoclonal to detect the binding of IgNAR V regions displayed on phage to the antigen(s) of interest.

4. Notes

1. A very wide range of dilutions of MS-222 is reported for anesthesia and its action is highly dependent upon water temperature and quality, in addition to the species of fish and their size. We generally use approximately 0.1% (w/v) in artificial seawater, but the smallest dose that gives the required level of anesthesia should be identified. The methods described in this chapter can also be used with other shark species to generate IgNAR V region
libraries; however, the antigen dose and the PCR primer anchor sequences may need to be optimized according to the species of fish under investigation. Similar anesthesia and PBL preparation methods have also been utilized in bony fish (not known to have the IgNAR isotype) but are beyond the scope of this chapter.

2. When resuspending the RNA, start with 20 μL DEPC-treated water and keep adding in 20-μL aliquots until the RNA pellet is completely dissolved. This should ensure that the minimum volume of DEPC-treated water is used.

3. In a few instances, we have detected a high number of frameshifted clones in the final library or clones carrying stop codons. We believe these sequences are amplified from contaminating genomic DNA carrying nonfunctionally rearranged V region sequences. Usually this is not a major problem because these clones should be eliminated during selection, but to reduce their occurrence and improve the functional size of libraries, RNA can be treated with RNase-free DNase prior to RT-PCR.

4. We use RT-PCR beads simply due to their convenience but any commercially available reverse transcriptase can be employed.

5. The primers NAR F4 For1 and F4 For2 were originally designed to anneal to the FR 4 of type I and II IgNAR V regions, respectively. These sequences differ from one another by a few base pairs. Subsequent work has shown that both primers will anneal to both IgNAR V region types, so we now mix the primers in a 1:1 molar ratio and use this primer mixture for first-strand cDNA synthesis. Both of these primers have a NotI site incorporated into their handles to allow subsequent cloning into the phagemid vector, pHEN2.

6. The primers used for the amplification of IgNAR V regions are designed to enable easy cloning of amplicons into the plasmid pHEN2 utilizing the incorporated NeoI and NotI sites. If a different phage display vector is used, the handles of the primers may need to be altered to incorporate the required restriction sites and to ensure that inserts are ligated into the correct reading frame.

7. NotI cuts linear DNA more efficiently than circular DNA. Therefore, NeoI is added first to the plasmid DNA and incubated for 1 h at 37°C to generate a linearized plasmid that can be rapidly cleaved by NotI, which is added thereafter.

8. For library construction, we never treat digested vector with calf alkaline phosphatase. To ensure all plasmid is properly digested with NeoI and NotI, we ligate 2 μL of vector DNA overnight in the absence of insert. We then transform the mixture into electroporation-competent cells. In this way we can evaluate the background arising from the presence of uncut
vector DNA or the re-ligation of vector DNA cut at a single site. If backgrounds are deemed too high, the digestion of vector DNA is repeated.

9. For transformation, 50 μL samples of electrocompetent *E. coli* TG1 cells are thawed on ice, mixed with 10 μL of ligation solution, pipetted into a chilled 0.2 cm electroporation cuvette, and pulsed at 2.5 KV, 200 Ω, and 25 μFd. We purchase electrocompetent cells from Stratagene for convenience and their very high transformation efficiency (~1 × 10^10 cfu/μg pUC18). Electrocompetent cells can be prepared using methods described by Sambrook et al. (13) and elsewhere in this volume. In brief, bacteria are grown to mid-log phase, chilled, and repeatedly washed with a low-salt buffer to reduce the ionic strength of the cell suspension. Cells are finally resuspended in small volumes (2–3 mL) of 10% (v/v) glycerol, flash-frozen, and stored at −80°C. Cells prepared this way should have a transformation efficiencies in the order of 10^8 cfu/μg pUC and can be stored for approximately 6 months.

10. We pick individual colonies straight from dilution plates into a PCR mix using sterile toothpicks then cycle as follows: 3 min at 95°C; 25 cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 72°C; and 3 min at 72°C.

11. We generally begin panning by coating immunotubes with antigen in the concentration range of 10–100 μg/mL and washing ten times with PBST before phage elution. To provide increasingly stringent selection, and thus increase the chance of obtaining high-affinity clones, we decrease the concentration of antigen 10- to 100-fold and increase the number of PBST washes by 5 cycles to 10 cycles with each round of panning. If fewer than 100 colonies are obtained after a selection, we repeat the selection with slightly less stringency.

12. Blocking of the immunotubes can be shortened by incubating for 2 h at room temperature, however, we find that nonspecific binding is significantly reduced by blocking overnight.

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The Isolation of scFvs Against Small Target Molecules

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Summary

Phage display has the capacity to rapidly isolate recombinant antibodies against protein targets and other molecules of significant size. However, there is no obvious lower limit to the power of the selection methods: this chapter describes how the techniques of phage display can be adapted to allow the isolation of antibodies against very small compounds. Antibodies generated in this way have many uses including the detection and quantitative analysis of the target chemical moiety in samples such as foods, water, and body fluids.

Key words: Carriers, Conjugation chemistry, Biacore, Hapten, Pesticide, Analyte

1. Introduction

Immunoassays are widely used for the analysis of low molecular weight compounds or haptens in biological samples, and are often the only viable analytical method available. The performance of these assays is critically dependent on the characteristics of the antibody used, and the identification of suitable antibodies is often a major hurdle in assay development. Although the main drive in the development of recombinant antibody technologies has been the desire to develop novel therapeutic antibody activities suitable for clinical applications, the production of anti-hapten antibodies for diagnostic applications can benefit considerably from the recombinant approach. Recombinant antibody technologies provide access to a much greater repertoire of antibody activities than traditional antibody production techniques, allowing selection of antibodies with characteristics that are critical for optimal
assay performance. They provide a much faster route for identification of the antibody of interest, and the means for continuous supply of the antibody. They also facilitate the modification or manipulation of the antibody for improved compatibility with novel assay platforms.

The vast majority of hapten immunoassays use the competitive assay design, in which the analyte competes with a labeled or immobilized version of the target analyte for binding to limited amounts of the antibody. Two-antibody reagent excess or sandwich assays are not commonly used for haptens because haptens are generally too small to bind to two antibodies simultaneously. In a competitive immunoassay, the lower limit of detection that can be achieved is directly influenced by the affinity of the antibody for its antigen. Hence, the higher the affinity of the antibody, the lower is the detection limit that can be theoretically achieved. As the rates of association of antibodies and antigens do not vary enormously, the rate of dissociation or the off-rate of the antigen is particularly important in determining assay performance (1, 2). Antibody specificity is also critical for optimal hapten assay performance. It is often the case that the biological sample being analyzed for hapten contains compounds that are structurally very similar to the target hapten and might also bind to the antibody. These irrelevant compounds might even be in much higher concentration than the target compound, as, for example, the concentration of dehydro-epiandrosterone sulfate in human serum samples for testosterone analysis (3). Alternatively, it may be desirable to measure a family of closely related structures simultaneously, in which case, an antibody with broad specificity would give optimal assay performance.

Anti-hapten recombinant antibodies that were first described were derived from preexisting hybridomas; now, these reagents are mostly isolated from immune antibody libraries, i.e., antibody libraries generated from the immune cells of hyperimmunized animals. Thus, affinity maturation of the antibody takes place in vivo and the chances of isolating the high-affinity antibodies developed are increased by eliminating the risky and time-consuming fusion process. Also, the immune repertoire of almost any species can be tapped, even those where hybridoma technology has not been described (e.g., chicken, llama), is not freely available (e.g., rabbit), or is not very robust (e.g., sheep). Naïve libraries have been used for isolation of anti-hapten antibodies, but they generally yield lower-affinity antibodies than are needed for sensitive assay development. In theory, it is possible to isolate high-affinity antibodies from naïve libraries but, in practice, an in vitro affinity maturation step is often required. A synthetic library has been described that was constructed on the basis of existing information on the structure of the antigenic site of anti-hapten antibodies (4). This may prove a useful starting point for isolation of anti-hapten antibodies.
The simplest and most widely used antibody libraries are scFv libraries displayed on the surface of filamentous phage. Fab libraries have also been used for isolation of anti-hapten antibodies. The same principles apply to the panning process regardless of the nature of the binder. As haptens are small molecules, they fit inside the antigen-binding site of an antibody. Hence there are no avidity effects on binding of free hapten to antibodies or antibody fragments, regardless of their valency. However, when libraries are panned on solid phase, avidity effects may come into play if the haptens are immobilized in close proximity on the solid surface and if the phage particles display more than one copy of a monovalent antibody fragment, or divalent/trivalent antibody fragments. These avidity effects can make it more difficult to isolate high-affinity binders and should be avoided. Thus, phagemid vector systems are recommended because resulting phage particles display only one copy of the recombinant antibody (on average). When using scFv systems, a standard long linker (16 amino acids) between the two variable domains is preferred, as this favors the formation of monomeric scFv units.

Haptens are by definition not immunogenic on their own and must be conjugated to a carrier protein for use in immunizations. There is a great body of literature on the importance of conjugation procedure for the outcome of the immunization. If a highly specific antibody is desired, the point of attachment of the hapten to the carrier protein must be selected so that a unique chemical feature of the hapten faces away from the carrier protein. Alternatively, if broad specificity is required, a feature of the hapten that is common to all members of the group should be accessible to the immune system. The need for a bridging group must also be considered so that the hapten is held away from the surface of the protein and can fit into the antigen-binding site of the antibody. But a word of caution, bridging groups can also be immunogenic and may give rise to antibodies that bind the conjugate but not the free hapten.

The same factors must also be considered when preparing hapten for panning experiments. If panning is to be carried out on a solid phase, the hapten must be immobilized onto the solid phase by covalent attachment or by adsorption of a hapten-protein conjugate and care must be taken with the point of attachment and with the use of bridging groups. If an immune library is to be panned, it is recommended that a different carrier protein and/or a different bridging group and/or different conjugation chemistry is used for immobilization of the hapten than was used in preparation of the immunogen. This should help avoid selection of antibody fragments that bind the carrier protein or bridging group. If panning is carried out in solution phase, it will be necessary to label or tag the hapten (e.g., through biotinylation) and similar considerations will be necessary.
This chapter describes the procedure commonly used for solid-phase panning in and elution from microtubes and also the less commonly used Biacore-based panning (Fig. 1). It also gives the protocol for solution-phase panning, which is recommended if suitable tagged haptens are available. Conjugation and tagging of haptens are not discussed, because of the great diversity of the chemical structures of haptens, and readers are referred to some excellent textbooks on the subject of conjugation (6).

2. Materials

1. Immunotubes (Nunc).
2. Hapten of interest conjugated to a suitable carrier protein or tag (bovine serum albumin [BSA], ovalbumin [OVA], biotin, etc.).
3. Coating buffer: 35 mM NaHCO₃, 15 mM Na₂CO₃, pH 9.6.
4. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate, pH 7.4.
5. PBS containing Tween 20 at 0.01 or 0.1% (v/v), designated as PBST at the higher concentration.
6. Carrier protein (BSA or OVA).
7. scFv phage display library of known titer.
8. Tris-buffered saline (TBS): 150 mM NaCl and 20 mM Tris-HCl, pH 7.4.
9. TBS containing 0.05% (v/v) Tween 20 (TBST).
10. Dynabeads M-280 magnetic streptavidin beads (Invitrogen).
11. Elution buffers: 100 mM triethylamine or 100 mM glycine–HCl, pH 2.2. Elution buffers are best prepared fresh on day of use and unused buffer discarded.
12. Neutralization buffer: 1.5 M Tris–HCl, pH 8.8.
13. Unconjugated hapten.
14. Anti-hapten antibody.
15. CM5 sensor chips (Biacore).
16. Amine-coupling kit containing N-hydroxy-succinimide (NHS), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC), ethanolamine hydrochloride, and 10 mM sodium acetate, pH 4.5 (Biacore).
17. Hapten of interest in carboxylated form.
18. Dimethylformamide (DMF).
19. Regeneration buffer: 100 mM NaOH.
20. HBS-EP: 150 mM NaCl, 10 mM Hepes (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid), 3 mM EDTA, and 0.005% surfactant P20, pH 7.4 (Biacore).
21. *Escherichia coli* XL1Blue (Stratagene). Carries a tetracycline-resistance gene.
22. LB agar plates (10 g/L tryptone, 5 g/L NaCl, 5 g/L yeast extract, and 10 g/L agar) supplemented with antibiotics as appropriate.
23. Antibiotic stocks: 100 mg/mL carbenicillin (dissolve in water and sterilize through a 0.22 μm filter), 50 mg/mL kanamycin (dissolve in water and sterilize through a 0.22 μm filter), 20 mg/mL tetracycline (in 100% ethanol).
24. Superbroth medium: 10 g/L 3-(N-morpholino) propanesulfonic acid (MOPS), 30 g/L tryptone, and 20 g/L yeast extract, pH 7.0.
25. VCSM13 helper phage (Stratagene). Carries a kanamycin-resistance gene.
26. 5× PEG/NaCl: 20% (w/v) polyethylene glycol (PEG) 8000, and 2.5 M NaCl.
27. Maxisorp enzyme-linked immunosorbent assay (ELISA) plates (Nunc).
28. ELISA wash buffer: 150 mM NaCl and 0.05% (v/v) Tween 20.
29. Tissue paper.
30. PBS/BSA: PBS with 0.1% (w/v) BSA.
31. Horseradish peroxidase (HRP)-conjugated anti-M13 monoclonal antibody (Amersham Bioscience).
32. Tetramethyl benzidine (TMB) peroxidase substrate (DAKO).
33. 1 M H₂SO₄.
34. 96 well tissue culture plates (high capacity; Qiagen).

3. Methods

3.1. Solid-Phase Panning on Immunotubes

1. Coat the immunotube by adding 1 mL of hapten-OVA conjugate (see Note 1) at 1 µg/mL in coating buffer and incubating overnight at 4°C with constant agitation on a rotating platform (see Note 2).
2. Discard tube contents and wash three times with PBS/0.01% Tween 20 by filling the tube to the top and then removing immediately.
3. Block the tube with 4 mL of PBS containing 3% (w/v) OVA (see Note 1) for 1 h at room temperature.
4. While the tube is blocking, dilute the phage library in 1.5% OVA/1.5% BSA to a concentration of 2 × 10¹¹ cfu/mL (see Note 1) and incubate at room temperature for 1 h. This should reduce nonspecific binding of phage to the conjugating protein.
5. Pour the blocking solution from the immunotube and blot dry on tissue paper. Add the phage suspension (2 × 10¹¹ phage) to the tube and incubate for 1 h at room temperature (see Note 2) with constant agitation.
6. Discard the phage solution and wash the tube five times with PBST and then five times with PBS (see Note 2).
7. Elute bound phage from the panning tube as described in Subheading 3.3.

3.2. Solution Phase Selection

1. Prepare an aliquot of 2 × 10¹¹ cfu of the phage library in 200 µL TBST containing 3% (w/v) BSA. Add to a polypropylene tube followed by an equal volume of biotinylated hapten diluted in TBST (see Note 2).
2. Incubate for 1 h at room temperature with constant agitation (see Note 2).

3. Meanwhile wash 100 μL (approximately 10^7 beads) Dynabeads M-280 magnetic streptavidin beads three times with TBST following the manufacturers instructions. To block the beads, resuspend them in 100 μL TBST with 3% (w/v) BSA and leave at room temperature for 30 min.

4. Remove the buffer from the beads using a magnetic capture device and add the phage-hapten mixture from step 1. Incubate for 30 min at room temperature with constant agitation (see Note 3).

5. Wash the beads five times with TBST and then five times with TBS (see Note 2).

6. Elute bound phage from the magnetic beads as described in Subheading 3.3.

3.3. Elution of Bound Phage

3.3.1. Nonspecific Elution with Triethylamine or Glycine (see Note 4)

1. Add 1 mL of either 100 mM triethylamine or 100 mM glycine-HCl to the washed selection tube/Dynabeads and incubate for 10 min at room temperature with constant agitation (see Note 5).

2. Immediately transfer the contents of the tube into a fresh tube containing 200 μL neutralization buffer.

3. Eluted phage should now be titrated (Subheading 3.5) and amplified for subsequent rounds of panning (Subheading 3.6). An aliquot should be retained after amplification for ELISA screening (Subheading 3.7).

3.3.2. Specific Elution with Free Hapten

1. Add 1 mL of unconjugated “free” hapten in PBS to the washed panning tube and incubate at room temperature to elute the bound phage (see Note 6 for a discussion of appropriate concentrations of hapten).

2. Transfer eluted phage to a clean tube for titration and amplification for subsequent rounds of panning.

3. Bound phage may also be subsequently eluted using one of the nonspecific methods above (Subheading 3.3.1).

3.3.3. Specific Elution with Free Anti-hapten Antibody

1. Add 1 mL of existing anti-hapten antibody (whole antibody, Fab, or scFv) in PBS to the panning tube and incubate at room temperature to elute bound phage with lower affinity than existing antibody (see Note 7).

2. Transfer eluted phage to a clean tube for titration and amplification for subsequent rounds of panning.

3. Phage that remain bound to the selecting surface may then be eluted using one of the nonspecific methods described above (Subheading 3.3.1).
Selection of phage-displayed antibodies based on their kinetic behavior at each round of biopanning can enable high-affinity antibodies to be easily identified (7). By using the surface immobilization and fluidic capabilities of Biacore biosensors and instruments with a sample recovery facility and capturing those antibodies with a naturally long off-rate, it is possible to select phage displaying high-affinity antibodies without the need for harsh elution reagents that may have undesired effects on phage stability.

1. Activate the surface by mixing EDC and NHS solutions from the amine-coupling kit in the ratio 1:1 and depositing 50 μL of the mixture on the surface of the sensor chip for 20 min.
2. Repeat step 1.
3. Prepare the amine surface by placing 50 μL of 1 M ethylenediamine, pH 8.5, in contact with the activated surface for 1 h.
4. Deactivate remaining activated groups by placing 50 μL of 1 M ethanolamine on the surface for 20 min.
5. Dissolve 1 mg carboxylated hapten in 250 μL of DMF and mix with 225 μL of 10 mM sodium acetate, pH 4.5, containing 5 mg of EDC and 2 mg of NHS.
6. Place 50 μL of this solution in contact with the amine surface for 2 h.
7. Place the coated chip into the instrument and condition the immobilized surface with repeated injection of 25 μL of regeneration buffer to remove any noncovalently bound material.
8. Equilibrate the coated chip with HBS-EP at a flow rate of 10 μL/min until a steady baseline is observed on the Biacore instrument.

1. Dilute the phage library to $2 \times 10^{11}$ cfu/mL in HBS-EP.
2. Pass diluted phage library (40 μL) over the chip surface at a flow rate of 1 μL/min.
3. Rinse unbound phage off by passing HBS-EP over the surface at the same flow rate.
4. Dissociate bound phage by continuous flow of HBS-EP over the chip surface for up to 24 h. Collect aliquots of the eluate at various time intervals (1, 2, 4, 8, and 24 h) during this period.
5. Remove remaining phage from the chip surface by passing 10 μL of glycine-HCl buffer over the chip. Neutralize phage eluted in this fraction with 200 μL of neutralization buffer.
6. Regenerate the chip surface by passing 25 μL of regeneration buffer over the chip and washing with HBS-EP until a steady baseline is obtained.
7. Titer and amplify dissociated phage fractions (Subheadings 3.5 and 3.6) and use for subsequent rounds of panning against hapten immobilized onto the biosensor.
1. Grow *E. coli* XL1Blue on a selective LB agar plate containing tetracycline at 10 μg/mL.

2. Prepare a dense suspension of bacteria from the plate and inoculate 2 μL into 2 mL of Superbroth. Add tetracycline to a final concentration of 10 μg/mL.

3. Incubate at 37°C while shaking at 250 rpm until the optical density at 600 nm is approximately 1.0.

4. Take the phage eluate that is to be tested and prepare serial dilutions in Superbroth through the range 10^{-1}–10^{-5}.

5. Add 1 μL of each dilution of the phage eluate to individual 50 μL aliquots of the *E. coli* culture. Incubate at room temperature without agitation for 15 min. Plate the entire infected culture on LB agar plates containing 100 μg/mL carbenicillin (see Note 9).

6. Incubate the plates overnight at 37°C.

7. Count the number of colonies on each plate (ignore plates with >300 colonies) and calculate the phage titer.

1. Inoculate 5 mL of Superbroth with 5 μL of a suspension of *E. coli* XL1Blue prepared as above. Add tetracycline to a final concentration of 10 μg/mL. Incubate at 37°C while shaking at 250 rpm until the optical density at 600 nm is approximately 1.0.

2. Add the neutralized phage eluate to the 5 mL *E. coli* culture and incubate at room temperature for 15 min without shaking.

3. Add 2 mL of prewarmed Superbroth, 1.6 μL of carbenicillin stock (100 mg/mL), and 12 μL of tetracycline stock (10 mg/mL) (see Note 9).

4. Incubate at 37°C for 60 min while shaking at 250 rpm.

5. Add 2.4 μL of carbenicillin stock.

6. Incubate at 37°C for 60 min while shaking at 250 rpm.

7. Add 10^{12} pfu VCSM13 helper phage, 46 μL of carbenicillin stock, 46 μL tetracycline stock, and Superbroth to a final volume of 100 mL.

8. Incubate at 37°C for 90 min while shaking at 250 rpm.

9. Add 140 μL kanamycin stock and incubate overnight at 37°C while shaking at 250 rpm.

1. Centrifuge the overnight culture at 3,000 × g for 15 min at 4°C.

2. Transfer the supernatant to a fresh ice-cold centrifuge bottle containing 25 mL of 5× PEG/NaCl and mix well by repeated inversion.

3. Incubate on ice for 30 min.
4. Pellet the phage by centrifugation at 10,000 × g for 30 min at 4°C and discard the supernatant.

5. Resuspend the phage in 2 mL PBS containing 1% (w/v) BSA by pipetting up and down repeatedly.

6. Transfer the resuspended phage to two 1.5 mL tubes and centrifuge at 13,000 × g for 5 min at 4°C. Filter the supernatant through a 0.22 μm filter.

7. Titrate the phage as described in Subheading 3.5 using phage dilutions of 10⁻⁵–10⁻¹¹.

8. If phage are not to be used immediately for another round of panning, they should be stored in an equal volume of glycerol at −20°C. Aliquots for screening by ELISA should be stored at 4°C for no longer than 3 days.

3.8. Screening of Phage-Displayed Antibodies by ELISA

3.8.1. Polyclonal Phage ELISA

1. Coat wells of a Maxisorp 96 well plate with hapten-OVA conjugate at 10 μg/mL in coating buffer at 4°C overnight (see Note 10).

2. Wash the plate four times with ELISA wash buffer, and remove any excess buffer by inverting the plate and tapping against a wad of clean tissue paper.

3. Block the wells by adding 200 μL/well PBS containing 3% OVA (w/v). Incubate for 60 min at 37°C (see Note 1).

4. Wash the plate as described above.

5. Add 20 μL of PEG-precipitated phage from each panning round into triplicate wells followed immediately by 80 μL of 3% OVA (w/v) in PBS. Shake briefly and incubate for 60 min at 37°C (see Note 11). An aliquot of phage from the original library prior to panning should also be assayed. As a further control, phage samples should be tested in wells coated and blocked with 3% OVA.

6. Wash the plate as described above.

7. Dilute an aliquot of the HRP-conjugated anti-M13 antibody 1:1,000 in PBS/BSA. Add 100 μL of the diluted reagent to each well and incubate for 60 min at 37°C.

8. Wash the plate as described above. Repeat the wash.

9. Add 100 μL TMB substrate/well to each well and incubate at room temperature for 20 min with constant agitation.

10. Stop the reaction with 1 M H₂SO₄ (100 μL/well). Read the optical density reading of each well at 450 and 690 nm. Subtract the OD₆₉₀ from the OD₄₅₀ reading to determine the value for each well.

3.8.2 Phage ELISA of Individual Clones

The agar plates used to titrate phage eluted at each round of selection, prior to amplification, provide a resource for testing the properties of individual (monoclonal) phage-displayed scFv antibodies.
All culture steps are carried out in 96 well tissue culture plates to enable mass screening.

1. Pick individual, well-defined clones from agar plates and transfer to the wells of a tissue culture plate. Each well should be preloaded with 400 μL Superbroth supplemented with carbenicillin at 100 μg/mL (see Note 9) and 1% (w/v) glucose. A maximum of 93 clones should be picked, leaving three wells without bacteria as negative controls.

2. Incubate overnight at 37°C with shaking at 250 rpm.

3. Subculture 40 μL of the overnight culture to the wells of fresh plates, each well containing 360 μL Superbroth (supplemented with carbenicillin and glucose as above) and 5 × 10⁸ pfu VCSM13 helper phage.

4. Incubate at 37°C for 2 h with shaking at 250 rpm.

5. Pellet the cells by centrifugation at 1,500 × g for 20 min at 4°C and discard the supernatant.

6. Resuspend each pellet in 400 μL Superbroth containing carbenicillin at a final concentration of 100 μg/mL and kanamycin at a final concentration of 50 μg/mL.

7. Incubate overnight at 37°C with shaking at 250 rpm.

8. Pellet the cells by centrifugation at 1,500 × g for 20 min at 4°C, and transfer 320 μL of the supernatant containing the phage to a fresh 96-well culture plate. Add 80 μL of PBS containing 3% OVA (w/v) and mix.

9. Proceed as for polyclonal phage ELISA (Subheading 3.8.1). Those clones with an optical density three times greater than the negative control should be reassayed in triplicate against both the hapten-carrier conjugate and the carrier molecule alone to better characterize their properties.

### 3.8.3. Determination of the ELISA Titer of Individual Phage Preparations for Use in Competition Assays (see Note 12)

1. Coat wells as described in Subheading 3.8.1, steps 1–4.

2. Prepare serial dilutions of phage in PBS containing 3% OVA (w/v) (see Note 13). Add 100 μL of each dilution to triplicate wells of the ELISA plate, incubate at 37°C for 60 min, and wash plate as before.

3. Detect bound phage as described in Subheading 3.8.1, steps 7–10.

4. The titer is defined as that dilution of phage required to generate an OD of approximately 1.0.

### 3.8.4. Competition Phage ELISA (see Note 13)

1. Coat wells as described in Subheading 3.8.1, steps 1–4.

2. Prepare logarithmic dilutions of the free hapten in PBS starting at 10 μg/mL.

3. According to the titer determined in Subheading 3.8.3, dilute the phage preparation in PBS containing 3% OVA (w/v).
4. Add 25 μL of each dilution of the hapten to triplicate wells of the ELISA plate. Add 25 μL of PBS/OVA in triplicate to control wells.

5. Immediately add 100 μL of diluted phage preparation to each well of the ELISA plate. Mix on a plate shaker, incubate for 60 min at 37°C without agitation, and then wash as before.

6. Detect bound phage as described in Subheading 3.8.1, steps 7–10.

7. For each concentration of free hapten, determine the percentage phage that have bound by dividing the mean OD of the sample ($B$) by the mean OD of the control wells ($B_0$) and multiplying by 100. Plot the $%B/B_0$ against the concentration of competing hapten and thereby determine the concentration of free hapten that results in 50% inhibition ($I_{50}$) (see Note 14).

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4. Notes

1. When an immune library is being panned, it is advisable to use a carrier protein for immobilization that is quite distinct than anything present in the immunogen. Thus, if the immunogen contained a hapten-BSA conjugate, the library can be panned against hapten-OVA. OVA is then also used to block the selecting surface (e.g., immunotube). We have found it beneficial to dilute the phage library in a solution containing both carriers (in this example, BSA and OVA) to prevent the recovery of phage that bear carrier-reactive antibodies.

2. For selection of high-affinity antibodies, the coating concentration of the hapten (concentration of hapten derivative in solution phase screening) should be reduced and the number of washes should be increased with each round of panning. Only phage bearing high-affinity scFvs will have a chance to bind to the small number of haptens present, especially if the binding time is limited. Increased washing will remove low-affinity or nonspecific binders. A typical panning protocol is given in Table 1. Some investigators suggest that stringent conditions should be used from the first round of selection. However, if scFvs of high affinity are present at low abundance in the library, they may be lost in early rounds of selection when their numbers are low. In our view, progressive increase in stringency during selection allows scFvs with the desired properties to be enriched and retained through the panning process.

3. The phage library can be preincubated in an immunotube coated with streptavidin (10 μg/mL) to deplete streptavidin binders.
4. The c-myc tag commonly incorporated into vectors used for phage scFv library generation has a trypsin proteolytic site. With these vectors, nonspecific elution of bound phage can be achieved by trypsin digestion (TPCK-treated protease [Sigma]: 500 μL of a solution of 1 mg/mL in PBS for 10 min at room temperature with constant agitation). This is considered to give a more complete elution of bound phage than extremes of pH. Trypsin elution can also be used to reduce the transfer through to subsequent selection rounds of nonspecifically bound helper phage that do not bear recombinant antibody. Strains of helper phage are available (e.g., KM13) that have a trypsin-cleavage site engineered within the gp3 minor coat protein. Because intact gp3 is required for phage infectivity, only those phage carrying an antibody fragment fused to a noncleavable product of gp3 will be infective and hence undergo amplification for later rounds of selection.

5. Phage particles will be damaged by prolonged exposure to low or high pH, so the timing here is important.

6. Addition of free hapten can be used in two ways to select hapten-specific, high-affinity scFv-bearing phage. High affinity is mostly associated with a low off-rate, so trapping of those phage that dissociate more easily (fast off-rate) with an excess of free hapten in solution (e.g., 10–100 μg/mL) will theoretically leave immobilized phage that are bound with high affinity. These can then be eluted using low or high pH (8). One difficulty with this approach is that high-affinity binders

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**Table 1**

**Typical panning conditions for high-affinity scFv selection**

| Panning round | Hapten concentration | Binding time and conditions | Washes |
|---------------|----------------------|---------------------------|--------|
| 1             | 10 μg/mL             | 120 min at RT followed by overnight at 4°C | 5× PBS/0.1% Tween 20 |
|               |                      |                           | 5× PBS |
| 2             | 1 μg/mL              | 60 min at RT              | 10× PBS/0.1% Tween 20 |
|               |                      |                           | 10× PBS |
| 3             | 0.1 μg/mL            | 30 min at RT              | 20× PBS/0.1% Tween 20 |
|               |                      |                           | 20× PBS |
| 4             | 0.1 μg/mL            | 30 min at RT              | 20× PBS/0.1% Tween 20 |
|               |                      |                           | 20× PBS |

*Overnight in PBS at 4°C*

*Molecular weight of hapten is approximately 300 AMU. RT room temperature*
will be contaminated with nonspecific binders, so it will work best after some selection has already taken place. An alternative approach is to use decreasing concentrations of hapten (e.g., 10 μM to 0.1 pM) and prolonged incubation times to elute hapten-specific scFv-bearing phage (9). Phage present in the solution phase at the end of the incubation period should be mainly hapten binders. Extending the incubation time will ensure elution of those scFv-bearing phage with high affinity and lowering the hapten concentration will favor the high-affinity binders.

7. Incubation with free antibody can be used to select for scFv-bearing phage that bind with higher affinity than a given antibody. As low-affinity binders dissociate from the surface, their places are taken by the higher-affinity antibodies. Thus phage bearing scFvs with lower affinity than the antibody will be in the solution phase. If left for long enough, phage that remain bound should be higher affinity than the antibody and can be eluted.

8. Direct immobilization of the hapten to the sensor chip surface generally gives a much more robust surface than immobilization of a hapten-protein conjugates. The latter can be carried out according to the immobilization wizard provided by the manufacturer. Direct attachment of hapten to all flow cells of the sensor chip is performed on the bench outside of the instrument because relatively large amounts of hapten have to be used during the coating and residues in the tubing might cause interference especially with high-affinity binders. A two-step procedure is given for covalent attachment of a carboxylated hapten to the carboxymethylated surface of a CM5 sensor chip (10). Use of the carboxymethylated C1 sensor chips has also been recommended to allow interactions to take place closer to the chip surface and to avoid avidity effects (7). Free amino groups are initially generated on the chip surface and the carboxyl groups of the hapten then react with the amino groups using carbodiimide chemistry. The procedure will have to be modified depending on the chemical structure of the hapten.

9. For phage display vectors having different antibiotic resistance, replace carbenicillin with the appropriate antibiotic.

10. Alternatively, the plate can be coated at 37°C for 90 min.

11. Each sample should be assayed in triplicate by making three separate dilutions rather than a single 300-μL dilution and splitting into three wells.

12. The specificity and affinity of binders for their target are best determined by competition ELISA. For true competition, it is necessary to use limiting amounts of the binding agent
in the assay. Hence it is essential to titer each preparation of binding agent prior to the competition assay.

13. Competition phage ELISA can be carried out with polyclonal phage pools to monitor the panning procedure or with individual phage clones to compare scFv clones. A successful panning procedure will show a decrease in $I_{50}$ after each round of panning.

14. If the expected decrease in $I_{50}$ (i.e., increase in affinity) is not seen, it is worth checking a sample of clones from each panning to confirm that the full-length scFv gene insert is still present. This is done by colony PCR. Following each round of panning, ten clones are picked, phagemid DNA is isolated, and PCR is carried out using primers that anneal to regions flanking the scFv reading frame. While not all clones may have the complete scFv sequence in the early rounds of panning, all clones from later rounds should have a full-length insert. Truncated and deleted scFvs are indicators of non-antigen-driven selection and indicate that specificity to the target antigen is being lost.

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Chapter 8

Display and Selection of scFv Antibodies on HEK-293T Cells

Mitchell Ho and Ira Pastan

Summary

We describe a human cell display strategy to isolate high-affinity single-chain antibody fragments (scFvs) specific for CD22 for the treatment of B-cell malignancies. Our strategy uses flow cytometry and human embryonic kidney 293T (HEK-293T) cells that are widely used for transient protein expression. Flow cytometry enhances the screen’s sensitivity thereby allowing us to isolate high-affinity scFvs. Using human cell display, one could isolate and engineer scFvs, single domains, Fabs, or whole IgGs for increased affinity and other biological functions.

Key words: Mammalian cell display, Antibody engineering, Antibody affinity maturation, Single-chain Fv or scFv, Phage display, Yeast display, Cell display, HEK-293T, Flow cytometry, B-cell malignancy

1. Introduction

Creating very effective therapeutic monoclonal antibodies (mAbs) to the targets in cancers and infectious diseases is an important and challenging task. Twenty-one therapeutic mAbs have been approved by the US Food and Drug Administration, and hundreds of mAbs are in clinical trials (1). The levels of antibody affinity obtained from hybridomas or from human antibody libraries are frequently insufficient for effective clinical use, in part because of the in vivo affinity ceiling (2); therefore, an in vitro improvement of antibody affinity is often required. For the past two decades, phage display (3, 4) has been used for in vitro antibody affinity maturation and, more recently, cell display systems using bacterial and yeast cells (5, 6) have been developed (7, 8). Very recently we showed that single-chain antibodies (scFvs) can be displayed on the surface of human embryonic kidney 293T (HEK-293T)
cells and used for affinity maturation. We call this method “mammalian cell display.” Our strategy is adapted from Wittrup’s yeast cell display used previously to isolate high-affinity antibodies in yeast cells, except we use HEK-293T cells because these cells are already widely used for transient protein expression. Mammalian cell display is a useful method for the isolation of scFv (9) and whole IgG (10) with high affinity and other specific biological functions. HEK-293T cell display relies on the transient transfection of antibody-encoding DNA to promote very high levels of antibody expression in human cells. Moreover, the expressed mouse or human antibodies could contain the posttranslational modifications that are required for antibody function. It has been suggested that mammalian cell display could be used to express recombinant antibody fragments that cannot be expressed in *Escherichia coli* (11). We have used this HEK-293T human cell display strategy to increase the affinity of antibodies that bind CD22 (9) and to isolate a human scFv that binds mesothelin from nonimmune human antibody libraries (Ho and Pastan, unpublished data). CD22 is an adhesion molecule expressed in B cells and overexpressed in B-cell leukemias and lymphomas. Mesothelin is highly expressed in several human solid tumors, including virtually all mesotheliomas and pancreatic adenocarcinomas, and approximately 70% of ovarian cancers and 50% of lung adenocarcinomas (12).

To display recombinant antibody on the cell surface, we have fused the scFv to the transmembrane domain of human platelet-derived growth factor receptor (PDGFR) (Fig. 1). The expression vector contains the cytomegalovirus (CMV) promoter (P<sub>CMV</sub>), the nucleotide sequence encoding the murine Ig κ chain signal peptide (METDTLLLWVLLWVPGSTGD), the scFv, a c-myc tag, and the transmembrane domain (amino acids Ala513-Arg561) of PDGFR. The c-myc epitope tag at the carboxyl terminus of the scFv can be used to measure levels of the scFv present at the surface of the transfected cells. In our published work, we expressed a scFv (clone RFB4) directed against CD22 on HEK-293T cells. Surface localization of the scFv-PDGFR fusion was verified by confocal fluorescence microscopy (Fig. 2) and flow cytometry (9). Location of the scFv at the cell surface enabled binding of biotinylated CD22-Fc and detection with streptavidin-conjugated reagents. Cells expressing the scFv-PDGFR fusion protein were thereby colabeled (Fig. 2d) by a mixture of the CD22-Fc (Fig. 2b) and the anti-c-myc antibody (Fig. 2c).

In this protocol, we describe the transfection of HEK-293T cells with a scFv library enabling surface display of the recombinant antibodies. Our methods use fluorescence-activated cell sorting (FACS) to select and collect cells that bind the target protein through interaction with cognate scFvs. scFvs identified in this way can be recovered and analyzed until highly enriched scFv clones with desired properties are isolated.
Display and Selection of scFv Antibodies on HEK-293T Cells

1. scFv library.
2. Primers for subcloning the antibody library into the SfiI and SacII sites of pDisplay vector.
3. High Fidelity Tgo DNA polymerase master mix (Roche).
4. DNA purification kit for isolation of polymerase chain reaction (PCR) products.
5. SfiI and SacII (New England Biolabs), and 10× digestion buffer provided with the enzymes.
6. pDisplay (Invitrogen) vector for library construction and as a negative control.

Fig. 1. (a) Diagram of expression plasmid for display of scFv on mammalian cells. $P_{CMV}$, cytomegalovirus promoter; $IgK$SP, murine Ig κ chain signal peptide; $VH$, heavy chain variable region; $VL$, light chain variable region; $Linker$, a flexible synthetic linker between VH and VL; $myc$, an epitope tag to measure the scFv expression level; $PDGFR$, the transmembrane domain of human platelet-derived growth factor receptor; $P_{SV40/ori}$, SV40 promoter and origin facilitating episomal replication in mammalian cells expressing SV40 large T-antigen; $Neo/Kan^{R}$, neomycin- and kanamycin-resistance gene. (b) Schematic illustration of surface display on mammalian cells. An additional ten-amino acid epitope tag ($c$-myc) was fused to the C terminus of the scFv, allowing quantitation of fusion display with mAb 9E10 independent of antigen binding. Fusion to the N-terminal portion of the PDGFR transmembrane domain was used to anchor scFv on the human (HEK-293T) cell surface.
7. T4 DNA ligase.
8. *E. coli* TOP10 (Invitrogen) at a competency of ≥10⁹ colony forming units (cfu)/µg DNA (Invitrogen) (*see Note 1*).
9. SOC medium (Invitrogen).
10. LB agar plates containing 100 µg/mL ampicillin.
11. DNA isolation kits (mini and maxi scale) (Qiagen).

### 2.2. Cell Culture and Transfection

1. HEK-293T cells (GenHunter) (*see Note 2*).
2. Growth medium for HEK-293T cells: Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum, L-glutamine, non-essential amino acids, and penicillin-streptomycin.
3. 100 mm tissue culture dishes for HEK-293T transfections.
4. Purified DNA comprising an antibody library that has been cloned into the mammalian expression vector pDisplay (Invitrogen) (*see Note 3*).
5. Serum-free DMEM containing glutamine but lacking antibiotics.
6. Transfection reagents: Lipofectamine reagent and PLUS reagent (Invitrogen).
7. 20% (v/v) FBS in DMEM containing glutamine and antibiotics.

### 2.3. Flow Cytometry

1. Phosphate-buffered saline (PBS).
2. Trypsin (0.25% [w/v]) and ethylenediamine tetraacetic acid (EDTA; [1 mM]) in PBS.
3. Target protein (e.g., biotinylated CD22-Fc; see Note 4).
4. Antibody for detection of the c-myc expression tag: 9E10 mAb (culture supernatant from hybridoma cell line 9E10, ATCC) (see Note 5).
5. Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody.
6. Streptavidin-R-phycoerythrin (PE) (Invitrogen) for visualization of biotinylated target protein (e.g., biotinylated CD22-Fc).
7. Flow cytometers for analysis (FACScalibur, Becton Dickinson) and cell sorting (FACSVantage SE flow cytometry, Becton Dickinson) (see Note 6).

### 2.4. Recovery of scFv-Coding DNA

1. Hirt’s Solution (13): 0.6% (w/v) sodium dodecyl sulfate (SDS) and 10 mM EDTA in 5 mL of double-distilled water (see Note 7).
2. 5 M NaCl.
3. Phenol:chloroform:isoamyl alcohol (25:24:1).
4. Glycogen (20 mg/mL).
5. Absolute and 70% (v/v) ethanol.
6. 3 M sodium acetate, pH 7.0.
7. DpnI restriction enzyme and 10× buffer (New England Biolabs).
8. Falcon 2052 tubes.
9. Competent *E. coli* TOP10, SOC, and LB agar with ampicillin, as above.
10. Glycerol.

### 3. Methods

The following procedure describes a HEK-293T human cell display system to identify scFv antibody proteins that bind CD22. This method may be adapted to identify antibodies that bind other targets. The strategy consists of transfecting HEK-293T, a human cell line, with an scFv antibody library (in this example,
an anti-CD22 scFv library) and adding appropriate target protein (in this example, the extracellular domain of CD22 fused to human Fc protein). The detection of cells that bind the target proteins is achieved by FACS: the cells transfected with the scFv library are sorted by FACS, and only cells in which the scFv binds to the target protein are collected. The plasmid DNAs are isolated from these cells and amplified in bacteria. These scFv DNAs are enriched by further rounds of transfection and FACS until scFvs with increased affinity for target proteins can be isolated (see Note 8).

A scFv library should be subcloned into appropriate sites of the pDisplay vector to allow expression. To do this, oligonucleotides bearing SfiI and SacII sites are used to amplify inserts from a scFv library using plasmid DNA from the library as a template. The 5' SfiI and 3' SacII sites facilitate cloning of the scFv sequences into pDisplay, downstream of the immunoglobulin κ chain leader sequence. The expression vector encodes a c-myc epitope tag and a transmembrane domain of PDGFR downstream of the scFv so that the product is anchored at the surface of the cell.

1. Design primers to amplify the scFv genes. The 5' forward primer must contain a SfiI site. The 3' reverse primer must contain a SacII site. Ensure that the sites are placed appropriately with respect to upstream and downstream sequences in the pDisplay vector. For the anti-CD22 scFv library, we designed the following 5' and 3' primers:

\[
\text{Dis}_\text{SfiI} \text{IF} \ 5' \ GGGGCCCAGCCGGCCATGGAAGTGCAG\text{CTGGTG} \ 3'
\]

\[
\text{Dis}_\text{SacII} \text{IR} \ 5' \ CTG CCGCGG AGCTTTGATTTCCAG \ 3'
\]

The SfiI and SacII sites are underlined.

2. Amplify the scFv genes. Use forward and reverse primers as described above at final concentrations of 10 μM, and 10 ng of library DNA as the template for PCR. The template and oligonucleotides are mixed with an equal volume of the Tgo master mix to generate a 50 μL reaction and then cycled using the following profile: one cycle at 95°C for 5 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min.

3. Isolate the PCR products, digest with SfiI and SacII and gel purify. Ligate into pDisplay vector that has also been cut with SfiI and SacII and gel purified.

4. Transform the ligation mix into competent E. coli TOP10 cells.

5. Recover the bacterial cells in SOC and plate to LB agar plates containing ampicillin following instructions with the cells. By serial dilution of the transformation mix, establish the size of the display library.
6. Each transformation should produce a cell surface display library containing approximately $1 \times 10^6$ independent clones. We also make a plasmid containing a wild-type scFv as a control.

7. Expand the library by growing on in liquid culture and isolate plasmid DNA.

### 3.2. Transfection

It is important to have adequate controls, as well as a sufficient number of cells for sorting and recovery of plasmid DNA. As well as transfecting with the scFv library, conduct control transfections with pDisplay containing a wild-type scFv antibody against the target of interest (e.g., CD22) in cases where a scFv library is a panel of mutants is being screened for elevated affinity of interaction with the target protein. In other experimental designs, a scFv of irrelevant specificity might constitute an appropriate control.

1. Plate HEK-293T cells to five 100 mm tissue culture dishes at $2 \times 10^6$ cells per plate, 24 h before transfection (see Note 9).

2. Prepare DNA in serum-free DMEM (4 µg library or control DNA in 750 µL of DMEM for a single dish of cells) and mix well.

3. Add 20 µL of PLUS reagent to the 750 µL of DNA solution, mix well, and incubate at room temperature for 15 min.

4. Dilute Lipofectamine reagent with serum-free DMEM in a second tube (30 µL Lipofectamine reagent in 750 µL serum-free DMEM for a single dish) (see Note 10).

5. Combine the DNA solution and the diluted Lipofectamine to create a solution of 1.5 mL total volume ready for transfection of a single dish of cells. Mix well, and incubate at room temperature for more than 15 min (see Note 11).

6. While complexes are forming, replace the medium on the HEK-293T cells with 5 mL of serum-free DMEM per 100 mm dish.

7. Gently add the DNA–Lipofectamine mixture to the dishes (1.5 mL mixture per dish). Gently shake the dish several times.

8. Incubate the dishes in a CO$_2$ incubator for 5 h.

9. Gently add 6 mL of 20% FBS in DMEM to each dish and return the dish to a CO$_2$ incubator.

10. Add 10 mL of 10% FBS in DMEM growth medium into each dish 24 h after transfection.

11. After incubation for 72 h, the cells are ready for sorting or analysis by flow cytometry (see Note 12).

### 3.3. FACS Sorting

The specific example described here assesses binding affinity of surface-displayed scFvs for CD22, but the method can be adapted to detect the binding of any target protein.
The cells are incubated with 0.1–1 μg/mL of biotinylated CD22-Fc and 20 μg/mL of mAb 9E10 (anti-c-myc) in 500 μL of growth medium. Control cells containing wild-type anti-CD22 scFv help determine the parameters such as compensation in FACS analysis and sorting. After incubation for 1 h at 4°C, the cells are washed with PBS and incubated with detection reagents conjugated to fluorochromes and are run on FACS sorting or analytical instruments. Those cells that are most intensely stained—in our experiments, we select the top 0.1% positive cells—are collected (Fig. 3). Plasmids are recovered from the selected cells and transformed into E. coli to recover plasmid scFv DNA for sequencing and further rounds of selection.

1. Collect the transfected HEK-293T cells from the 100 mm Petri dishes, 72 h after transfection. In a typical experiment, this would recover approximately 1 × 10^7 cells per dish. To recover the cells, first remove the medium from all plates and wash the cells once with 2 mL of PBS.

2. Add 4 mL of trypsin-EDTA buffer to the test and control plates.

3. Incubate for 5 min at room temperature.

4. Add 10 mL of complete DMEM into each dish. Collect cells from the library (test) transfections and combine into a single 50 mL tube. Collect the cells from the control transfections into a 15 mL tube.

5. Centrifuge the test and the control cells at 500 × g in a tabletop centrifuge for 3 min at room temperature to pellet the cells.

6. Aspirate off the supernatant.

Fig. 3. Enrichment of mammalian cells displaying an improved scFv variant by kinetic selection and flow cytometric sorting. The dot plot shows only 50,000 cells of the total cell population (10^7). Each dot represents an individual observed cell. The cells were labeled with no antigen (a) or 1 nM antigen (CD22-Fc) (b) for separation. FITC fluorescence (binding to the c-myc tag) represents the number of surface expressions on an individual cell. The PE fluorescence represents binding to the antigen (CD22). A sort window (c) was drawn to include the top 0.1% of total cells in terms of ratio of PE-FITC fluorescence. Cells that fell within the window were collected. (Reproduced from ref. 9 with permission. Copyright 2006 National Academy of Sciences, USA).
7. Resuspend the test cells in 10 mL of complete DMEM by gentle agitati
don of the tubes and pipetting up and down four times.
8. Add biotinylated CD22-Fc to a final concentration of 0.1–1 µg/mL and mAb 9E10 to 20 µg/mL (see Note 13).
9. Resuspend the control cells expressing the wild-type/irrelevant scFv separately in 5 mL of complete DMEM and determine the concentration of cells recovered using microscopy.
10. Sample four aliquots, each of 5 x 10^5 control cells into 1 mL of complete DMEM in separate tubes, labeling the tubes 1A, 1B, 1C, and 1D.
11. Add biotinylated target protein to 1B to a final concentration of 0.1–1 µg/mL, mAb 9E10 to 1C to a final concentration of 20 µg/mL, and both biotinylated target protein and 9E10 (as specified) to 1D. Resuspend the cells by gentle agitati
don of the tubes.
12. Incubate test samples and control cells for 60 min on ice.
13. Add 4 mL of complete DMEM to each control tube.
14. Centrifuge all samples for 5 min at 500 x g in a tabletop centrifuge.
15. Wash the cells twice with PBS by resuspension and centrifugation.
16. Incubate the test and control cells on ice with 1:200 dilutions of FITC-labeled goat anti-mouse antibody, and streptavidin–R–PE conjugate.
17. Incubate all samples on ice for 30 min.
18. Wash the cells with 5 mL of complete DMEM.
19. Centrifuge the cells for 5 min at room temperature at 500 x g in a tabletop centrifuge.
20. Remove and discard the supernatants.
21. Wash the cells twice with 5 mL of PBS.
22. Resuspend the test cells in 2 mL of complete DMEM. Resus
dpend the control cells in 1 mL of complete DMEM.
23. Incubate all tubes on ice in the dark.
24. Set the parameters on a FACSVantage SE using the control cells to minimize overlap of FITC and PE fluorescence signals.
25. Using these parameters, collect PE and FITC double-positive cells from the test sample by FACS, isolating those cells for which the ratio of PE:FITC fluorescence lies in the top 0.1%. Collect the cells that fall within the sort window as described in Fig. 3 (see Note 14).

### 3.4. scFv Recovery

1. Pipette half of the recovered PE and FITC double-positive cells into a microcentrifuge tube. This should be a volume of approximately 1.5 mL.
2. Microcentrifuge for 5 min at 500 x g.
3. Discard the supernatant and add the remaining volume (typically 1.5 mL) of collected cells to the same tube.
4. Microcentrifuge again for 5 min at $500 \times g$.
5. Discard the supernatant (see Note 15).
6. Add 400 μL of Hirt’s Solution.
7. Incubate at room temperature for 20 min.
8. Add 100 μL of 5 M NaCl, mix by inversion, and leave at 4°C overnight (see Note 16).
9. Microcentrifuge the cells at maximum speed for 5 min at room temperature.
10. Carefully transfer the supernatant into a new labeled microcentrifuge tube.
11. Phenol:chloroform extract the supernatant twice.
12. Transfer extracted supernatant to a fresh microcentrifuge tube.
13. Add 1 μL of glycogen solution and fill the tube to the top with absolute ethanol to precipitate the DNA.
14. Microcentrifuge at maximum speed for 5 min at room temperature to pellet the DNA.
15. Remove the supernatant and resuspend the pellet in 100 μL of double-distilled water.
16. Add 10 μL of 3 M sodium acetate, pH 7.0, and 300 μL of absolute ethanol.
17. Microcentrifuge at maximum speed for 5 min at room temperature to pellet the DNA.
18. Remove the supernatant and resuspend the pellet in 100 μL of double-distilled water.
19. Add 10 μL of 3 M sodium acetate, pH 7.0, and 300 μL of absolute ethanol.
20. Microcentrifuge the tubes for 20 min at maximum speed at 4°C.
21. Wash the pellet with 1 mL of 70% ethanol.
22. Resuspend the pellet in 10 μL of double-distilled water.
23. (Optional) Perform a DpnI digestion using the 10 μL of DNA. Incubate overnight at 37°C (see Note 17).

### 3.6. Transformation

1. Incubate the entire 10 μL sample of isolated DNA (with or without DpnI digestion) with 100 μL of high-competence *E. coli* TOP10 cells on ice for 20 min in Falcon 2059 tubes.
2. Transfer tubes to a 42°C water bath for 1 min and return to ice.
3. Add 1 mL of SOC and incubate on a shaker at 37°C for 1 h.
4. Plate the entire transformation mix onto LB agar plates containing ampicillin.
5. Incubate overnight at 37°C.
6. Count and record the number of colonies that grow (see Note 18).
7. Pick 10–20 individual clones after analysis and inoculate into individual 50 mL liquid cultures. Grow overnight at 37°C.
8. Scrape the remaining colonies from the plates and inoculate into a 500 mL liquid culture. Grow overnight at 37°C.
9. Make glycerol stocks by sampling 1 mL from each culture, adding 300 µL of 60% glycerol, and storing at −80°C.
10. Prepare DNA on a maxiprep scale from each culture using Qiagen reagents, according to the manufacturer’s protocols.
11. Transfect isolated DNA from the large-scale culture into HEK-293T cells and repeat the FACS sorting using the procedure described above. The concentration of target protein should be reduced in successive rounds to enrich for high-affinity binders. DNA from the 50 mL cultures can be used as described in Subheading 3.7.

In this protocol, the goal is to use DNA prepared from individual colonies to identify scFvs with the capacity to bind the target protein with highest affinity.

1. Use DNA isolated from 50 mL cultures set up with individual bacterial colonies.
2. Transfect 4 µg of each DNA sample into separate cultures of HEK-293T cells using the protocols above.
3. Perform analytical FACS to identify scFv clones that bind the target with high affinity (see Subheading 3.8).
4. Sequence the scFv fragments with primers Dis_SfiIF and Dis_SacIIR (see Note 19).

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4. Sequence the scFv fragments with primers Dis_SfiIF and Dis_SacIIR (see Note 19).

3.7. Identification of Binders

In this protocol, the goal is to use DNA prepared from individual colonies to identify scFvs with the capacity to bind the target protein with highest affinity.

1. Use DNA isolated from 50 mL cultures set up with individual bacterial colonies.
2. Transfect 4 µg of each DNA sample into separate cultures of HEK-293T cells using the protocols above.
3. Perform analytical FACS to identify scFv clones that bind the target with high affinity (see Subheading 3.8).
4. Sequence the scFv fragments with primers Dis_SfiIF and Dis_SacIIR (see Note 19).

3.8. Determination of Affinity Constants (K_D) by Flow Cytometry

K_D is measured by determining the concentration of target required for half-maximal binding to scFv present at the surface of transfected HEK-293T cells. This is determined from the mean fluorescence intensity of the HEK-293T cells when no antigen is bound and the concentration of target that gives maximal intensity. We have shown that this is easily established by setting up a series of concentrations of target that are then used to label the cells. The mean fluorescence intensity of the target-binding population of cells is then measured by flow cytometry.
1. Culture HEK-293T cells transfected with the scFvs of interest and cells transfected with a control scFv as described above. Remove the media from all plates and wash the cells once with 2 mL of PBS per plate.
2. Add 4 mL of trypsin-EDTA buffer to test and control plates.
3. Incubate for 5 min at room temperature.
4. Add 10 mL of complete DMEM into each dish and collect cells into separate 15 mL tubes.
5. Centrifuge the test and control cells at 500 × g in a tabletop centrifuge for 3 min at room temperature to pellet the cells.
6. Aspirate off the supernatant.
7. Resuspend 1 × 10^5 cells from each sample in 1 mL aliquots of FACS buffer by gentle agitation of tubes and pipetting up and down four times.
8. Add biotinylated antigen to the cells at a range of concentrations (e.g., 0.1–100 nM) (see Note 20).
9. Incubate cells for 60 min on ice.
10. Wash twice.
11. Add 1:200 streptavidin-PE and incubate on ice for 30 min.
12. Wash twice with PBS and analyze by flow cytometry.
13. Use the geometric mean of the output as the mean fluorescence intensity.
14. Determine equilibrium constants by using the Marquardt–Levenberg algorithm for nonlinear regression with GraphPad Prism following the developer’s instructions.

4. Notes

1. Any bacterial strain of high competence (≥10^9 cfu/μg DNA) will suffice, providing it is capable of carrying the pDisplay vector.
2. HEK-293T cells stably express the large T-antigen of SV40 (simian virus 40), allowing episomal replication of plasmids containing the SV40 origin. Any cell lines expressing SV40 large T-antigen may suffice. HEK-293T cells are grown in 10% FBS in DMEM. The cells must be in good condition before the transfection. HEK-293T cells should be split every 2 days to keep them growing.
3. The yield of pDisplay-derived plasmids from E. coli culture is low. At least 500 mL of E. coli culture is required to obtain enough DNA (200 μg of plasmid DNA) to transfect 50
dishes. Qiagen Maxi prep column-grade DNA works well in transfection.

4. We prefer to use an Fc fusion protein because Fc fusion proteins are highly expressed in mammalian cells used for synthesis (HEK-293T or HEK-293F [Invitrogen]) and because they can be purified in one step on a protein A column (14). However, other formats of target proteins may also be suitable.

5. This antibody is used to detect expression levels of scFv.

6. Many FACS core facilities may have these two machines because they are widely used. However, other brands of cytometer may suffice.

7. The QIAprep Spin Miniprep Kit can be used as an alternative solution to recover scFv plasmid DNA from HEK-293T and other mammalian cells.

8. When using mammalian cell display, there are three major points that must be considered. These are:
   
   (a) The size of the library to be screened;

   (b) The transfection efficiency of the mammalian host cells; and

   (c) Episomal replication of the scFv library in these cells.

An appropriate antibody library will be in a plasmid with a promoter such as CMV that drives high levels of protein expression in mammalian cells. The library sizes we have used so far range from $10^3$ to $10^6$. The plasmid must also contain an origin for episomal replication, such as polyoma or SV40, which matches the elements used to drive replication (that is, polyoma large T-antigen or SV40 large T-antigen). We recommend using the commercially available plasmid pDisplay that contains the SV40 origin for replication suitable for simple vector rescue in cell lines expressing the large T-antigen (e.g., HEK-293T, COS1, COS7, and CHO-Tag). Episomal replication is important because it is required to amplify the transfected plasmid DNA in the mammalian host cells. This ensures that most of the plasmids can be recovered from sorted cells. Flow cytometry allows millions of cells to be screened rapidly. In a typical screen, we sort at least $1 \times 10^7$ cells and collect approximately 10,000 cells in the gate. Hence, mammalian cell display provides a high-throughput approach to the identification of novel high-affinity antibodies for possible clinical applications.

For permanent display of scFv on human cells, we recommend that stable cell lines be cloned by transfecting the scFv libraries into cells such as HEK-293F.

9. In our laboratory, we transflect five to ten dishes per screen and two control plates.
10. The diluted Lipofectamine reagent is not stable. Make the dilution just before the next step.

11. During the incubation, DNA–lipofectamine complexes will be formed.

12. A time course experiment indicated that the expression level of the transfected scFv is maximal 48–72 h after transfection.

13. The dilution and amount of the antibody used to measure affinity needs to be determined empirically.

14. In a sort of more than $1 \times 10^7$ cells, we typically collect approximately 10,000 cells.

15. All cells should be collected at the bottom of one microcentrifuge tube.

16. Plasmid DNA can be isolated from the sorted HEK-293T cells using the QIAprep Spin Miniprep Kit following the manufacturer’s instructions. We have found that as few as 100 cells are enough for successful plasmid isolation.

17. In theory, plasmids replicating episomally in mammalian cells due to SV40 large T are not methylated. After isolation of the plasmid DNA from the cells, the DNA is digested with $DpnI$, which only digests methylated DNA, thereby removing any contaminant bacterial plasmids. Thus, if the mammalian host cells contain episomally replicating library DNA (which is desired), then there will be many colonies on the selective plates. If the host cells do not contain episomally replicating scFvs, there will be no or only a few colonies on the plates. However, we find the difference between $DpnI$-digested DNA and nondigested DNA from HEK-293T cells is two-fold or less. Therefore, we consider this digestion an optional step.

18. A typical number of colonies from a successful screen ranges from 1,000 to 10,000. We typically obtain about one clone per sorted cell.

19. We typically see highly enriched clones from a small library (diversity ~1,000) after only a single round of FACS sorting. For a large library (diversity ~$10^6$), the best enrichment is achieved at the third or fourth round of FACS sorting.

20. The dilution and amount of the target used to measure affinity needs to be determined empirically. For initial measurements, we routinely use 0.1, 0.5, 1, 5, 10, 50, and 100 nM of antigen (e.g., CD22-Fc). It is recommended that probing with anti-c-myc reagents should be used to assess whether levels of each scFv displayed on transfected cells are equivalent so that data from the target titration is informative.
Acknowledgments

This work was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research.

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Isolation of scFvs that Inhibit the NS3 Protease of Hepatitis C Virus by a Combination of Phage Display and a Bacterial Genetic Screen

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Summary

The need for inhibitors for enzymes linked with microbial infection, specifically the NS3 protease of hepatitis C virus (HCV), inspired us to develop a unique, rapid and easy color-based method described herein. The NS3 serine protease of HCV has a role in processing viral polyprotein and it has been implicated in interactions with various cell constituents, resulting in phenotypic changes including malignant transformation. NS3 is currently regarded a prime target for antiviral drugs.

We established a genetic screen that is based on coexpression of NS3, a β-galactosidase reporter that is cleavable by NS3, and potential inhibitors within the same bacterial cell. A single-chain antibody (scFv) library was prepared from spleens of NS3-immunized mice and the screen was used to isolate a panel of protease-inhibiting scFvs. Candidate scFvs were validated for inhibitory activity using an o-nitrophenyl-β-galactoside (ONPG) hydrolysis assay.

The methods can be used more generally to isolate protease-inhibiting cytoplasmic intrabodies able to inhibit proteases or other activities that can be linked with the phenotype of Escherichia coli.

Key words: Immune antibody phage display library, Single-chain antibodies, Protease inhibitors, Genetic screen, Hepatitis C virus, NS3 protease, β-Galactosidase

1. Introduction

Hepatitis C virus (HCV) causes acute or chronic hepatitis, cirrhosis, and hepatocellular carcinoma (1, 2). There have been advances in the therapy of HCV, but even the recent combination of PEGylated alpha-interferon and ribavirin fails to eliminate infection in nearly 50% of those carrying HCV (3, 4). This high frequency of treatment
failure points to the need for more specific, less toxic, and better-directed antiviral therapies for HCV (4–6).

HCV is a single-stranded RNA virus with a 9.6 kb genome that encodes a single, large open-reading frame that is translated to a large polyprotein precursor of 3,010–3,033 amino acids (4, 7). Most processing of the precursor is directed by the virally encoded NS3, a serine protease that is dependent on the NS4A cofactor for efficient cleavage activity; NS3 is therefore essential for replication of the virus (8, 9). In addition, expression of NS3 has been found to interfere with signal transduction pathways, promote cell proliferation, and cause cell transformation (10–14). The NS3-4A protease activity has been also implicated in blocking the host cell’s ability to mount an innate antiviral response. Due to its essential role in viral replication and its effects on the physiology of the infected cell, NS3 presents a prime target for antiviral therapy.

All NS3-inhibiting antibodies identified thus far have been isolated by their binding to NS3 and, in a separate step, testing for the inhibition of catalysis (15–18). We opted for an approach that would facilitate the direct isolation of NS3-inhibitory scFvs. A bacterial genetic screen for inhibitors of NS3 catalysis has been developed by our group and applied to the isolation of scFv inhibitors of NS3 protease activity (19). The genetic screen is based on coexpression of NS3, an engineered, β-galactosidase reporter construct that can be cleaved by NS3, and potential inhibitors within the same bacterial cell. Inhibitory scFvs thus generate a blue phenotype as NS3 activity is blocked and the β-galactosidase reporter retains its activity. Expression of non-inhibitory scFvs leads to a background of white colonies on agar plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

Preliminary to our studies, an immune scFv library was prepared using spleens of NS3-immunized mice. The library was subjected to limited affinity selection by phage display, and the enriched scFv population was subcloned into an expression vector, pMALc, for efficient production of intracellular antibodies in Escherichia coli (20). The scFv library was then transferred into the screening strain of bacteria (those expressing NS3 and the reporter) and blue colonies were identified as potential NS3 protease inhibitors. The inhibitory potential of the scFvs was confirmed by a qualitative β-galactosidase activity assay.

Although our studies have centered on isolating intracellular inhibitors of the HCV NS3 protease, our methods provide a general approach for isolation of intracellular antibodies with properties that can be linked to a phenotype in E. coli.
2. Materials

The commercial vendors listed below are the ones we usually use. We do not endorse or guarantee their products.

1. Sterile, double-distilled water (SDDW) (see Note 1).

2. DNA modifying enzymes: NcoI, NotI, BstBI, Bsu36I, BsaIII, SfiI, EcoRI, BspHI, and SphI restriction enzymes (see Note 2), T4 DNA ligase (New England Biolabs), and alkaline phosphatase (Roche).

3. Agarose (Sigma).

4. QIAquick gel extraction kit, QIAGEN plasmid mini/maxi kit (Qiagen).

5. DNA elution buffer: 10 mM Tris (HCl), pH 8.0 (see Note 1).

6. Microcon-polymerase chain reaction (PCR) ultrafiltration device/Montage PCR centrifuge filter device (Millipore).

7. PCR amplification: high-fidelity thermostable DNA polymerase (such as Vent DNA polymerase, New England Biolabs), dNTP mix of 2.5 mM each, 10× PCR buffer with or without MgCl₂, MgCl₂ if required.

8. Competent *E. coli* strains suitable for cloning, such as XL1-Blue or TG1.

9. 2× YT medium: 16 g/L tryptone, 10 g/L yeast extract, and 5 g/L NaCl. For solid media, add Bacto agar to a final concentration of 1.8–2% (w/v) before autoclaving. Tryptone, yeast extract, and Bacto agar can be obtained from Difco (Becton Dickinson Microbiology Systems).

10. YTAG medium: 2xYT medium supplemented with 100 mg/L ampicillin and 1% (w/v) glucose.

11. YTAK medium: 2xYT medium supplemented with 100 mg/L ampicillin and 50 mg/L kanamycin.

12. LB medium: 10 g/L Bacto-tryptone, 5 g/L yeast extract, and 10 g/L NaCl. For solid media, add Bacto agar to a final concentration of 1.8–2% (w/v) before autoclaving. Tryptone, yeast extract, and Bacto agar can be obtained from Difco (Becton Dickinson Microbiology Systems).

13. LBAG medium: LB medium supplemented with 100 mg/L ampicillin and 0.4% (w/v) glucose.

14. LBAK medium: LB medium supplemented with 100 mg/L ampicillin and 50 mg/L kanamycin.

15. Antibiotics prepared as stock solutions in SDDW: 100 mg/mL ampicillin, 50 mg/mL kanamycin (Sigma).

16. 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-gal; Sigma) prepared as a stock solution at 20 mg/mL in either dimethyl
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sulfoxide or dimethyl formamide. Protect from light and store at −20°C. Spread 40 μL of stock to the surface of each selective agar plate and allow to dry before seeding bacteria.

17. α-Nitrophenyl β-D-galactoside (ONPG) (Sigma).

18. Z-buffer: 60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, and 50 mM 2-β-mercaptoethanol, pH 7.

3. Methods

3.1. Construction of an Immune Anti-NS3 Antibody Phage Display Library

An immune anti-NS3 murine scFv library was prepared using standard protocols (see earlier chapters in this volume). The library was prepared using splenocytes from mice that were immunized with recombinant NS3 as the source for antibody variable domains. All routine phage-display techniques were essentially as described (21). DNA encoding the assembled scFvs was digested with *Nco*I and *Not*I and ligated into a phage display vector, pCC14 (22). The ligated DNA was introduced into TG1 *E. coli* cells by electroporation, resulting in a library of 1.5 × 10⁷ clones.

3.2. Isolation of NS3-Inhibiting scFvs

3.2.1. Enrichment of the Immune Anti-NS3 Library for NS3-Binders

A single cycle of affinity selection of the phage antibodies was carried out using 25 μg of a fusion protein comprising maltose binding protein (MBP) and NS3. The fusion protein was immobilized on 100 μL of amylose resin (New England Biolabs). The affinity selection was carried out to enrich NS3 binders from the immune anti-NS3 library and by reducing the size of the library, thereby enable screening on indicator plates as will be described below. The output from the selection cycle was approximately 10⁵ clones.

3.2.2. Cloning of NS3-Binders into the pMALc-NN Vector

Pool affinity-selected phage and subclone the scFv inserts into pMALc-NN (20) (see Note 3). pMALc-NN is a vector for efficient cytoplasmic expression of MBP fusion proteins. The MBP-scFvs are under the control of an IPTG-inducible tac promoter, and the plasmid carries an ampicillin-resistance cassette and the *colE1* origin of replication, and is thus compatible with pMGT14, the vector for the genetic screen. The polylinker region of pMALc-NN contains *Nco*I and *Not*I restriction sites (see Note 4) that allow direct subcloning from many common phagemid vectors. In case your vector is based on different restriction sites, design PCR primers as described in Note 5.

1. Digest 10 μg of DNA from the scFv library with 10–20 U of *Nco*I and *Not*I restriction enzymes. Incubate for 1–2 h at 37°C (see Notes 6 and 7).

2. Load the digested DNA on a preparative 1.5% agarose gel for agarose gel electrophoresis. Excise the scFv-coding fragment (typically 750 bp) from the gel using a scalpel blade and carefully trim away any excess agarose.
3. Extract the DNA fragment using the QIAquick Gel Extraction Kit. Recover the DNA in 30–50 μL of DNA elution buffer.

4. Estimate the concentration of the recovered DNA using spectrophotometry. The NanoDrop ND-1000 spectrophotometer (NanoDrop) is particularly well suited to this task. The digested DNA is ready for ligation. Store at −20°C.

5. Digest 10 μg of the pMALc-NN vector DNA with 10–20 U of NeoI and NotI restriction enzymes. Incubate for 1–2 h at 37°C (see Notes 6 and 7).

6. Load the digested vector DNA on a preparative 1% agarose gel. Recover the 6,100 bp vector fragment from the gel as described above and repeat steps 3 and 4.

7. Set up 5–10 identical DNA ligation reactions that each contain:
   - 80 ng of purified NeoI- and NotI-digested scFv-coding insert
   - 100 ng of purified NeoI- and NotI-digested vector DNA
   - 2 μL of 10× ligation buffer
   - 1 μL of T4 DNA ligase
   - SDDW to complete a final volume of 20 μL.
   In all ligations, the molar ratio of vector:insert should be between 1:3 and 1:5. Incubate the reactions at 16°C for 16 h, or as recommended for cohesive ligation by the supplier of the T4 DNA ligase.

8. Combine the ligation reactions and load the entire sample to a Microcon-PCR device to purify and exchange from ligase buffer into SDDW. Following the first spin, add 500 μL of SDDW to the cartridge and spin again. Recover the DNA in a final volume of 50 μL.

9. Introduce the ligated DNA into TG1 E. coli cells by electroporation (see Note 8) and spread to YTAG agar plates. Leave for 16 h at 30°C.

10. Using a cell scraper or a disposable inoculation loop, scrape the cells from each plate into 10 mL of LBAG medium. Combine the cells and collect them by centrifugation. Resuspend the cells in 10 mL of LBAG medium containing 20% (v/v) sterile glycerol. Store the library glycerol stock in 1 mL aliquots at −80°C.

A genetic screen for NS3 protease activity was established as described (19). The screen is based on phenotypic changes that result from the expression of enzyme, substrate, and potential inhibitor, each under the control of a different promoter, within the same E. coli cell. The plasmid pMGT14 was designed as a vector for expression of the enzyme, a recombinant form of the HCV NS3 protease, and its substrate, an engineered derivative of the lacZ gene encoding an NS3-cleavable β-galactosidase enzyme. Coexpression of a scFv in E. coli cells carrying pMGT14 results in a phenotypic change that makes it possible to identify...
antibodies able to inhibit NS3. Construction of the pMGT14 plasmid vector was carried out in few steps as described below and illustrated in Fig. 1. We applied the screen to identify NS3-inhibiting antibodies directly from a large pool of scFv clones.

Fig. 1. Steps in the construction of plasmid pMGT14 for the bacterial genetic screen. The plasmid pMGT14 carries the coding information for both the substrate (NS3-cleavable β-galactosidase expressed under the control of the trpR promoter) and the enzyme component (MBP-scNS3 expressed under the control of the araBAD promoter) of the genetic screen. Construction of this plasmid carrying both lacZ with the inserted NS3 cleavage site (lacZNS5A/B) and MBP-scNS3 was done in a few steps of PCR, digestion, and ligation.
Design of the reporter system was based on the observation that certain sites within the *E. coli lacZ* gene are permissive for insertion of short sequences without totally compromising β-galactosidase activity (23). We reasoned that it should be possible to insert short sequences that encode for protease recognition sites at these positions. Protease-mediated cleavage would thus result in loss of β-galactosidase activity; conversely, inhibition of the protease would retain β-galactosidase activity.

To follow the construction steps listed below, it would be necessary to obtain plasmids from the author but similar constructs could be prepared from other *lacZ* expression plasmids in which the *lacZ* gene lies under the control of a weak constitutive promoter. Sites for protease recognition sequences can be chosen from information in the literature (23–25).

We chose the *trpR* promoter-controlled *trpR-lacZ* fusion construct from the plasmid pIB13. This carries the control region and the first 77 codons of *E. coli trpR* gene fused to the eighth codon of *lacZ* (26). The construct was transferred onto a low-copy p15A replicon plasmid that carries a kanamycin-resistance gene, plasmid pACYC177 (New England Biolabs). The resulting pEB13 plasmid is compatible with high-copy number plasmids that are based on the *colE1* replicon and that carry scFv with an independent (ampicillin) antibiotic marker.

Plasmid pEB13-Sfi carries two *SfiI* restriction sites between *lacZ* codons 282 and 283. In this plasmid, the open reading frame of the *lacZ* gene is disrupted by an in-frame TAA stop codon located between the *SfiI* sites (Fig. 2). *E. coli* cells that carry pEB13-Sfi form colorless colonies on indicative X-gal plates. Cloning between the *SfiI* sites of a DNA duplex for insertion of a protease recognition site results in restoration of the *lacZ* open reading frame and formation of blue colonies on indicative X-gal plates. Plasmid

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**Fig. 2. Cloning the NS5A/B site into pEB13-Sfi.** (a) The nucleotide sequence created around *lacZ* codons 282–283 (as marked above the text) in plasmid pEB13-Sfi. The two *SfiI* sites are indicated by underlining. The engineered stop codon is indicated in bold. (b) Insertion of the DNA duplex from the phosphorylated primers NS5A/B-SE and NS5A/B-AS into *SfiI*-digested pEB13-Sfi. The sequence encoding the NS5A/B site is highlighted in bold. (c) Nucleotide and amino acid sequence created around *lacZ* codons 282–283 (as marked above the text) in plasmid pEB13-NS5A/B. The sequence of the NS5A/B site is highlighted in bold.
pEB13-Sfi may serve as a general-purpose substrate plasmid for the insertion of any desirable sequence that specifies a protease cleavage site as a staggered DNA duplex. In the case described in this chapter, a sequence specifying the NS5A/B site was inserted, yielding a β-galactosidase construct that was cleavable by NS3. pEB13-Sfi can be prepared from pEB13 (Subheading “The trpR-lacZ Expression Vector pEB13”) as follows:

A primer set (LacZ-70-BACK, LacZ-282-FOR, LacZ-Sfi-282–280-BACK, and LacZ-520-FOR; Table 1) was designed for the insertion of a SfiI restriction site followed by TAA stop codon and a second SfiI restriction site between codon 282 and 283 of the lacZ gene carried on plasmid pEB13.

1. Set up two PCR amplification tubes of 25 μL total volume each, with 1× PCR buffer, 200 μM of each dNTP, 1 U high-fidelity thermostable DNA polymerase (such as Vent DNA polymerase), and 1 ng pEB13 plasmid DNA as template.

Table 1
Oligonucleotide primers

| Primers for construction of pEB13-Sfi |
|---------------------------------------|
| LacZ-70-BACK  5’ CCGGAAGCTGGCTGGAGTGC 3’ |
| LacZ-282-FOR  5’ GGCCCTCTATCGATAATTTCAC 3’ |
| LacZ-Sfi-282–283-BACK  5’ GAAATTATCGATGAGGGCCAACGTGGCCGGAATTTTTGGCCTCTGGGGCCCGTGGTGGTTATGCC 3’ |
| LacZ-520-FOR  5’ CAGCCGGGAAGGGCTGGTCTT 3’ |

B. Primers for NS5A/B site duplex (should be 5’ phosphorylated)

| NS5A/B-SE  5’ AGCTAGCGAGGACGTCGTCTGCTGCTCGATGTCCTACACTG 3’ |
| NS5A/B-AS  5’ TGTAGGACATCGAGCAGCAGACGTCCTCGCTAGCTCGT 3’ |

C. Primers for SphI-NotI sites duplex (should be 5’ phosphorylated)

| pEB13-dup-SE  5’ AATTGCATGCTGCAGGCGGCCGCGCGCCGCGC 3’ |
| pEB13-dup-AS  5’ AATTGCAGGCCGCGCCTGCAGGCGCATGC 3’ |

D. Primers for MBP-scNS3 cloning

| NS3-Nco-BACK  5’ TCAGTACCATGGGCGCCTATCGCTCGATGTCCTACACTG 3’ |
| NS3-Not-FOR  5’ GGGAAAGCGGCCGCGTACCTACGAGATGTTTCCATAGA 3’ |
| MalE-BspHI-BACK  5’ CCCTAATCATGAAAACGTGAAGAAGGCGTAA 3’ |

E. Primers for recloning of scFvs

| MALc6  5’ GACGCGCAGACTAATTCGAGC 3’ |
| MALc5  5’ CTGCAAGGCGATTAAGTTGGG 3’ |
To each tube add the following primer mixes in a final primer concentration of 1 μM each. The number in parentheses represents the expected size of the PCR product:

**Tube 1:** Primers LacZ-70-BACK and LacZ-282-FOR (see Note 9) to amplify the 5’ region of lacZ (~650 bp).

**Tube 2:** Primers LacZ-Sfi-282–280-BACK and LacZ-520-FOR to amplify the 3’ region of lacZ (~750 bp).

2. Proceed to thermal cycling according to the following conditions:
   - 5 min at 95°C (denaturation step)
   - cycles of: 94°C for 0.5 min; 55°C for 1 min; and 72°C for 1 min
   - Final elongation: 5 min at 72°C (final extension), hold at 4°C.

3. Analyze a 2 μL aliquot on a 1.5% agarose gel against an appropriate size marker. Reactions should yield a product with the length noted at step 1. If the results are satisfactory, separate the remaining PCR products on a 1.5% preparative agarose gel. Proceed to gel extraction and reamplification if needed (see Note 10) and estimate the concentration of the recovered DNA.

4. Set up an overlap-extension PCR tube of 50 μL total volume with 1× PCR buffer, 200 μM of each dNTP, 1.5 U high-fidelity thermostable DNA polymerase, 50 ng of each PCR product from the previous step as template, and the LacZ-70-BACK and LacZ-520-FOR primers at a final concentration of 1 μM each, to obtain the SfiI-TAA-SfiI containing lacZ gene (~1,400 bp).

5. Proceed to thermal cycling according to the following conditions:
   - 5 min at 95°C (denaturation step)
   - 30 cycles of: 94°C for 0.5 min; 55°C for 2 min; and 72°C for 2 min
   - Final elongation: 5 min at 72°C (final extension), hold at 4°C.

6. Repeat step 3.

7. Digest the purified PCR product with 10–20 U of BsulI restriction enzyme for 1–2 h at 37°C. Add 4–20 U BssHII restriction enzyme and incubate for an additional 1–2 h at 50°C.

8. Purify the digested PCR product using a Microcon-PCR device, recover it in 50 mL of DNA elution buffer, and quantify as described above.

9. Digest 2–3 mg of the lacZ gene-containing pEB13 vector DNA with 10–20 U of BsulI and BssHII restriction enzyme as described above. Chill the digested vector gradually at room temperature for 10–15 min and then on ice for 1–5 min.
Add 1 U of alkaline phosphatase and incubate for 20–30 min at 37°C.

10. Load the digested vector DNA onto a preparative 1% agarose gel. Recover the fragment of the linear vector from the gel according to the correct vector size, proceed to gel extraction, and estimate the concentration of the recovered DNA.

11. Set up a DNA ligation reaction that contains:
   - 50–100 ng of Bsu36I- and BssHII-digested and dephosphorylated vector DNA
   - 50–100 ng of Bsu36I- and BssHII-digested PCR product
   - 2 µL of 10× ligation buffer
   - 1 µL of T4 DNA ligase
   - SDDW to complete a final volume of 20 µL.

   Incubate at 16°C for 16 h, or as recommended by the supplier of T4 DNA ligase for cohesive ligation.

12. Transform the ligated DNA into competent cells, seed on agar medium containing X-gal and kanamycin. Look for white (colorless) colonies the next day and confirm by sequencing purified plasmid DNA that the intended changes have been introduced.

In plasmid pEB13-NS5A/B, the lacZ reading frame in pEB13-Sfi (Subheading “Construction of Plasmid pEB13-Sfi”) was restored by ligating an oligonucleotide duplex between the SfiI site, introducing an NS3 cleavage site. E. coli cells that carry pEB13-NS5A/B form blue colonies on X-gal-supplemented agar plates.

A double-stranded DNA duplex was prepared by mixing the phosphorylated primers NS5A/B-SE and NS5A/B-AS (Table 1). The duplex carries the coding sequence for the NS5A/B site flanked by staggered ends that are compatible for ligation into the SfiI-digested plasmid pEB13-Sfi. Insertion of the duplex resulted in the loss of both SfiI sites in the resulting plasmid, pEB13-NS5A/B (see Fig. 2).

1. To prepare a DNA duplex (see Note 11), incubate 300 pmol of each primer at 95°C, gradually decrease the temperature to the appropriate annealing temperature (see Note 12) at a rate of 0.1°C/s. Incubate at the annealing temperature for an additional 5 min.

2. Purify the DNA duplex and buffer-exchange it into 25 µL SDDW using a Microcon-PCR device.

3. To insert the duplex, digest 2–3 µg of the pEB13-Sfi vector with 20–40 U of SfiI restriction enzyme for 3–4 h at 50°C (see Note 13). Chill the digested vector gradually at room temperature for 10–15 min and then on ice for 1–5 min.
Add 1 U of alkaline phosphatase and incubate for 20–30 min at 37°C.

4. Gel-purify the DNA and perform a ligation as described in steps 10 and 11 of Subheading “Construction of Plasmid pEB13-Sfi.”

Transform the ligated DNA into competent cells, and plate on plates containing X-gal and kanamycin. Look for blue colonies the next day and confirm by sequencing purified plasmid DNA that the intended changes have been introduced.

Plasmid pEB13-NS5A/B carries the reporter gene, but not the gene for the NS3 protease. The NS3 sequence was recovered from pBAD-MBP-scNS3 (see Subheading “Construction of Plasmid pBAD-MBP-scNS3” below). To prepare plasmid pEB13-NS5A/B, a DNA duplex carrying restriction sites for SphI and NotI was cloned into the unique EcoRI site of plasmid pEB13-NS5A/B as follows: the duplex was assembled from phosphorylated primers pEB13-dup-SE and pEB13-dup-AS (Table 1) that together carry sites for SphI and NotI flanked by two EcoRI restriction sites. This provides SphI and NotI restriction sites for subcloning a DNA fragment bearing the gene for HCV-NS3 protease under the control of the araBAD promoter and the regulatory araC gene from pBAD-MBP-scNS3.

1. Repeat steps 1 and 2 of Subheading “Construction of pEB13-NS5A/B Vector” with the pEB13-dup-SE and pEB13-dup-AS primers.

2. Digest the purified PCR product with 10–20 U of EcoRI restriction enzyme for 1 h at 37°C and purify using the Microcon-PCR device again.

3. To insert the duplex, digest 2–3 μg of the pEB13-NS5A/B vector with 10–20 U of EcoRI restriction enzyme for 1 h at 37°C. Add 1 U of alkaline phosphatase and incubate for 20–30 min at 37°C.

4. Gel-purify the DNA and perform a ligation as described in steps 10 and 11 of Subheading “Construction of Plasmid pEB13-Sfi.”

5. Transform the ligated DNA into competent cells, seed on X-gal- and kanamycin-containing plates. Look for blue colonies the next day and analyze plasmid DNA.

In this construction, the NS3 protease gene was isolated and placed under the control of the E. coli araBAD promoter. This was achieved in two steps. The first step was to clone the NS4-NS3 (scNS3) protease of the 1b HCV genotype from plasmid pYB43 (27) into pMALc-NN vector (20). The resulting plasmid is pMALc-NN-scNS3, in which the NS3 protease in a single-chain
form is stabilized for efficient expression by fusion to MBP (MBP-scNS3), and expression is controlled by the tac promoter (19).

In the second step, the MBP-scNS3 protease was subcloned into a pBAD plasmid vector, placing expression under the control of the araBAD promoter. This was carried out by transferring the scNS3 from the pMALc-NN-scNS3 into the pBAD-TOPO DNA vector (Invitrogen). The resulting plasmid is pBAD-MBP-scNS3, where expression of MBP-scNS3 is controlled by the araBAD promoter. Construction of plasmid pBAD-MBP-scNS3 was carried out as follows:

A primer set (MalE-BspHI-BACK and NS3-Not-FOR, Table 1) was designed for the insertion of the BspHI and NotI restriction sites at the 5’ and 3’ ends of the MBP-scNS3 gene, respectively. BspHI generates a terminus that can be ligated to NeoI-cut DNA but the resulting product loses both BspHI and NeoI sites.

1. Set up a PCR amplification tube of 50 µL total volume with 1× PCR buffer, 200 µM of each dNTP, 1.5 U high-fidelity thermostable DNA polymerase, 1 ng template pYB43 plasmid, and the MalE-BspHI-BACK and NS3-Not-FOR primers at a final concentration of 1 µM each. The amplicon should be ~1,800 bp in size.

2. For PCR amplification, agarose gel electrophoresis, and purification, repeat steps 2 and 3 of Subheading “Construction of Plasmid pEB13-Sfi.”

3. Digest the purified PCR product with 10–20 U of BspHI and NotI restriction enzymes for 1 h at 37°C (see Note 14) and purify using a Microcon-PCR device.

4. Digest 2–3 µg of pBAD-TOPO DNA vector (Invitrogen) with 10–20 U of NeoI and NotI restriction enzymes for 1 h at 37°C (see Note 7). Add 1 U of alkaline phosphatase and incubate for 20–30 min at 37°C.

5. Gel-purify the DNA and perform a ligation as described in steps 10 and 11 of Subheading “Construction of Plasmid pEB13-Sfi.”

6. Transform the ligated DNA into competent cells, seed on LBAG plates, and look for positive clones by colony PCR (see Note 15). Check the plasmids for errors by DNA sequencing.

The final plasmid of the genetic screen, pMGT14, carries both the substrate (lacZ-NS5A/B) and the enzyme (MBP-scNS3) components for the screen. Hence we introduced the arabinose-regulated MBP-scNS3 fragment into the pEB13-NS5A/B-SphI-NotI vector between the SphI and NotI sites. The enzymatic activity of the engineered β-galactosidase in the presence or absence of MBP-scNS3 was tested in an ONPG hydrolysis assay. We found that the NS3-cleavable β-galactosidase enzyme had 12% β-galactosidase enzymatic activity in comparison with the wild-
type lacZ gene product encoded by pEB13. Nevertheless, E. coli cells that expressed the mutant enzyme formed blue colonies on X-gal indicator plates. The efficiency of substrate cleavage was, to some extent, arabinose dose dependent. However, cleavage was efficient even at the lowest concentration of inducer. This was in accord with our design of a system where the enzyme concentration will surpass that of the substrate.

Construction of pMGT14 was carried out as follows:

1. Digest 2–3 \( \mu \)g of the MBP-scNS3 gene-containing pBAD-MBP-scNS3 plasmid DNA with 10–20 U of SphI and 10–20 U of NotI restriction enzymes for 1–2 h at 37°C.

2. Load the digested products on a preparative 1% agarose gel, and recover the araBAD-MBP-scNS3 fragment (~3,100 bp) for gel extraction. Estimate the concentration of the recovered DNA.

3. Digest 2–3 \( \mu \)g of the pEB13-NS5A/B-SphI-NotI vector DNA with 10–20 U of SphI and 10–20 U of NotI for 1–2 h at 37°C. Add 1 U of alkaline phosphatase and incubate for 20–30 min at 37°C.

4. Load the digested vector DNA on a preparative 1% agarose gel, recover the vector fragment (~7,800 bp), proceed to gel extraction, and estimate the concentration of the recovered DNA.

5. Set up a DNA ligation reaction that contains:
   - 50–100 ng of purified SphI- and NotI-digested DNA
   - 50–100 ng of purified SphI- and NotI-digested and dephosphorylated vector DNA
   - 2 \( \mu \)L of 10× ligation buffer
   - 1 \( \mu \)L of T4 DNA ligase
   - SDDW to complete a final volume of 20 \( \mu \)L
   
   Incubate at 16°C for 16 h, or as recommended by the ligase supplier for cohesive ligation.

6. Transform the ligated DNA into competent TG1 cells, plate on X-gal-, arabinose-, and kanamycin-supplemented plates and place plates in a 37°C incubator for 16 h. Verify the clones by colony PCR and by DNA sequencing.

7. Grow 3 mL cultures of E. coli TG1 carrying pMGT14 in LBAK in culture tubes shaking at 250 rpm, 37°C. At an OD\(_{600nm}\) of 0.6, induce the cultures with arabinose at 0, 0.002, 0.02, and 0.2% (w/v) for 4 h at 30°C.

8. Measure the absorbance at OD\(_{600}\) (for future calculations).

9. Collect the cells by centrifugation in glass or polypropylene test tubes (see Note 16) and resuspend in 1 mL of Z-buffer.
10. Gently permeabilize the cell membrane by adding dropwise 50 μL chloroform and 50 μL of 0.1% SDS. Vortex at full speed for 20 s and leave for 5 min at 28°C.

11. Add 200 μL of ONPG substrate (4 mg/mL in SDDW solution).

12. Stop the reaction after 10 min at room temperature with 1/2 volume of 1 M Na₂CO₃.

13. Centrifuge the tubes for 2 min at 3,500 × g at room temperature and carefully transfer the cell-free supernatant into a cuvette (do not carry over any chloroform). Record the absorbance at 405 or 420 nm.

14. Calculate the β-galactosidase units according to Miller (28) (β-galactosidase units = \[\frac{\text{OD}_{420} [\text{step 13}] \times 1,000}{\text{OD}_{600} [\text{step 8}]} \] × t × v, in which t represents the reaction time in minutes, and v represents the sample volume in milliliters).

15. Prepare competent TG1-pMG14 cells (see Note 6).

A single cycle of affinity selection of the scFv library by phage display was used initially to enrich for NS3 binders. The enriched scFv library was cloned into the plasmid pMAlc-NN for the expression of intrabodies fused to MBP. The intrabody library was then introduced into pMGT14-carrying E. coli TG1 cells and screened for potential NS3 protease-inhibiting clones. The transformants were plated to yield individual colonies on indicator X-gal-supplemented agar plates and screened for blue colonies. The screen was carried out as follows:

1. Grow 100 μL of E. coli TG1 carrying pMALc-NN-scFv from Subheading 3.2.2, step 10 in 100 mL of YTAG in a 1 L Erlenmeyer flask, shaking at 250 rpm at 37°C overnight.

2. Prepare plasmid DNA using a QIAGEN plasmid maxi kit.

3. Transform the supercoiled plasmid library into the competent E. coli TG1 that carry pMG14 (see Note 8).

4. Plate the transformed cells on 2xYT agar plates supplemented with 0.004% (w/v) X-gal, 100 μg/mL ampicillin, 50 μg/mL kanamycin, 0.2% arabinose, and 0.05 mM IPTG.

5. Incubate the plates for 2–3 days at 25°C, until blue colonies appear.

6. Pick the blue colonies using a pipette tip. Touch the tip into a PCR mix for colony PCR (see Note 15) using primers MALc6 and MALc5 (Table 1). Pipette the rest of the colony into 3 mL of LBAK in 13 mL culture tubes and grow with overnight shaking at 250 rpm at 37°C. Prepare a glycerol stock (see Subheading 3.2.4, step 10).

7. Digest the PCR products with NeoI and NotI and reintroduce the candidate scFv inserts into pMAlc-NN DNA prepared by digestion with NeoI and NotI (see Subheading 3.2.4).
Introduce them also into chemically competent *E. coli* for preparing plasmid DNA that can be later analyzed by DNA sequencing. In addition, introduce the clones back into chemically competent *E. coli* TG1 carrying pMGT14 to validate the results of the initial screen (see Subheading 3.3.3, steps 4 and 5).

The potential of the selected scFvs to inhibit NS3 catalysis is evaluated quantitatively by measuring the β-galactosidase level that serves as an indicator for NS3 enzymatic activity level in the genetic screen. An ONPG hydrolysis assay is an example for a test that can be carried out on cultures of the selected clones. scFvs with the ability to inhibit NS3 activity should yield higher levels of β-galactosidase activity when compared with control noninhibitory scFvs (i.e., those picked from colorless colonies).

1. Grow cells in 3 mL of LBAK in culture tubes shaking at 250 rpm at 37°C. At an OD$_{600nm}$ of 0.6, induce with 0.2% (w/v) arabinose for 4 h at 30°C.

2. Repeat Subheading “Construction and Testing the Final Genetic Screen Plasmid, pMGT14”, steps 8–14.

3. Subsequently, the inhibitory scFvs should be expressed and purified, and evaluated in vitro for the inhibition of protease activity. For HCV NS3 protease inhibitors, we used a colorimetric assay we developed for that purpose (27). Commercial assays for NS3 protease activity that can be used to evaluate potential protease inhibitors exist as well (see http://www.anaspec.com/pdfs/71126.pdf).

4. Notes

1. Buffers and media are made in distilled, de-ionized water (Mili-Q biocel, Millipore), that are sterilized by autoclaving (SDDW).

2. If a restriction enzyme is only partially digesting, it may be necessary to add more enzyme units and/or longer incubation time than recommended in this protocol.

3. Plasmid pMALc-NN can be obtained from the corresponding author upon request. As an alternative, plasmid pMALc2x can be purchased from New England Biolabs and its polylinker modified for subcloning scFvs as described (20).

4. Whenever cloning between the *Nco*I and *Not*I restriction sites is referred to, the design of the recombinant gene should follow the rule: the sequence CCATGGCC should come before the first codon of the cloned open reading frame, and the sequence GCGGCCGCA should come after the last codon (without a stop codon).
5. For subcloning scFvs from a phage display vector where the scFvs were not initially cloned as *Nco*I-*Not*I fragments, use the following PCR primers to append those sites to your scFvs: 5′ end sense primer: ATATATCCATGGCC nnn nnn nnn nnn nnn nnn nnn nnn (nnn triplets are codons of the 5′ end of the VH domains under investigation) 3′ end antisense primer will be the antiparallel of 5′ nnn nnn nnn nnn nnn nnn nnn nnn GCGGCCGCATATAT (here nnn triplets are codons of the 3′ end of your VL domains) To avoid preparing large sets of family-specific primers that may be required for amplifying library-derived clone pools, a different solution is recommended: replace the *Nco*I and *Not*I restriction sites of pMALc-NN with a pair of sites suitable for direct subcloning from your own phage system.

6. For detailed protocols for DNA fragments digestion and separation, as well as for plasmid DNA purification and transformation, see Sambrook et al. (29).

7. Supercoiled plasmid DNA requires up to five-fold more *Not*I restriction enzyme for complete digestion than linear DNA. Simultaneous digestion with *Not*I and *Nco*I, *Bsp*HI, or *Sph*I ensures the complete digestion by *Not*I.

8. A protocol for preparing electrocompetent *E. coli* cells and for electroporation can be found in the literature (21).

9. Since in antibody engineering, rearranged antibody genes are recovered from messenger RNA (mRNA), the antisense BACK primers according to the gene are actually the primers used on the mRNA template. Hence in the antibody-engineering field, the BACK primers are the sense primers while the forward (FOR) primers are the antisense primers.

10. If no product or multiple products appear on the gel, optimize PCR conditions by modification of the MgCl₂ concentration, adding DMSO in different concentrations, varying the annealing temperature, and modification of the number of PCR cycles or the duration of each step in the cycling program.

11. When ordering primers for DNA duplex, it is necessary to order them with a 5′ phosphorylation to enable ligation with the vector.

12. The annealing temperature for the PCR program should be at least 5°C less than the lower melting temperature (TM) value of the primers.

13. The action of the *Sfi*I restriction enzyme is inhibited by dcm methylation of the restriction site, hence use a dcm⁻ *E. coli* strain for plasmid preparation or otherwise add an extra 20–40 U of *Sfi*I restriction enzyme and incubate for an additional 3–4 h at 50°C.
14. The action of the BspHI restriction enzyme is inhibited by dam methylation, hence use a dam - E. coli strain for plasmid preparation or otherwise add an extra 5–20 U of the restriction enzyme and incubate for an additional 1–2 h at 37°C.

15. For colony PCR, the bacterial clones picked from the agar plates are used as the template, hence the PCR program should start with 5 min of heating to 94–98°C to lyse the cells before the polymerization cycles. The best primer set would comprise one compatible with the insert and one with the vector.

16. As chloroform is used at a later stage in the method, tubes need to be made of a solvent-resistant material at this stage.

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Chapter 10

Guided Selection Methods Through Chain Shuffling

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Summary

We provide procedures for the panning of fully humanized Fab antibodies using guided selection. Human heavy and light chain genes are amplified. A parental light chain is cloned into a phage display vector and combined with the heavy chain library. After several rounds of panning, positive clones are identified and the heavy chain sequences that are recovered are combined with light chains for further selection by phage display. Human Fab antibodies are obtained that bind the same epitope as the parental antibody.

Key words: Guided selection, Chain shuffling, Human antibody fragment, Phage display, Antibody library

1. Introduction

Rodent monoclonal antibodies (mAbs) have significant limitations when used as therapeutic antibodies in humans, the most important being the immune response against rodent immunoglobulins and insufficient activation of human effector function. To overcome these hurdles, various so-called humanization strategies have been designed and implemented. These include the linking of rodent variable regions with human constant domains to form chimeric antibodies (Abs), grafting rodent complementarity-determining regions onto human Ab frameworks, and further approaches to full humanization (1–4).

Fully human mAbs minimize the risk of recognition as a foreign protein and hence fail to elicit an immune response when
administered to patients (5). Current strategies for the production of fully human mAbs include:

- The use of transgenic mice (Xenomice) (6) that have been generated by the introduction of segments from human immunoglobulin loci into mice that are unable to produce murine Abs as a result of gene targeting.

- Human-human hybridoma techniques (7), although this is complicated by the difficulty in generating stable hybridomas that secrete human Abs. It is also ethically unacceptable to immunize human patients.

- The use of Hu-PBL-SCID mice in which human peripheral blood lymphocytes (PBLs) are transplanted into immunodeficient SCID mice to reconstruct the human immune system in a rodent background (8).

- Recombinant human antibody techniques based on the construction and screening of libraries of human Abs from naïve or immunized donors, or of synthetic origin.

  The last approach makes possible the large-scale screening of Ab gene repertoires using phage display, ribosome display (9), yeast surface display (10), and “peptides-on-plasmid” display (11); all have been successfully used for the selection of Abs, but phage displaying remains the most widely utilized strategy.

Since 1994, guided selection through chain shuffling has been extensively used for the humanization of mouse mAbs (12, 13). One of the advantages of this strategy over the direct selection by phage display from antibody libraries is that antigen binders may be more frequently selected from genes present in a large antibody library. It also makes possible the generation of recombinant human mAbs that bind to the same epitope as a conventional mouse mAb produced by hybridoma technology (12–15). This chapter describes methods for the guided selection of a Fab antibody of fully human sequence. In overview, the gene encoding the light chain of the rodent mAb of interest is inserted into a phagemid display vector. A human heavy chain library encoding Fd fragments (the heavy chain variable domain and the first constant region domain) is inserted to create a hybrid Fab display library that is part rodent (light chain) and part human (heavy chains). Panning on the target antigen is used to extract binders, their attachment to the target being guided by the rodent light chain and any human Fd that complements this specificity. The rodent light chain is then replaced with human light chains and selection is repeated to isolate Fabs that are completely human in sequence and possess the same specificity of the parental antibody (see Note 1).
2. Materials

1. PBLs from patients or healthy individuals. Preferably, the donors have a high titer of Abs against the target antigen (see Note 2).

2. Reagents and molecular biology kits for RNA isolation, complementary DNA (cDNA) synthesis (Invitrogen), and extraction of DNA from agarose (Qiagen).

3. Reagents for PCR (TaKaRa) and primers for amplification of human Fd and light chain repertoires.

4. Restriction enzymes appropriate to the phage display vector chosen for the experiment and DNA ligases.

5. Phage display: pComb3, pComb3H, pComb3X, or another phagemid vector appropriate for Fab display.

6. Fd and/or light chain polymerase chain reaction (PCR) products from the rodent (parental) mAb of interest (see Note 3).

7. Host bacteria: *Escherichia coli* TOP10F', TG1, XL1-Blue, or other suitable strains for phage infection and propagation.

8. Bacteriological media for growth and selection of *E. coli*: SOC, 2xYT, or other media.

9. Disposable plasticware: 176 cm² cultivation dishes, 96 well enzyme-linked immunosorbent assay (ELISA) microtiter plates.

10. Buffer solutions: 20% (w/v) polyethylene glycol 8000, 15% NaCl (w/v) for phage precipitation; phosphate-buffered saline (PBS); 1% (w/v) bovine serum albumin (BSA) in PBS; 0.05 M bicarbonate buffer, pH 9.6; washing buffer (PBS-T: 0.05% [v/v] Tween-20 in PBS); elution buffer (0.1 M glycine–HCl, pH 2.2, containing 0.1% [w/v] BSA); and 2 M Tris base.

11. Apparatus: thermal cycler for PCR, electroporation apparatus.

12. Helper phage: M13KO7 (New England Biolabs) or helper phage appropriate for the display system in use.

13. Target antigen, cell lysate, etc., to which the parental mAb binds (see Note 4).

14. Isopropyl β-D-1-thiogalactopyranoside (IPTG).

15. Primary and secondary antibodies for immunological assay: horseradish peroxidase-labeled mAb or polyclonal Ab against phage protein pIII, goat polyclonal anti-human F(ab')₂ (IgM + IgG + IgA H + L), or goat anti-human IgG. Parental mAb for control study.
3. Methods

3.1. Amplification of Human Fd and Light Chain Sequences

1. Separate PBLs by density gradient centrifugation, and recover total RNA using an RNA isolation kit. Assess RNA quality by agarose gel electrophoresis and spectrophotometry. Prepare first-strand cDNA using a cDNA synthesis kit (see Note 2).

2. Carry out PCR amplifications in 50 μL volumes containing 5 μL of cDNA synthesis product, 0.2 μM of each primer, 200 μM dNTPs, 5 μL of 10× PCR buffer, and 1 U of Taq Plus polymerase. Primers annealing to the heavy and light chain variable domain sequences should be combined with the corresponding primers for constant region sequences. Typical thermal cycling conditions are 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min (30 cycles); followed by 72°C for 10 min (see Notes 5 and 6).

3. Analyze the amplified DNA fragments on a 1% (w/v) agarose gel, excise, and purify with gel extraction reagents.

3.2. Construction and Preparation of Hybrid Fab Phage Display Libraries

1. Prepare the display vector by restriction digestion and ligate into relevant sites the digested light chain amplification product derived from the parental rodent mAb. In the pComb3X vector used in our laboratory, the light chain products are inserted as SacI/XbaI fragments.

2. Digest the purified human Fd PCR fragments with appropriate enzymes for ligation into the display vector. For pComb3X, the products are digested with SpeI and XhoI. Ligate the Fd fragments into the vector that carries the light chain gene of the parental mAb (see Note 7).

3. Following ligation, transform the DNA into electrocompetent E. coli XL1-Blue or another appropriate strain by electroporation.

4. After electroporation, add 3 mL SOC medium and shake the culture at 225 rpm for 1 h at 30°C.

5. Remove aliquots for plating onto selective agar to determine the library size and diversity. The remaining bacteria are plated on large 176 cm² dishes of agar containing appropriate antibiotics and glucose to repress expression of the Fab protein. E. coli XL1-Blue transformed with pComb3X are selected on agar containing 100 μg/mL carbenicillin, 20 μg/mL tetracycline, and 1% (w/v) glucose. Incubate overnight at 30°C.

6. Scrape the bacteria from one plate into 200 mL of 2xYT liquid medium containing 100 μg/mL carbenicillin, 20 μg/mL tetracycline, and 1% (w/v) glucose (see Notes 8 and 9).

7. Shake the culture for 1.5 h at 30°C then add 10¹² pfu of M13KO7 or helper phage appropriate for the display system in use. Incubate without shaking for 2 h at 37°C.
8. Pellet the bacteria by centrifugation at $3,500 \times g$ for 15 min at 4°C. Discard the supernatant and resuspend the pellet in 200 mL 2xYT containing 100 μg/mL carbenicillin, 20 μg/mL tetracycline, and 70 μg/mL kanamycin. Incubate the culture with shaking at 30°C overnight.

9. Pellet the bacteria by centrifugation at $10,000 \times g$ for 15 min at 4°C.

10. Collect the supernatant and precipitate the phage by addition of polyethylene glycol 8000 and NaCl to final concentrations of 4% and 3% (w/v) respectively. Incubate on ice for 30 min and centrifuge at $3,500 \times g$ for 30 min to collect the phage.

11. Resuspend the phage pellets in 2 mL PBS containing 1% (w/v) BSA and microcentrifuge for 3 min to pellet bacterial debris. Transfer the supernatant to fresh tubes and store at 4°C.

12. Titrate the library by preparing serial dilutions, infecting into an appropriate strain of *E. coli*, and plating on selective agar containing the antibiotic against which the phagemid vector confers resistance.

3.3. Panning the Hybrid Fab Phage Display Library (see Note 10)

1. Coat a single well of an ELISA microtiter plate with 100 μL of the target antigen at a concentration of 50 μg/μL in 0.05 M bicarbonate buffer, pH 9.6.

2. Wash the well twice with PBS and block the plastic by completely filling the well with 1% (w/v) BSA in PBS. Incubate at 37°C for 1 h.

3. Add 100 μL of the hybrid Fab library (typically $10^{12}$ cfu) and incubate the plate at 37°C for 2 h.

4. Remove the unattached phage and wash the well with PBS-T five times. In later rounds of selection, increase the number of washes to 10 (second round), and 15–20 (third round).

5. Wash the plate once more with distilled water and elute adherent phages by adding 100 μL of elution buffer with incubation for 10 min at room temperature.

6. Pipette the elution buffer up and down several times, remove to a fresh tube, and neutralize by adding with 6 μL of 2 M Tris base.

7. Prepare a fresh culture of *E. coli* XL1-Blue or other strain suited to infection and propagation of phage. Grow the bacteria into log phase and sample a 2 mL aliquot.

8. Add the sample of neutralized phage and incubate for 15 min at room temperature to allow infection to take place.

9. Plate the bacteria onto 176 cm² dishes of selective agar and incubate overnight at 30°C. The bacterial colonies can be used for the preparation of phage and the next round of panning.
3.4. Construction and Panning of Human Phage Fab Libraries

The preceding methods should yield hybrid antibodies that carry a single light chain sequence derived from the parental rodent mAb, and human Fd sequences that are able to impart affinity toward the target antigen of interest. In the steps that follow, human light chain sequences are sought that can replace the contribution of the rodent light chain.

1. Take the human light chain PCR products generated earlier (Subheading 3.1) and digest them with enzymes appropriate for ligation into the phage display vector. In the pComb3X vector, light chain sequences are inserted between the SacI and XbaI sites, so the light chain products should be digested with these restriction enzymes and purified.

2. Purify phagemid DNA from the pComb3X/hu-Fd clones that emerged from panning (Subheading 3.3). Excise the rodent light chain by digesting with the same enzymes as in step 1 (e.g., SacI and XbaI).

3. Construct a completely human Fab display library by ligating the light chains into the prepared vector.

4. Prepare phage that display the human Fab fragments.

5. Use panning on antigen-coated plastic to isolate human Fabs with affinity for the target antigen of interest.

3.5. Preparation of Fab-pIII Fusion Proteins for Analysis (see Note 11)

ELISA with clonal stocks of phage can be used to confirm the specificity of displayed Fabs for the target antigen of interest. However, ambiguities in the results can be resolved by testing the properties of a soluble form of the recombinant Ab. The Fab display vector pComb3X, in common with many vectors for the display of single-chain Fv fragments (scFvs), carries an amber stop codon between coding sequences for the Ab and the phage protein pIII. Expression in amber-suppressing strains of E. coli (e.g., XL1-Blue, TG1) results in the Ab-pIII fusion protein that is essential for display of the immunoglobulin on phage. Induction of high-level expression with IPTG results in sufficient levels of synthesis for ELISA analysis using bacterial lysates (see below), although much of the fusion protein accumulates in the bacterial membrane. Transfer of the phagemid vector to a non-suppressing E. coli host (e.g., HB2151) will result in expression of the Ab alone.

1. After the final round of panning, pick single E. coli XL1-Blue colonies for storage at −70°C in glycerol-supplemented medium.

2. Plate out individual isolates and grow each one separately at 30°C in 2 mL of 2xYT medium containing 100 μg/mL carbenicillin, 20 μg/mL tetracycline, and 1% (w/v) glucose, or antibiotics suitable for the bacterial strain and display vector in use. Grow until an absorbance at 600 nm of approximately 0.6 is achieved.

3. Pellet the bacteria from each culture by centrifugation at 3,500 × g for 10 min at 4°C and resuspend the cells in 10 mL
aliquots of 2xYT containing 100 μg/mL carbenicillin and 20 μg/mL tetracycline.

4. Add IPTG to each culture to a final concentration of 1 mM and shake each culture overnight at 30°C.

5. Harvest the cells by centrifugation at 3,500 × g for 10 min at 4°C and wash each pellet once by resuspension in 2 mL PBS.

6. Pellet each stock again by centrifugation and resuspend in 1 mL aliquots of PBS. Lyse the cells by three to four cycles of freeze–thawing.

7. Pellet intact cells and debris by centrifugation at 10,000 × g for 5 min. Recover each supernatant; the supernatant contains sufficient Fab-pIII fusion protein for testing in ELISA or another analytical method to check for binding to the antigen of interest.

8. Binding of the Ab to the target antigen can be detected with reagents against the pIII component, detection tags carried on the construct (e.g., the HA influenza epitope carried on pComb3X, or c-myc carried on many other vectors) or human Ab epitopes carried on the light and/or heavy chains. The parental rodent mAb should be used as a control (see Note 12).

4. Notes

1. Suboptimal interaction of the human Fd and light chain components may mean that the human Fab possesses a lower affinity for the target antigen than the parental rodent Fab. To reduce the chances of this outcome, we suggest two approaches:

   (a) The construction in parallel of two hybrid libraries: the first carrying the rodent light chain and a library of human Fd sequences; the second, the inverse, in which the rodent Fd is paired with a library of human light chains. After screening both with the antigen, the human sequences from each library are pooled and the Fd/light chain pairing with the highest affinity for the antigen is extracted by panning.

   (b) A step-by-step approach, as described in this protocol. We suggest that the parental light chain is used initially to guide the selection of human Fd sequences because the complementarity-determining regions of the heavy chain are likely to play a dominant role in the interaction of Fab and antigen.

2. Bone marrow, lymph nodes, or PBLs from patients or healthy individuals with high serum titers against the target antigen make good sources of total RNA for PCR. For targets that are
nonimmunogenic in a human donor, sequences can be taken from a naïve human library.

3. Guided selection can begin with either the light chain or Fd from the parental mAb but, in our published studies, the parental light chain sequence was paired with a repertoire of human Fd chains. The method could be adapted by using just the variable domain sequence from the parental mAb, linking this with a human light chain constant domain to avoid problems of poor interaction between rodent and human constant domains.

4. Problems may arise if fusion proteins or cell lysates are used for panning because the selection may drift toward the recognition of molecules with some degree of cross-reaction with the intended target. If highly purified antigen is available, this makes the best target for panning.

5. The construction of a large human phage antibody library with good diversity is based on the amplification of all human Ab genes. To construct the antibody library successfully, the first step is to amplify as many genes as possible by choosing a reliable set of oligonucleotide primers. Other chapters in the volume provide this information and it is widely available in the published literature. Cleavage sites for restriction enzymes used in cloning must be present in the primers, in the appropriate reading frames. Examples of primer sets that amplify of human Ab sequences with good efficiency can be found in our published work (15).

6. To avoid the loss of rare Ab genes during amplification, multiple combinations of forward and reverse primers may be required. In our experience, primers can be divided into distinct groups and, in some cases, they can be mixed for PCR; see other chapters in this volume.

7. Rare clones may be lost during amplification, ligation, and panning. It may help to generate several Fab libraries for panning.

8. How big does a library need to be? This will depend on whether the Ab sequences are derived from an immune individual or from a naïve library. An immune Ab library with >10^8 cfu may be sufficient for extraction of clones of value but if the sequences are derived from a naïve human source, it is likely that the library will have to be perhaps two orders of magnitude bigger to be of use. One advantage of guided selection is that it is quite feasible to pair a rodent light chain with 10^7 human Fd sequences, select, and then combine the extracted Fds with a further 10^7 human light chains (14). It would be completely impractical to attempt construction of a library in which all possible combinations of 10^7 unique Fd sequences and 10^7 light chains were available for selection.
9. For successful construction of a phage display library, it is vital to plan an evaluation strategy. We suggest that the following checks should be included:

(a) Careful estimation of the overall size of the library.
(b) Assessment of the success of insertion of PCR products. We propose that >90% of clones tested should carry the insert being ligated.
(c) Use of DNA sequencing to analyze the diversity of the inserted sequences.

10. Ideas for panning strategies can be taken from this volume and the literature. We emphasize the value of halving the coating concentration of target antigen, round by round, to drive the isolation of positive clone(s) of high affinity. We also advocate increasing the washing times with successive panning rounds in order to increase stringency.

11. Various types of assays can be used for analysis of the specificity and affinity of Ab recovered from panning. Phage ELISA (see other chapters in this volume) is an easy and convenient method for the identification of the positive clones in an initial evaluation. Greater precision comes from the analysis of purified, soluble, monomeric Ab fragments. This can be logistically difficult if large numbers of clones are to be screened: this method provides a convenient intermediate-level approach.

12. The following methods should be considered for an analysis of the specificity and affinity of selected Abs:

(a) Direct ELISA to provide initial on the specificity of Ab-antigen binding.
(b) Competitive ELISA. Assays in which Ab binding is made competitive with the parental mAb can assess whether the recombinant Ab recognizes the same epitope as the parental mAb.
(c) Western blotting.
(d) Immunohistochemistry using cells or tissues along with flow cytometry.
(e) Determination of association and dissociation rate constants, $K_{on}$ and $K_{off}$. Surface plasmon resonance can be used to obtain this information.
(f) Use in animal models of disease to analyze the biological properties of the selected Ab in vivo.
(g) DNA sequencing and analysis.
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Sequential Antigen Panning for Selection of Broadly Cross-Reactive HIV-1-Neutralizing Human Monoclonal Antibodies

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Summary

Many phage display techniques drive selection toward the isolation of highly specific antibodies. However, the identification of monoclonal antibodies that are cross-reactive has implications for the development of diagnostics, therapeutics, and vaccines against pathogens or cancer cells that are able to rapidly generate variants and escape mutants. To identify human monoclonal antibodies with high activity against HIV and broad-spectrum activity, we developed a technique termed sequential antigen panning. This methodology could be used to isolate recombinant antibodies against any antigen that shares epitopes with other antigens.

Key words: Selection Fab; HIV gp120; gp140; Cross-reaction

1. Introduction

Phage display is a powerful methodology for selection of binders specific for any given target (1–4). After longer than a decade of development, antibody phage display has become a relatively mature methodology and has been successful in various applications (5, 6). The results from panning a phage-displayed antibody library depend largely on antigen property (solubility and purity), antibody library quality (library size and diversity, antibody format, phage fusion partner, etc.), and panning method (solid-phase or solution-phase panning). Monoclonal antibodies with high affinity (nanomolar or even picomolar) for the target of interest can be isolated from phage-displayed antibody libraries (7).
Identification of broadly cross-reactive HIV-1-neutralizing human monoclonal antibodies has implications for the development of HIV-1 therapeutics and vaccines. HIV-1 has evolved various mechanisms for evading immune surveillance (8, 9). The ability of the virus to rapidly generate mutants to escape immune responses and drugs has become a fundamental problem in prevention and treatment of HIV-1 infections. We have aimed to develop antibody-based inhibitors of viral entry that have potential for HIV-1 therapy and prophylaxis. To identify Env-specific human monoclonal antibodies with high affinity and broad-spectrum activity in viral neutralization, we developed a methodology, termed sequential antigen panning (SAP), by sequentially changing antigens during panning and screening (10). Using SAP and recombinant Env ectodomain gp140s from 89.6 and IIIB (clade B) as antigens for panning, and gp140s from 89.6, IIIB and JR-FL (clade B) as antigens for screening, we identified from an HIV-1 immune antibody Fab library a panel of human monoclonal antibodies that are broadly cross-reactive with Env from different clades (10–12). The SAP methodology that was used could also be used for any antigen that shares common epitopes with other antigens. Examples include but are not limited to rapidly mutating viruses and cancer cells, as well as proteins that share common structural elements. Finally, variations of this methodology can be devised, including strategies to use more antigens in different orders during panning and screening.

2. Materials

2.1. Biotinylation of Antigens

1. Phosphate-buffered saline (PBS): 9.0 g/L NaCl, 144 mg/L KH₂PO₄, 795 mg/L Na₂HPO₄, pH 7.4.
2. 1 M NaHCO₃: 84.01 g/L NaHCO₃, pH 8.6.
3. 2 M glycine: 150.14 g/L glycine.
4. H₂O: molecular biology grade.
5. Freshly prepared 10 mM biotin stock: weigh 2.2 mg of EZ-Link Sulfo-NHS-LC-Biotin (Pierce) (molecular weight: 556.59), dissolve in 400 μL H₂O (see Note 1).
6. Microcon YM-10: regenerated cellulose 10 kDa molecular weight cut-off (Millipore) (see Note 2).

2.2. Preparation of Phage Library

1. 2YT medium: 16 g/L Bacto-tryptone, 10 g/L Bacto-yeast extract, 10 g/L NaCl, pH 7.0; autoclaved for 20 min at 15 psi on liquid cycle.
2. *Escherichia coli* TG1: suppressor strain (K12, Δ[lac-pro], supE, thi, hsdD5/FtraD36, proA+B+, lacIq, lacZ M15) for propagation of phage particles.

3. M13KO7 helper phage (Invitrogen).

4. Ampicillin: 200 mg/mL stock, filter-sterilized through a 0.2 μm filter.

5. 20% (w/v) glucose: filter-sterilized through a 0.2 μm filter.

6. PEG/NaCl: 20% (w/v) polyethylene glycol (PEG) 6000, 117 g/L NaCl. Autoclave for 20 min at 15 psi on liquid cycle (see Note 3).

7. 2YTAG agar plates: 1.5% (w/v) agar in 2YT medium, autoclaved for 20 min at 15 psi on liquid cycle. After cooling to 42–45°C, supplement to final concentration of 200 μg/mL with ampicillin and 2% (w/v) glucose, and pour the medium onto 9 cm petri dishes. Keep the plates at 4°C when agar has solidified.

8. Glycerol: autoclaved for 20 min at 15 psi on liquid cycle.

### 2.3. Panning of Phage Library

1. 5% and 3% MPBS: 5% and 3% (w/v) skim milk in PBS.

2. Freshly prepared 100 mM triethanolamine (TEA): 700 μL triethanolamine (7.18 M original concentration) in 50 mL H2O, diluted prior to use.

3. 1 M Tris–HCl, pH 7.5: 121.14 g/L Tris base, adjust pH to 7.5 using 1 N HCl.

4. PBST: 0.1% (v/v) Tween-20 in PBS.

5. Streptavidin-coated Dynabeads M-280 (Invitrogen).

6. Magnetic separation apparatus: Dynal magnetic stand (Invitrogen).

7. Head-to-tail rotator.

8. Bioassay plates: Bioassay petri dish, 245 mm (VWR).

9. Recombinant gp140<sub>89.6</sub>, gp140<sub>IIIB</sub>, and gp120<sub>JR-FL</sub>: produced by recombinant vaccinia viruses and purified by a combination of lentil lectin affinity chromatography using Sepharose 4B (Pierce) followed by size-exclusion chromatography using a HiLoad 16/60 Superdex 200 prep-grade column (Amersham Bioscience).

10. HIV-1 immune antibody Fab library: constructed using pComb3H phagemid vector and 30 mL of bone marrow obtained from three long-term nonprogressor patients whose sera exhibited the broadest and most potent HIV-1 neutralization among 37 HIV-infected individuals.

### 2.4. Phage ELISA

1. Coating buffer: 8.401 g/L NaHCO<sub>3</sub>, 29.25 g/L NaCl, pH 8.3.

2. Horseradish peroxidase (HRP)-conjugated monoclonal mouse anti-M13 antibody (GE Healthcare).
3. Methods

3.1. Biotinylation of the Antigens

1. Take the vial of biotin reagent from the −20°C freezer and let it equilibrate to room temperature before opening the vial in step 4.

2. Prepare the antigens. Adjust the concentration of the antigens (recombinant gp140 proteins) to 0.1–1 mg/mL in a suitable buffer (e.g., a phosphate-based buffer). Avoid buffers containing Tris or glycine—if the original buffer contains Tris or glycine, change the buffer to PBS or another suitable buffer before biotinylation.

3. Add 1/10 (v/v) of 1 M NaHCO₃ to the antigen solution.

4. Prepare 10 mM biotin stock: gently open the biotin bottle, weigh 2.2 mg biotin reagent, and dissolve it in 400 µL of molecular biology-grade water.

5. Add a 20-fold molar excess of biotin to the antigens.

6. Incubate at room temperature for 30 min.

7. Add 1/20 (v/v) of 2 M glycine to the mixture to terminate the reaction.

8. Dialyze the mixture against PBS (change the buffer three to four times) or use Microcon YM-10 to remove free biotin if dealing with a small volume.

9. Check the efficiency of the biotinylation reaction by dot blotting.
1. Inoculate 500 μL of a glycerol stock of E. coli TG1 carrying an HIV-1 immune human antibody Fab library (see Note 4) into 500 mL 2YT medium supplemented with 200 μg/mL ampicillin and 2% glucose, grow at 37°C for approximately 3 h with shaking at 250 rpm till the optical density at 600 nm reaches 0.6.

2. Transfer 25 mL of culture into a 50 mL centrifuge tube (see Note 5), add 4 mL M13KO7 helper phage (>1 × 10^{11} cfu/mL) to the tube, and gently mix by inverting the tube twice (see Note 6). Keep the tube stationary at 37°C for 30 min. Keep the remaining 475 mL of the culture growing for 3 h, centrifuge at 3,300 × g for 20 min, resuspend the cell pellet in 2YT medium containing 15% glycerol, and store the glycerol stock at −80°C for future use.

3. Centrifuge the helper phage-infected culture at 3,300 × g for 10 min, resuspend the cell pellet in 500 mL 2YT medium supplemented with 200 μg/mL ampicillin and 35 μg/mL kanamycin (2YTAK medium) (see Note 7), and grow the cells at 30°C for 16–20 h with shaking at 250 rpm.

4. Spin down the cells at 11,000 × g for 10 min (see Note 8), divide the supernatant between two 500 mL centrifuge bottles, add 1/5 (v/v) of PEG/HCl (50 mL) to each bottle, and mix well. Keep the bottle at 4°C for 1 h or more.

5. Centrifuge the solution at 11,000 × g for 30 min, remove the supernatant (see Note 9), resuspend the phage pellet in each bottle in 20 mL sterile H₂O, and combine. Add 1/5 (v/v) PEG/HCl (8 mL) to the phage suspension, mix well, and keep the mixture at 4°C for 20 min or more.

6. Centrifuge the phage solution at 11,000 × g for 10 min, remove the supernatant, centrifuge again for 2 min, and remove the residual supernatant.

7. Resuspend the pellet in 5 mL PBS in 15 mL tubes, and centrifuge at 11,600 × g for 10 min to remove cell debris. Transfer the supernatant containing phage particles to a fresh tube.

8. Titer the phage library. Prepare serial ten-fold dilutions of the phage in PBS, then take 1 μL of 10^{-6}, 10^{-7}, and 10^{-8} dilutions and infect each to 1 mL of log-phase E. coli TG1 cells by incubating at 37°C for 30 min without shaking. Spread 50 μL of cells of each infection mix onto 2YTAG plates, incubate the plates at 30°C overnight, count the colonies, and calculate the phage titer (see Note 10). Alternatively, the phage titer can be estimated by measuring the optical density at 280 nm (1 OD_{280nm} = 2.33 × 10^{12} cfu/mL).

9. Keep the phage library at 4°C for short-term storage or add 15% glycerol and keep it at −80°C for long-term storage (longer than a year).
3.3. Sequential Antigen Panning of Phage Library

1. Take $5 \times 10^{12}$ to $1 \times 10^{13}$ cfu phage particles from the phage library to a sterile 1.5 mL microfuge tube, add 500 μL of 5% MPBS and 100 μL pre-washed streptavidin-M280-Dynabeads, and bring the final volume up to 1 mL by adding PBS. Incubate the solution at room temperature for 1 h with gentle mixing on a head-to-tail rotator.

2. Separate the Dynabeads from the solution by letting the tube sit in a magnetic separator for a few minutes. Transfer the solution to a fresh 1.5 mL microfuge tube.

3. To the 1 mL solution of phage, add biotinylated recombinant gp140<sub>89.6</sub> to a final concentration of 50 nM, and incubate the antigen/phage mixture at room temperature for 2 h with gentle mixing on a head-to-tail rotator.

4. Add 100 μL prewashed streptavidin-M280-Dynabeads to the mixture, and keep rotating for 30 min.

5. Separate the Dynabeads from the solution as described in step 2; wash the beads ten times with PBST and ten times with PBS, using the magnetic separator to recover the biotinylated gp140<sub>89.6</sub> with any attached phage after each wash.

6. Elute bound phage from the Dynabeads by adding 1 mL of freshly prepared 100 mM TEA and incubating the beads at room temperature for 10 min with gentle shaking on a head-to-tail rotator. Separate the beads from solution, transfer the eluted phage to a 50 mL centrifuge tube containing 0.5 mL of 1 M Tris HCl, pH 7.5, and vortex the eluted phage for 10 s.

7. Add 10 mL log-phase <i>E. coli</i> TG1 culture to the eluted phage, keep the tube still, and incubate the phage/cell mixture at 37°C for 30 min.

8. Centrifuge the mixture at 3,300 × g for 10 min, remove the supernatant, and resuspend the cell pellet in 1 mL 2YT medium. Titer the recovery of phage from the first round of panning by preparing serial ten-fold dilutions from an aliquot of the mixture, and spreading 50 μL from the $10^{-4}$, $10^{-5}$ dilutions onto 2YTAG plates and incubating the plates at 30°C overnight. Spread the rest of the bacterial mixture onto a bioassay plate containing 2YTAG agar, and incubate the bioassay plate at 30°C overnight.

9. Prepare phage for the second round of panning. Follow the methods in Subheading 3.2, but with decreased scale and a simplified procedure. Briefly, add 5 or 6 mL 2YT medium containing 15% glycerol to the bioassay plate and scrape the colonies of bacteria into a suspension. Take 100 μL, inoculate into 100 mL 2YTAG medium (<i>see Note 11</i>), and grow to log-phase (OD<sub>600nm</sub> = 0.5–0.6) (~2 h). Take 10 mL of the culture and infect with 1.6 mL M13KO7 helper phage. Centrifuge the cells, resuspend the cell pellet in 100 mL 2YTAK, and
grow at 30°C overnight. Centrifuge the culture, precipitate phage particles from the supernatant by adding 1/5 (v/v) PEG/HCl, and recover by centrifuging at 11,000 × g for 10 min. Resuspend the phage in 2 mL PBS and centrifuge at 11,600 × g for 10 min to remove bacterial debris. Keep the amplified phage at 4°C.

10. Second round of panning against 10 nM biotinylated gp140$_{89.6}$ (see Note 12): repeat steps 1–8 except that the input of phage can be reduced to $1 \times 10^{11}$-1 × $10^{12}$ cfu and the antigen concentration can be reduced to 10 nM. In addition, the washing stringency is increased to 20 washes of captured Dynabeads with PBST and 10 washes with PBS.

11. Prepare phage for the third round of panning by repeating step 9.

12. Third round of panning against 50 nM biotinylated gp140$_{IIIB}$: Repeat steps 1–8. The input of phage can be maintained at that used for the second round of panning.

13. Prepare the phage library for the fourth round of panning. Repeat step 9.

14. Fourth round of panning against 10 nM biotinylated gp140$_{IIIB}$: Repeat step 10, except that the antigen is 10 nM biotinylated gp140$_{IIIB}$ and the washing stringency is increased to 20 washes with PBST and 10 washes with PBS.

15. Prepare the phage library for the fifth round of panning. Repeat step 9.

16. Fifth round of panning against 2 nM biotinylated gp140$_{89.6}$: Repeat step 10 except that the antigen concentration is decreased to 2 nM.

17. Prepare the phage library for the sixth round of panning. Repeat step 9.

18. Sixth round of panning against 2 nM biotinylated gp140$_{IIIB}$: Repeat step 16 except that the antigen is 2 nM biotinylated gp140$_{IIIB}$ (see Note 13).

3.4. Polyclonal Phage ELISA

1. Coating. Prepare 1 μg/mL gp140$_{89.6}$ and gp140$_{IIIB}$ in coating buffer, then coat each well on MaxiSorp plates with 100 μL of solution (see Note 14). Incubate the plates at 4°C overnight.

2. Blocking. Wash the plates four times with PBST, then add 200 μL of 3% MPBS per well and incubate the plates at 37°C for 1 h.

3. Polyclonal phage. Take aliquots of the phage stocks prepared after each round of panning and dilute into 3% MPBS. Remove the blocking buffer from the plates, then add 100 μL containing $1 \times 10^9$ cfu phage from each stock to each well. Incubate the plates at 37°C for 2 h.
4. Detecting antibody. Remove the phage solutions, wash the plates four times with PBST, and add 100 μL per well of a 1:5,000 dilution of an HRP-conjugated anti-M13 monoclonal antibody. Incubate the plates at 37°C for 1 h.

5. Substrate. Remove the detecting antibody solution, wash the plates four times with PBST, and add 100 μL per well ABTS. Allow color to develop at room temperature over 10–20 min, and then measure the optical density at 405 nm.

3.5. Preparation of Monoclonal Phage (see Note 15)

1. Inoculate well-isolated single colonies from titration plates into 100 μL 2YTAG medium in a sterile 96 well plate (“seed plate”). Grow at 37°C overnight with shaking at 220 rpm.

2. Transfer 20 μL overnight culture from each well to 180 μL 2YTAG medium containing 10⁹ cfu/mL M13KO7 helper phage in a sterile U-bottom 96 well plate (see Note 16). Grow at 37°C for 2 h with shaking at 220 rpm to allow the bacteria to reach log phase. Keep the seed plate at 4°C for short-term storage (less than a month) or −80°C after adding 15–20% glycerol for long-term storage.

3. Spin down the bacteria in the U-bottom plate at 1,800 × g for 10 min, pipette off the supernatants, add 200 μL 2YTAK medium to each well, and resuspend the bacterial pellets.

4. Grow the bacteria at 30°C overnight with shaking at 200 rpm.

5. Spin down the bacteria in the U-bottom plate at 1,800 × g for 10 min. The supernatant can be used directly in monoclonal phage ELISA (step 3 in Subheading 3.6).

3.6. Monoclonal Phage ELISA

1. Coating. Dilute recombinant gp140 89.6, gp140 IIIB, and gp120 JR-FL to 1 μg/mL in coating buffer, and apply 100 μL per well to MaxiSorp plates. Incubate the plates at 4°C overnight.

2. Blocking. Wash the plates four times with PBST, and add 200 μL of 3% MPBS to each well. Incubate the plates at 37°C for 1 h.

3. Monoclonal phage. Remove the blocking buffer, and add 50 μL of 3% MPBS to each well. Transfer 50 μL of monoclonal phage-containing culture supernatant to each well and incubate the plates at 37°C for 2 h.

4. Detecting antibody. Remove the solutions containing monoclonal phage and wash the plates four times with PBST. Add 100 μL of a 1:5,000-diluted HRP-conjugated anti-M13 monoclonal antibody and incubate the plates at 37°C for 1 h.

5. Substrate. Remove the detecting antibody solution, wash the plates four times with PBST, and add 100 μL per well ABTS. Allow color to develop at room temperature over 10–20 min and then measure the optical density at 405 nm (see Note 17).
3.7. Expression and Purification of Soluble Fab Fragments

1. Prepare phagemid DNA from clones chosen from ELISA results. Transform DNA into electrocompetent *E. coli* HB2151, plate the transformed cells onto 2YTAG agar plates, and incubate at 37°C overnight.

2. Pick single colonies from fresh transformation plates and inoculate into 3- to 5 mL aliquots of 2YTAG medium. Grow at 37°C for 6–8 h with shaking at 250 rpm.

3. Transfer each culture to SB medium (inoculation: 1–5% [v/v]) supplemented with 200 μg/mL ampicillin and 20 mM MgCl₂. Grow the bacteria at 37°C with shaking at 250 rpm until OD₆₀₀nm = 0.6–0.9.

4. Induce each culture by adding IPTG to a final concentration of 0.5 mM (range: 0.1–1.0 mM) and keep growing the bacteria at the reduced temperature of 30°C with shaking at 250 rpm for 12–20 h (see Note 18).

5. Centrifuge the culture at 3,300 × g for 15 min at 4°C. Resuspend the pellet in 10 mL PBS containing protease inhibitors.

6. Sonicate the bacteria on ice in a sonic disrupter for 180 s, pulsing at 50% duty cycle, with the output control set at 5.

7. Pellet the cellular debris by centrifuging at approximately 48,000 × g for 30 min at 4°C. Transfer the supernatant to a clean tube. The lysate can be stored for up to 1 month at −20°C.

8. Purify the Fab fragments by protein G affinity purification.

3.8. Immunoassay of Interaction Between Soluble Fabs and Recombinant gp140s or gp120s

1. Coating. Dilute recombinant gp140₀⁹₉₆, gp140₀²⁰₃, and gp120₀ifiant⁰ to 1 μg/mL in coating buffer, and coat at 100 μL per well to MaxiSorp plates. Incubate the plates at 4°C overnight.

2. Blocking. Wash the plates four times with PBST, then add 200 μL of 3% BSA in PBS to each well. Incubate the plates at 4°C for 1 h.

3. Primary antibody. Remove the blocking buffer and add 100 μL per well of serial three-fold dilutions of the soluble Fab antibodies, starting at a concentration of 20 μg/mL. Incubate the plates at 37°C for 2 h.

4. Secondary antibody. Remove the Fab solutions, wash the plates four times with PBST, then add 100 μL per well of a 1:2,500-diluted solution of HRP-conjugated goat anti-human IgG, F(ab')2 polyclonal antibody. Incubate the plates at 37°C for 1 h.

5. Substrate. Remove the secondary antibody solution, wash the plates four times with PBST, and add 100 μL per well ABTS. Allow the reaction to proceed at room temperature for 10–20 min then measure the optical density at 405 nm.
1. Sulfo-NHS-Biotin reagents are moisture sensitive. Store the vial of biotin reagent at −20°C with desiccant. To avoid moisture condensing onto the product, equilibrate the vial to room temperature before opening.

2. The molecular cut-off of the Microcon unit used for removing free biotin and buffer exchange into PBS depends on the size of the protein to be biotinylated. For recombinant gp140s, both YM-30 and YM-10 units can be used.

3. Mix well after autoclaving, and then keep the solution at room temperature.

4. The optical density at 600 nm should be below 0.1 after inoculation. The minimum cell number of the initial inoculum should be equal to the size of the library. For example, if the library size is $1 \times 10^{10}$, the inoculum should contain at least $1 \times 10^{10}$ E. coli TG1 cells.

5. Try to use disposable containers if possible when handling phage particles; 1 OD$_{600nm}$ E. coli TG1 culture = $8 \times 10^8$ cells per mL.

6. The ratio of phage to cells for infection should be approximately 20 to 1.

7. The cell pellet can be resuspended in 10 mL 2YTAK medium and then transferred to a 2 L flask containing 490 mL 2YTAK medium.

8. All centrifuge steps should be done at 4°C except where indicated.

9. All waste solutions from phage preparation need to be decontaminated by adding 10% Clorox bleach (final concentration) to the solutions and incubating for at least 2 h before disposal. This minimizes the risk of cross-contamination from one selection to another.

10. The phage library should have a titer of $10^{13}$–$10^{14}$ cfu/mL. If the titer is too high or too low, repeat the preparation of phage.

11. Keep the rest of the glycerol stock at −80°C.

12. Antigen can be altered in the second round of panning. For example, the second round of panning can be done against 50 nM gp140$_{IIIb}$, the third round of panning against 10 nM gp140$_{89.6}$, and the fourth round of panning against 10 nM gp140$_{IIIa}$. In our experience, sequentially changing antigens is more efficient than alternating antigens, which means that panning against the first antigen for two to three rounds followed by panning against the second antigen for another
two to three rounds is more efficient in selecting antibodies that are cross-reactive with both antigens.

13. Keep the titering plates after the fifth and sixth rounds of panning. They will be used to prepare monoclonal phages for screening.

14. MaxiSorp plates enhance the coating of gp140s/120s since they are envelope glycoproteins. Standard ELISA plates may be used for other antigens.

15. Prepare monoclonal phage after the fifth and sixth rounds of panning if they show enrichment in polyclonal phage ELISA for recombinant gp140_{89.6} and gp140_{IIIb}. If there is no enrichment after the sixth round of panning, perform seventh and eighth rounds of panning by repeating the fifth and sixth rounds of panning. If there is still no enrichment after the eighth round of panning, it is unlikely that cross-reactive clones will emerge from screening.

16. To avoid phage cross-contamination, U-bottom plates are recommended.

17. Identify phage clones that cross-react with all the antigens tested in the monoclonal phage ELISA (OD_{405nm} ≥ 1.0), and use corresponding cultures in the seed plate as inocula for the preparation of plasmid DNA. Send the plasmid DNA for sequencing and analyze the sequences. Express clones with unique sequences as soluble Fab fragments.

18. The final OD_{600nm} before induction, IPTG concentrations, and time for induction can vary between antibodies and should be optimized. If the Fab is toxic to the bacterial host, glucose should be used to suppress Fab expression while cultures are growing. The glucose needs to be removed before adding IPTG for induction; this can be achieved by harvesting the cells by centrifugation and resuspending the cells in fresh medium lacking glucose but containing IPTG at the desired concentration.

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Chapter 12

Selection of Antibodies Able to Rapidly Enter Mammalian Cells

Marie-Alix Poul

Summary

This chapter outlines a protocol for the selection by phage display of single-chain variable antibody fragments with dual properties-specificity for tumor cells and the ability to be internalized. The protocol is based on a direct incubation of living target cells with antibody phage display libraries under conditions that allow active endocytosis of phage particles by cancer cells as well as recovery of intracellular phage particles that retain infectivity. This “functional” selection helps avoid the isolation of irrelevant phages that may be obtained when selection is performed on heterogeneous material as a source of antigen. Internalizing antibodies recovered from human antibody repertoires are promising reagents for various therapeutic applications including the delivery of drugs to the cytosol of targeted cell populations.

Key words: Single-chain variable antibody fragment, Cancer cell targeting, Internalizing antibody

1. Introduction

This chapter outlines a protocol for the selection by phage display of tumor-specific internalizing antibodies. In principle, the method is suitable for any population of viable cells that are adherent. Isolating tumor-specific antibodies allows their use in applications such as induction of antibody-dependent cytotoxicity or inhibition of signaling pathways involved in tumor cell migration, growth, and/or survival. In addition, antibodies targeted toward epitopes that undergo internalization from the surface of cancer cells could be exploited to achieve specific intracellular delivery of chemotherapeutic drugs (1).

The principle for the isolation of internalizing antibodies is to incubate the library of phage-displayed antibodies with target
cells at 4°C. This allows the specific binding of phage through the single-chain antibody fragment (scFv) that they display. Unbound phage particles are then eliminated with gentle washes. Phage particles that are bound to targets that undergo rapid internalization through receptor-mediated endocytosis are obtained by:

1. Incubation of the target cells at 37°C for a short period of time (usually 15 min) to enhance specific internalization mechanisms.
2. Careful elimination of extracellular phage particles that remain bound to the cell surface or the extracellular matrix.
3. Recovery of fully infectious intracellular phages by cell lysis. If target cell-specific antibodies are required, a counter selection can be performed using an appropriate cell population (e.g., normal epithelial cells when scFvs are required against targets unique to carcinoma cells) to deplete the library of phage that are reactive with common, tissue-associated epitopes.

2. Materials

2.1. Selection and Amplification

1. Target and control cells: target cells need to be adherent and grown to sub-confluence in 10 cm-diameter cell culture dishes (see Note 1), in their usual culture medium.
2. Phosphate-buffered saline (PBS).
3. 0.05% (w/v) trypsin, 0.53 mM EDTA.
4. Antibody phage library: this protocol has been successfully applied with phagemid libraries (2) and phage libraries (3). A protocol using a phagemid library is given here.
5. Platform angle rocker, in a refrigerated environment (4°C).
6. Stripping buffer: 500 mM NaCl, 100 mM glycine, pH 2.5.
7. Neutralization buffer: 500 mM Tris-HCl, pH 7.4.
8. Hemocytometer.
9. Lysis buffer: 100 mM triethylamine prepared just before use.
10. *Escherichia coli* TG1. Use fresh colonies obtained from growth of the bacteria on minimal medium to ensure expression of the F pilus. Helper phage appropriate to the library chosen for the selection (e.g., M13KO7).
11. Negative control phage clone that does not bind to target cells. This can be an scFv against an irrelevant target. The phage or phagemid vector and helper phage used for viral production should be identical to that used for the library to be used in selection.
12. Positive control phage clone that binds and is internalized into the target cells, if available (see Note 2).
13. 2x TY medium.
14. Polyethylene glycol/NaCl for phage precipitation.

1. Round small glass cover slips.
2. 6 well tissue culture plates.
3. Glycine buffer: 500 mM NaCl, 50 mM glycine, pH 2.8.
4. Fixation solution: PBS containing 4% (w/v) paraformaldehyde. This should be freshly prepared by weighing 2 g of paraformaldehyde into 40 mL water. Heat to 60°C in a fume hood, add a few drops of 1 N NaOH to dissolve, let the solution cool to room temperature, then add 10 mL of 5x PBS. The solution can be kept for a week at 4°C.
5. Acetone, kept at –20°C.
6. Saturation buffer: PBS containing 1% (w/v) bovine serum albumin (BSA).
7. Anti-Fd bacteriophage biotin-conjugated rabbit IgG (Sigma). This has antibodies against the major phage coat protein, pVIII. Dilute 300 times.
8. Anti-c-myc monoclonal antibody, clone 9E10, assuming that scFvs in the library used for selection are tagged with this epitope. Used at a final concentration of 1 μg/mL.
9. Polyclonal anti-mouse IgG biotin conjugate.
10. Streptavidin conjugated with a fluorochrome (e.g., Texas Red).

3. Methods

3.1. Selection Protocol

3.1.1. Phage Binding and Internalization

1. Grow the target cells in a 10 cm-diameter cell culture dish (see Note 1) in an appropriate culture medium. Allow the cells to reach 80% confluence. Replace the culture medium 1 h before the panning so that the cells are metabolically active.
2. Wash the cells gently with 20 mL of cold sterile PBS once and cover the cells with 4 mL of cold culture medium containing an excess of cells suitable for counter selection, freshly trypsinized and maintained in suspension (see Note 3). It is important that media used at this stage are pre-cooled.
3. Add the phage library, typically 10^11–10^12 cfu for a library with diversity in the range 10^8–10^9. This ensures that each unique phage antibody is represented 100–1,000 times.
4. Incubate with gentle shaking for 1 h on a platform angle rocker at 4°C.
5. Remove the cells in suspension and the liquid. Wash the adherent cells carefully six times using 20 mL of cold PBS for each wash.

6. Cover the cells with 10 mL of culture medium that has been prewarmed to 37°C. Transfer the cells to an incubator at 37°C with 5% CO₂ and a humidified atmosphere. Incubate for 15 min.

7. Wash the cells carefully three times with 20 mL of cold PBS. Do not forget to wash the edge of the plate.

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3.1.2. Elimination of Phage Particles from the Cell Surface

1. Add 4 mL of freshly prepared stripping buffer to the culture dish (see Note 4). Incubate for 10 min at room temperature. Collect the supernatant into a 15 mL tube and neutralize with 1.5 mL of neutralization buffer. Keep this wash (termed GlyI; total volume, 5.5 mL) on ice.

2. Repeat this step twice to obtain GlyII and GlyIII washes.

3. Wash the cells twice with 20 mL PBS.

4. Trypsinize the cells. Add 1 mL trypsin-EDTA, incubate for 5 min at 37°C and inactivate the protease by adding 1 mL of complete medium. Collect cells into a 50 mL tube and count the cells using a hemocytometer (see Note 5).

5. Wash by adding 50 mL of cold PBS and pellet the cells by low-speed centrifugation.

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3.1.3. Recovery of Intracellular Phage

1. Add 1 mL of freshly prepared lysis buffer to the cell pellet and incubate for 5 min at 4°C. Collect the cell lysate in a 15 mL tube.

2. Neutralize immediately with 0.5 mL of neutralizing buffer. This is termed the intracellular fraction (total volume, 1.5 mL). Keep on the lysate on ice until required.

3. Grow a sample of *E. coli* TG1 until the bacteria are in mid-log phase. Infect a 10 mL aliquot of the bacteria TG1 with 1 mL of the intracellular fraction by adding the lysate and incubating for 30 min at 37°C without shaking.

4. Plate the bacteria onto a large Petri dish containing antibiotics appropriate for the phagemid system in use.

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3.1.4. Monitoring the Recovery of Phage Using Titration

1. Grow a sample of *E. coli* TG1 until the bacteria are in mid-log phase.

2. Inoculate 1 mL samples of the bacteria with 10- and 100 μL samples from GlyI, GlyII, and GlyIII stripping washes, and 10- or 100 μL samples of the intracellular fraction.

3. Allow infection to take place by incubating the bacteria for 30 min at 37°C without shaking.

4. Plate 10- and 100 μL samples from each infection mix onto agar plates containing an antibiotic appropriate for the phagemid system in use (see Note 6).
5. The selection protocol can be adapted to the cell model in use, aided by using negative (see Note 7) and positive control scFvs (see Note 8).

3.2. Amplification of Phage

1. After overnight incubation at 37°C, scrape the colonies from the large agar plate created from the intracellular fraction of phage using 5 mL of 2x TY liquid medium.

2. Inoculate 10 mL of 2x TY liquid medium with 100 μL of the bacterial suspension. The absorbance at 600 nm should be less than 0.2; if it is not, add more 2x TY. The remainder of the bacterial suspension can be frozen with glycerol as a cryoprotectant.

3. Grow the bacteria into exponential phase by incubation at 37°C with shaking.

4. Add helper phage appropriate to the phagemid system in use.

5. Adjust the volume of the culture to 50 mL with 2x TY and grow overnight at 30°C with vigorous shaking (220 rpm).

6. The next day, concentrate phage contained in the culture supernatant 20-fold by standard NaCl/PEG precipitation and resuspension in PBS.

7. Filter phage suspension through a 0.45 μm syringe filter. Titrate the phage; the recovery should be between 10^{12} and 10^{13} cfu/mL.

3.3. Further Rounds of Selection

1. The recovered phage can now be used in further rounds of selection using the protocol described above.

2. Using this functional selection, two to three rounds are usually sufficient to obtain phage with specificity for molecules at the surface of the cells of interest, and with the ability to undergo internalization.

3. After the second and third rounds of selection, pick between 96 and 192 individual colonies from the titration plates. Prepare individual phage stocks from each clone and check their binding to the target cells by monoclonal phage cell ELISA (4). At the same time, check the binding of phage to the cells used for counter selection (see Subheading 3.1.1, step 2).

3.4. Checking for Phage Internalization

1. Grow the target cells on coverslips to 50% confluence using six well tissue culture plates.

2. Dilute the phage sample under test to 5 × 10^{11} cfu in 2 mL of culture medium and add to the cells (see Note 9). If purified scFv or other forms of the recombinant antibody are available, they should be diluted to 10 μg/mL (see Note 10).

3. Incubate the phage or recombinant antibody with the cells for 2 h at 37°C. As a control, perform duplicate experiments at 4°C.
4. Wash the coverslips six times with PBS and three times for 10 min each wash with glycine buffer.

5. Neutralize with PBS and incubate the cells with fixative solution for 5 min at room temperature. As a control, omit washes with glycine buffer.

6. Permeabilize the cells with cold acetone (−20°C) for 30 s and then add saturation buffer.

7. Invert the coverslips and place onto a 100 μL drop of primary antibody. This primary antibody will be biotinylated anti-Fd phage antibody if the internalization of phage particles is being studied. For purified scFv carrying the c-myc detection tag, monoclonal 9E10 should be used. Incubate for 1 h at room temperature and then wash three times with PBS.

8. Reveal the location of the primary antibody using a streptavidin–fluorochrome conjugate (for detection of phage) or biotinylated anti-mouse Ig and streptavidin-fluorochrome conjugate (for detection of soluble scFv). Wash thoroughly.

9. Observe the location of the fluorescent signal using confocal microscopy.

4. Notes

1. Culture dishes are preferable to flasks because the efficiency of washing tends to be better and easier to perform. Depending on the target cell morphology, between 2 and 10 millions cells can grow on a 10 cm-diameter cell culture dish.

2. Endocytosis through the transferrin receptor (TfR) is a useful model for clathrin-mediated endocytosis, the major entry route for a number of extracellular soluble ligands. Phage displaying anti-human TfR scFvs that undergo internalization are available (4) and could be used to optimize the conditions for recovery of internalized phage particles if target cells display significant levels of TfR at the cell surface.

3. A depletion step is essential if binders specific to the target cells are to be recovered. The library can be depleted of specificities that recognize common cell surface components prior to selection (5, 6) (termed the pre-depletion step) or concurrently with selection as described in this protocol (termed co-depletion) (4). Pre- or co-depletion should be carried out at each round to maintain selective pressure. In the protocol described here, co-depletion should be done at 4°C using as many target-negative cells as possible (20–100 million) in a minimal volume of suspension. In a co-depletion step, the
time of incubation should be adjusted (at least 1 h) so that the target cells remain alive and can internalize bound phage particles when the temperature of incubation is increased to 37°C. The duration of the co-depletion step can be prolonged to as long as 12 h using minimal volumes of solution.

4. Stripping must remove extracellular binders efficiently but conditions must be relatively mild so that the target cells remain adherent during this step and so that internalized phage particles remain intracellular. The composition of the stripping buffer can be altered by addition of 200 mM urea and/or 2 mg/mL polyvinylpyrrolidone if the target cells can bear it.

5. Incubate with trypsin–EDTA sufficiently long for collection of all the cells while keeping the phage particles intracellular. Trypsin will also help remove phage bound to the extracellular matrix and, if the library has been rescued using a trypsin-cleavable helper phage like KM13 phage (as used with the Tomlinson I and J libraries), the treatment will inactivate nonspecific binders. Phage removed in this way will be eliminated by the next wash/centrifugation step. At least 70% of the cells should be recovered after the selection step. If not, consider a less stringent protocol by reducing the number or the duration of the washes.

6. After the first round, expect an output of $10^6$–$10^7$ cfu in the GlyI fraction and significantly less in GlyII and GlyIII fractions. Expect an output of approximately $10^4$ cfu in the intracellular fraction. If the number of phage in the intracellular fraction is higher than in the GlyIII fraction, it is a promising indication that the selection is specific for internalizing phage. Phage recoveries in the intracellular fraction should increase significantly (by one to three logs) from round 1 to round 3 (see Table 1). It is more accurate to normalize the output of phage against the number of target cells recovered after selection steps and counted just before lysis. From round 1 to round 3, GlyI titers might not change since the target(s) for phage binding might be present in low quantities at the cell surface and, thus, the system might be saturated with phage at the first round.

7. It is a good option to optimize the background to the cell model under investigation using a negative control (phage displaying an irrelevant scFv). Adapt the steps to the properties of the target cell:

– If the GlyIII titer is higher than that of the intracellular fraction, consider increasing the number of PBS washes before the temperature shift to 37°C and/or afterward. Also consider increasing the number of washes with stripping buffer (up to six) or using stripping buffer with increased stringency (see Note 4).
If the recovery of phage in the intracellular fraction is more than $10^6$ cfu at the first round of selection, increase the number of washes with stripping buffer, add an extra wash with PBS after cell trypsinization, and transfer the cell suspension to a new 50 mL tube after the first wash.

8. If the target cells express TfR, phagemids displaying anti-TfR internalizing scFvs can be used to optimize the recovery of internalized phages. Other phage might also be used as positive controls depending on the target cells (4, 6, 7). Mix $10^3$ cfu of positive control phage with $10^{12}$ cfu of negative control phage to mimic a phage library. If positive and negative control phage carry distinct antibiotic resistance markers (e.g., tetracycline-resistant Fd phage or kanamycin-resistant helper phage as negative controls versus ampicillin-resistant positive control phage), this can facilitate easy discrimination. Perform a round of selection and titer the intracellular fraction using plates with each antibiotic to discriminate. Ideally, the procedure should allow the recovery of at least one positive control phage against a background of between $10^4$ and $10^6$ cfu of negative control phage.

9. The volume of phage suspension should be limited to 1/10 of the total culture medium so that any traces of PEG present do not impair the internalization process. Phage particles can also be directly stained by covalent attachment of a fluorochrome. Options include the use of activated FITC (6-[fluorescein-5-\{and-6\}-carboxamido]hexanoic acid, succinimidyl ester [5\{6\}-SFX]) (Molecular Probes) to label lysine residues on the major phage coat protein, pVIII. Labeling in this way means that internalized phage can be directly observed by fluorescence.
microscopy after the cell surface has been stripped. To covalently stain phage, dilute a suspension of titer $5 \times 10^{12} \, \text{cfu/mL}$ with an equal volume of 0.1 M carbonate buffer, pH 9. Add 10 μL of freshly prepared FITC-succinimidyld ester solution (100 mg/mL in dimethylsulfoxide) and incubate for 1 h at room temperature, mixing end-over-end. Use PEG precipitation to remove all traces unconjugated FITC, resuspend the phage in 100 μL of PBS, and store at 4°C in the dark. Use flow cytometry to check that the conjugation did not impair the specificity of the phage antibody. In our unpublished work, we have observed internalization of phage bearing the anti-TfR scFv H7 as early as 5 min after incubation with the SKBR-3 cell line (unpublished).

10. Subclone the scFv sequence into a vector for expression of soluble antibody fragments in *E. coli* (e.g., pSYN). This can allow the production of monovalent scFv with a c-myc detection tag and a hexahistidine sequence for purification (8). For the production of bivalent (scFv)$_2$, subclone in a vector that imparts a cysteine and a hexahistidine tag at the carboxy terminus (6).

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Identification of the Specificity of Isolated Phage Display Single-Chain Antibodies Using Yeast Two-Hybrid Screens

Nicolaj Rasmussen and Henrik J. Ditzel

Summary

A method is described for the identification of the antigen recognised by an scFv isolated from an antibody phage display library using selection against a complex mixture of proteins (e.g. intact cells, purified cell surface membranes, and tissue sections). The method takes advantage of a yeast two-hybrid system that additionally allows for reorganization of post-translational modifications to the bait and target proteins. This technique is therefore especially useful for identifying surface-expressed antigens.

Key words: Phage display, scFv, Yeast two-hybrid, Antigen identification

1. Introduction

The development and use of combinatorial libraries displayed on the surface of filamentous phage has led to the isolation of many monoclonal antibodies of desired specificity (1-3). Traditionally, antibodies have been selected using antigens that have been purified and characterised, but it is also possible to isolate antibodies by panning on whole cells (4-6) or by using heterogeneous cell lysates (7). Due to the complexity of these antigen preparations, the specificities of the retrieved phage antibodies need to be determined after isolation. Several techniques in combination with mass spectrometry have been successfully utilised to identify the targets of antibodies of unknown specificity, including affinity purification and ion exchange or size exclusion chromatography (4, 8, 9). However, several of these techniques are dependent on the ability of the antibody to bind to an epitope that may be denatured, a feature not always present when the antibody is selected.
by panning on whole cells, or other complex antigens. Identification of the antigen recognised by the isolated antibody is often difficult, laborious, and not always successful with conventional techniques such as affinity purification or filter lift assays. In this chapter, we describe the use of a yeast two-hybrid system for the identification of the antigen recognised by a given single-chain antibody (scFv).

Conventional yeast two-hybrid systems utilise the ability of two interacting proteins to locate a transcription activating domain in the proximity of a regulatory DNA-binding site \((10, 11)\). This is accomplished by replacing the interacting domain of one of the two transcription-activating elements with a protein or protein fragment and replacing the interacting domain of the other transcription-activating element with a single protein, a protein fragment, or a whole library of different proteins. If the two fusion proteins interact, the function of the regulatory element will be reconstituted and drive the expression of a downstream reporter gene. The identity of the interaction partner can then be obtained by sequencing.

The CytoTrap yeast two-hybrid system monitors protein interactions at the plasma membrane through activation of a Ras-dependent signal pathway by the hSos protein (Fig. 1)(12). In contrast to conventional yeast two-hybrid systems or bacterial screening systems, this enables the expression of proteins requiring post-translational modifications in the cytoplasm such as glycosylation, an important feature of surface-expressed proteins. Using CytoTrap yeast two-hybrid screening, several protein-protein interactions have been identified, including protein interaction on the surface of intracellular compartments (13) and at the plasma membrane (14), as well as scFv-surface antigen interactions, as we recently reported (5).

![Fig. 1. CytoTrap yeast two-hybrid system, utilizing the Ras-signalling pathway for identification of the antigen recognised by the scFv. (1) Interaction of the bait and target protein locates hSos to the plasma membrane; (2) hSos activates Ras; (3) Ras activates a signal cascade permitting the mutant yeast strain cdc25H to grow at 37°C.](image-url)
2. Materials

1. Phage display vector DNA carrying the scFv of interest.
2. NotI and NeoI restriction enzymes (New England Biolabs).
3. Wizard SV gel and polymerase chain reaction (PCR) clean-up system (Promega).
4. pSos (Stratagene).
5. T4 DNA ligase (Invitrogen).
6. Escherichia coli XL1-Blue (Stratagene).
7. Electroporation apparatus (e.g. Biorad GenePulser Xcell) and cuvettes.
8. LB medium: 10 g/L NaCl, 10 g/L tryptone, and 5 g/L yeast extract, pH 7.0; autoclaved. Supplement with 20 g/L agar for solid medium.
9. LB agar supplemented with carbenicillin (50 µg/mL).
10. LB agar supplemented with chloramphenicol (30 µg/mL).
11. S.N.A.P. DNA midiprep isolation kit (Invitrogen).
12. Glycerol (autoclaved).
13. pMyr (Stratagene).
14. Temperature sensitive Saccharomyces cerevisiae strain cdc25H (Stratagene).
15. YPAD medium: 10 g/L yeast extract, 20 g/L Bacto peptone, 20 g/L dextrose, and 0.4 g/L adenine sulphate; autoclaved. Supplement with 20 g/L agar for solid medium.
16. LiSORB (100 mM lithium acetate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 M sorbitol).
17. Salmon sperm DNA (Invitrogen).
18. PEG/LiOAc (100 mM lithium acetate [from a stock at pH 7.5], 10 mM Tris-HCl [from a stock solution at pH 8.0], 1 mM EDTA [from a stock solution at pH 8.0], and 40% [w/v] polyethylene glycol [PEG] 3350).
19. Dimethyl sulfoxide (DMSO).
20. 1.4 M β-mercaptoethanol.
21. 1 M sorbitol.
22. 10× Dropout solution (see Note 1) 300 mg/L L-i.-isoleucine, 1,500 mg/L L-i.-valine, 200 mg/L L i.-adenine hemisulphate salt, 500 mg/L L i.-arginine HCl, 200 mg/L L i.-histidine HCl monohydrate, 500 mg/L L i.-lysine HCl, 200 mg/L L i.-methionine, 500 mg/L L i.-phenylalanine, 2,000 mg/L L i.-threonine, 500 mg/L L i.-tryptophan, 500 mg/L L i.-tyrosine, 1,000 mg/L L i.-glutamic acid, 1,000 mg/L L i.-aspartic acid, and 400 mg/L L i.-serine. Filter sterilise.
23. SD/glucose (−UL) agar plates (see Note 2): 1.7 g yeast nitrogen base without amino acids, 5 g ammonium sulphate, 20 g dextrose, and 17 g Bacto agar, added to water and made up to 900 mL. Autoclave and cool, but maintain molten before addition of 100 mL of 10× Dropout solution, then pour plates.

24. Acid-washed glass beads.

25. SD/galactose (−UL) agar plates (see Note 2): 1.7 g yeast nitrogen base without amino acids, 5 g ammonium sulphate, 20 g galactose, 10 g raffinose, and 17 g Bacto agar, added to water and made up to 900 mL. Autoclave and cool, but maintain molten before addition of 100 mL of 10× Dropout solution, then pour plates.

26. Yeast lysis solution: 2.5 M lithium chloride, 50 mM Tris-HCl, pH 8.0, 4% (v/v) Triton X-100, and 62.5 mM EDTA.

27. Phenol/chloroform (Invitrogen).

28. Absolute ethanol and 70% (v/v) ethanol.

29. 3 M sodium acetate, pH 5.2.

30. CytoTrap control plasmids pSos MAFB, pMyr MAFB, and pMyr Lamin C (Stratagene).

31. LB agar supplemented with tetracycline (40 μg/mL).

32. SB medium: 32 g/L tryptone, 20 g/L yeast extract, and 5 g/L sodium chloride, pH 7.5.

33. 2 M glucose, sterile.

34. 1 M magnesium chloride, sterile.

35. Liquid nitrogen for snap freezing.

3. Methods

3.1. Bait Plasmid Construction

1. Digest 5 μg of purified plasmid DNA containing the scFv sequence of interest using 5 U of NeoI and NotI for 3 h at 37°C (see Note 3).

2. Gel-purify the scFv fragment on a 1% agarose gel using Wizard SV gel and PCR clean-up reagents following the manufacturer’s recommendations.

3. Digest 5 μg of purified pSos with 5 U of NeoI and NotI for 3 h at 37°C.

4. Gel-purify the large fragment of the digested pSos vector on a 1% agarose gel using Wizard reagents.

5. Quantify the purified insert and vector.
6. Ligate 100 ng of purified insert with 100 ng purified vector using T4 DNA ligase overnight at room temperature in a final volume of 25 µL.

7. Electroporate 1 µL of the ligation reaction into 50 µL of electrocompetent E. coli XL1-Blue (see Subheading 3.6) using an apparatus such as a Biorad GenePulser Xcell. For this system, use 2 mm electroporation cuvettes and exponential decay with the following settings: 2.5 kV, 25 µF capacitance, and 200 Ω resistance. Plate the transformed bacteria onto LB plates containing carbenicillin and incubate overnight at 37°C.

8. Analyse the transformed bacteria by isolating plasmid DNA, and sequence using the sequencing primers Sos 5’ (CCAGGTTTTTC-CCAGT) and Sos 3’ (GCCAGGTTTTTC-CCAGT).

9. Pick a single clone in which the scFv has been successfully fused in-frame with hSos and inoculate into 100 mL LB media containing 50 µg/mL carbenicillin and 1% (w/v) glucose. Grow overnight with shaking at 37°C.

10. Purify the plasmid by using a midiprep kit. Store the DNA at −20°C.

3.2. Amplification of cDNA Library and Recloning for Two-Hybrid Screening

1. Take the complementary DNA (cDNA) library prepared from the tissue or cell line in which the target for the scFv exists and titre by making serial dilutions on LB agar plates containing chloramphenicol (see Note 4).

2. Plate approximately 20,000–30,000 clones on a series of 15 cm LB agar plates containing chloramphenicol, using sufficient plates to completely recover the library.

3. Incubate overnight at 37°C.

4. Add 6 mL LB medium to each plate and gently scrape the bacteria off with a cell spreader.

5. Wash each plate with an additional 2 mL LB media.

6. Pool the recovered cell suspensions.

7. Purify plasmid DNA from half of the amplified library using a S.N.A.P. midiprep kit.

8. Add sterile glycerol to the remaining library to a final concentration of 20% (v/v). Make 1 mL aliquots and store at −80°C.

9. Excise the inserts from the cDNA library, purify, and ligate into pMyr using appropriate restriction sites in the vectors as described in Subheading 3.1.

10. Electroporate into E. coli XL1-Blue, check the success of the construction by characterising colonies picked at random, and prepare plasmid DNA (midiprep) as described in Subheading 3.1.
1. Restreak the cdc25H strain of \textit{S. cerevisiae} on a YPAD plate and incubate at room temperature until colonies appear (approximately 4 days).

2. Pick four to five cdc25H colonies into separate 1.5 mL microfuge tubes, each containing 1 mL of liquid YPAD medium. Vortex vigorously until no cell clumps are visible. Generation of four to five independent yeast preparations in parallel is recommended to offset the ability of the cdc25H strain to produce revertants (see \textbf{Note 5}).

3. Transfer the yeast suspension into 500 mL flasks, each containing 100 mL YPAD medium. Incubate for 14–16 h at room temperature while shaking at 220–250 rpm.

4. After incubating for a maximum of 19 h, measure the OD$_{600}$ of the cultures to check that they have achieved values in excess of 1. If the cultures do not reach this density, the process must start again with \textbf{step 2}, or preferably from \textbf{step 1}.

5. Prepare dilutions of the cultures into 600 mL of YPAD in 2 L flasks to OD$_{600}$ values of 0.2. Incubate for 3 h at room temperature while shaking at 220–250 rpm.

6. Measure the OD$_{600}$ and confirm that it is over 0.7. Plate 75 \textmu L from each culture onto YPAD agar plates, seal and incubate the plates at 37°C to check for temperature-sensitive revertants. Observe the plates daily for 4–6 days. If at day 6 more than 30 colonies have appeared on one plate, the corresponding preparation of competent cells is unreliable and should be discarded.

7. Pellet the 600 mL cultures by centrifugation at 1,000 \times g for 5 min at room temperature. Discard the supernatant and resuspend the pellet in 100 mL sterile dH$_2$O by repeated pipetting. Spin the cells at 1,000 \times g for 10 min at room temperature.

8. Discard the supernatant and resuspend the pellet in 100 mL LiSORB. Incubate the cell suspension at room temperature for 30 min.

9. Meanwhile, set up 800 \textmu L aliquots of sheared salmon sperm DNA at 20 mg/mL sufficient for each independent yeast culture. Transfer the DNA to a boiling water bath and incubate for 10 min. After boiling, add 1,200 \textmu L LiSORB to each 800 \textmu L aliquot of salmon sperm DNA and mix by pipetting. Cool to room temperature.

10. Pellet the yeast cells by centrifugation at 1,000 \times g for 5 min at room temperature. Resuspend each pellet of yeast cells in 600 \textmu L LiSORB.

11. Add 1,200 \textmu L of salmon sperm DNA mixture from \textbf{step 9} to the 600 \textmu L suspension of yeast cells. Mix thoroughly by gentle pipetting.
12. Add 10.8 mL PEG/LiOAc solution and 1,060 μL DMSO to each cell preparation. Mix thoroughly but gently by pipetting. The cells are now ready for transformation.

3.4. Co-transformation

1. Add 40 μg of the scFv bait construct in pSos, 40 μg of the library of cDNA in pMyr, and 200 μL of 1.4 M β-mercaptoethanol to 10 mL of freshly prepared competent cdc25H yeast cells in a 50 mL conical tube.
2. Mix the contents thoroughly but gently by inversion to ensure a homogenous mixture.
3. Transfer the mixture into 20 separate microcentrifuge tubes.
4. The CytoTrap system provides a number of negative and positive controls for the screen that should be used. Prepare an additional negative control in a separate microcentrifuge tube by adding 2 μg of pSos plasmid, 2 μg of the library of cDNA in pMyr, and 10 μL of 1.4 M β-mercaptoethanol to 500 μL competent yeast cells.
5. Incubate the transformation mixtures for 30 min at room temperature.
6. Incubate the transformation mixtures for 20 min at 42°C.
7. Incubate the transformation mixtures for 3 min on ice.
8. Pellet the cells by centrifugation for 30 s at full speed in an appropriate microcentrifuge at room temperature. Discard the supernatant.
9. Resuspend the cells in 0.5 mL of 1 M sorbitol and plate the entire transformation mix from each tube on a series of 15 cm SD/glucose (−UL) agar plates (see Note 2). Disperse the suspension with acid-washed glass beads. Once the plate has dried, the beads can be removed.
10. Invert the plates and incubate for 48 h at room temperature.
11. Replica plate the transformants from test and control plates onto SD/galactose (−UL) agar plates to activate expression of myristylated target from the cDNA (see Notes 2 and 3). Incubate the plates at 37°C. Colonies should start to appear after 3 days.
12. After 6 days, evaluate the number of colonies on the negative control, which will give an estimate of the numbers of false positives from the cDNA library and of temperature-sensitive revertants (see Note 5). Confirm the success of the experiment by checking the outcome of transformations with the positive and negative control plasmids provided by the manufacturer of the CytoTrap system.
13. Also after 6 days, pick colonies from the library screen (SD/galactose [−UL] incubated at 37°C) and patch them onto an SD/glucose (−UL) agar plate. Incubate the plate at 22–25°C.
for 48 h. Return the original galactose plate to 37°C, as further colonies may appear over the next 4 days.

14. After the replica plate has been incubated for 48 h, pick colonies onto two fresh SD/glucose (−UL) plates and one SD/galactose (−UL) plate. Incubate one SD/glucose (−UL) and the SD/galactose (−UL) plate at 37°C for approximately 48 h. These are termed the “primary interaction test plates”. Keep the remaining SD/glucose (−UL) plate at 22–25°C as a replica-plating source plate.

15. After the 48-h incubation, evaluate the primary interaction test plates, identifying those patches that grow at 37°C on the SD/galactose (−UL) plate, but not on the SD/glucose (−UL) plates. Perform a secondary interaction test by replica-plating to SD/galactose (−UL) the transformants on the remaining SD/glucose (−UL) plate kept at 22–25°C. Candidates growing on the SD/galactose (−UL) plate, but not on the SD/glucose (−UL) plates, at 37°C in both tests should be considered putative positive clones and analysed further.

3.5. Verification of Interaction

3.5.1. Isolation of Recombinant pMyr from Yeast Cells for Identification of scFv Target

1. Inoculate each of the putative positive clones into 5 mL SD/glucose (−UL) liquid medium in a 50-mL conical tube.

2. Incubate the culture for 2–3 days at room temperature with vigorous shaking until the culture is saturated (OD_{600} > 1.0).

3. Spin the culture at 1,000 × g for 5 min at room temperature.

4. Discard the supernatant and resuspend the pellet in 0.3 mL yeast lysis solution.

5. Transfer the suspension to a 1.5 mL microcentrifuge tube.

6. Add 50 μL of acid-washed glass beads (0.5 mm) and 300 μL of phenol/chloroform.

7. Vortex vigorously for 1 min.

8. Spin the suspension at 14,000 × g for 5 min at room temperature.

9. Transfer the top aqueous phase to a new microcentrifuge tube.

10. Precipitate the DNA with 600 μL absolute ethanol overnight at −20°C.

11. Spin the suspension at 14,000 × g for 10 min at 4°C.

12. Discard the supernatant and wash the DNA pellet with 1 mL of 70% ice-cold ethanol.

13. Discard the supernatant and dry the DNA pellet.

14. Resuspend the DNA pellet in 40 μL of H₂O.

15. Precipitate the DNA with 4.8 μL of 3 M sodium acetate and 100 μL of absolute ethanol.

16. Repeat steps 11–13.
17. Resuspend the DNA pellet in 20 μL of H₂O.
18. Transform electrocompetent E. coli XL1-Blue (see Note 6) and select for the pMyr plasmid by plating on LB agar plates containing chloramphenicol (see Note 7).
19. Isolate the DNA from positive clones using a miniprep kit, and sequence the insert.

3.5.2. Co-transformation of Putative Positive Clones

1. Prepare competent yeast cells as described in Subheadings 3.3. Use 300 ng of bait (scFv construct in pSos vector) and target plasmid (cDNA candidate in pMyr, Subheading 3.5.1, step 17) for the transformation, and 0.1 mL of competent yeast cells (see Note 8).
2. Positive and negative controls can be included. For the positive control, use the combination of pSos MAFB and pMyr MAFB. For the negative control, use pSos MAFB and pMyr Lamin C.
3. If the results obtained are identical to the results in the initial screen, the interaction partner can be considered positive and the interaction further verified by enzyme-linked immunosorbent assay (ELISA) with purified scFv and its target or other suitable techniques.

3.6. Bacterial Transformation by Electroporation

3.6.1. Preparation of Electrocompetent E. coli XL1-Blue

1. Restreak E. coli XL1-Blue on a LB-agar plate containing tetracycline (40 μg/mL) and incubate at 37°C overnight.
2. Inoculate a single colony from the overnight plate into 20 mL of SB containing tetracycline (40 μg/mL). Incubate at 37°C overnight.
3. To prepare SOC medium inoculate 2 L of SB with 40 mL of 2 M glucose and 20 mL of 1 M magnesium chloride. Add the 20 mL of overnight bacterial culture and grow for approximately 4 h at 37°C until the OD₆₀₀ is 0.7–0.8.
4. Transfer the culture to four 500 mL centrifuge bottles and chill on ice for 20 min.
5. Centrifuge at 1,800 × g for 20 min.
6. Discard the supernatant and resuspend each pellet in cold 10% (v/v) glycerol. Pool two pellets into single centrifuge bottles and fill with cold 10% (v/v) glycerol.
7. Centrifuge at 2,500 × g for 20 min.
8. Discard the supernatant and resuspend each of the two pellets in cold 10% (v/v) glycerol. Add cold 10% (v/v) glycerol to 250 mL.
9. Centrifuge at 4,000 × g for 20 min.
10. Discard the supernatant and resuspend the pellets in cold 10% (v/v) glycerol. Transfer the resuspended pellets to 50 mL tubes and fill the tubes with cold 10% (v/v) glycerol.
11. Centrifuge at $4,000 \times g$ for 20 min.

12. Discard the supernatant and resuspend the pellets in 1 mL of cold 10% (v/v) glycerol. Make aliquots of 200 µL and freeze in liquid nitrogen. Store the competent cells at −80°C.

3.6.2. Transformation of Electrocompetent E. coli XL1-Blue

1. Mix 50 µL of electrocompetent E. coli XL1-Blue with 1 µL of DNA (e.g. from Subheading 3.5.1, step 17), and incubate for 1 min on ice (see Note 6).

2. Electroporate the cells using a Biorad GenePulser Xcell and 2-mm cuvettes using exponential decay with the following settings: 2.5 kV, capacitance 25 µF, resistance 200 Ω.

3. Immediately flush the cuvette with 3 × 1 mL of SOC media. Incubate the culture for 1 h at 37°C, shaking, and plate 1 µL and 100 µL into LB agar plates containing appropriate antibiotics.

4. Notes

1. For selection of yeast transformants carrying the pSos vector, leucine is omitted from the Dropout solution. Similarly, for selection of pMyr-based constructs, uracil is absent. For a non-selective experiment, these components can be added at 1,000 and 200 g/L, respectively.

2. The “−UL” designation of this medium indicates the absence of uracil and leucine and hence the agar is selective for yeast transformants that carry pSos and pMyr. In pSos, expression of bait protein—in this chapter, the fusion of the scFv of interest to hSos—is constitutive. In pMyr, expression of a myristylated target protein is regulated by the galactose-dependent GAL1 promoter.

3. The scFv of interest is cloned into the MCS of the pSos vector at the end of hSOS. When using scFv genes from a pHEN2 library, the scFv fragment can be directly excised from the pHEN2 vector using the restriction enzymes NcoI and NotI and subsequently ligated into NcoI- and NotI-digested pSos vector. When using phage display vectors other than pHEN2, it may be necessary to construct primers for the introduction of appropriate restriction enzyme sites through PCR. In planning the construction, the scFv must be ligated into the correct reading frame so that a protein fusion to hSos is created.

4. If a specific cell line or other RNA source is to be used for the construction of a cDNA library, we recommend using the ZAP cDNA synthesis kit (Stratagene). Several cDNA libraries
cloned into the pMyr vector are commercially available from Stratagene. cDNAs that are ligated into the correct reading frame are expressed in a galactose-dependent manner with an amino-terminal myristylation signal, directing the protein to the cytoplasmic membrane. If the co-transformed scFv bait construct interacts with the target protein, hSos will be co-localised to the membrane and can thereby activate Ras. This will result in activation of a signal cascade that permits mutant yeast strain cdc25H to grow at 37°C.

5. The cdc25H yeast strain can revert, acquiring the ability to grow at 37°C. Four to five independent preparations of competent cells are recommended to ensure obtaining at least one preparation with very few revertants that can be used successfully. This can be quite laborious (Subheadings 3.3 and 3.4). We have successfully used only one preparation, but the experiment has to look critically at the background of revertants so that sufficient putative positive clones are picked for further characterisation. In practice, we typically test two to three times as many yeast colonies as are present on the control plate to maximise the chances of isolating genuinely positive clones.

6. For the transformation, use freshly made electrocompetent cells or thaw a frozen aliquot on ice.

7. The procedure yields a mixture of intact plasmid DNA and fragmented chromosomal DNA. In consequence, the DNA is not suited to gel analysis, but it can be used to transform electrocompetent E. coli.

8. To verify the interaction between bait and target protein, transform yeast basically as performed for the library screen, but using only a single clone instead of the whole library.

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Generation of Bispecific and Tandem Diabodies

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Summary

Conventionally, antibody phage display has been used to isolate recombinant antibodies that are monovalent in their interaction with target antigens. These antibodies can be reengineered for expression in mammalian cell culture as full-length, monospecific immunoglobulins. An emerging branch of research has sought to generate bivalent recombinant antibodies by manipulating the length of the linker separating heavy- and light-chain variable domains in single-chain Fv proteins, thereby promoting inter-scFv interaction and the formation of “diabodies.” With careful control, this can generate scFv-based proteins able to bind two very distinct targets, “bispecific diabodies.” Further manipulation enables the assembly of higher-order complexes.

Key words: scFv, Linker, Cancer, Therapeutics

1. Introduction

A major goal of antibody (Ab)-based tumor targeting has been to specifically deliver a variety of payloads, such as radioisotopes, drugs, toxins, lymphokines, and enzymes to defined sites for imaging and therapy. Intact immunoglobulin G (IgG) molecules are large (150–180 kDa) glycoproteins that exhibit a slow systemic clearance, often leading to poor tumor-targeting specificity (Fig. 1). Smaller Ab-derived molecules include enzymatically produced 50 kDa Fabs and engineered 25 kDa single-chain Fvs (scFvs) consisting of heavy- and light-chain variable regions ($V_H$ and $V_L$) connected by a flexible peptide linker of 14–24 residues ($I$, $2$) (Fig. 1). Compared with IgG, Fabs and scFvs exhibit
significantly improved tumor specificity and intratumoral penetration (3–5). However, the rapid blood clearance and monovalent nature of these small molecules result in considerably lower quantitative tumor retention (6, 7).

In recent decades, attention has focused on the generation of scFv-based molecules with molecular weight in the range of the renal threshold for first-pass clearance. Construction of such molecules can be achieved by shortening the linker between the $\text{V_H}$ and $\text{V_L}$ domains in scFvs. Reduction of the linker length to fewer than 10–12 residues disfavors the formation of monomeric scFv molecules and promotes intermolecular $\text{V_H}/\text{V_L}$ pairings into 50 kDa noncovalent scFv dimer diabodies (8) (Fig. 2a). The prolonged tumor retention in vivo and higher tumor-to-blood ratios reported for diabodies compared with scFv monomers result from both their reduced kidney clearance and their higher avidity (9, 10). The diabody format can also be used for generation of recombinant bispecific antibodies (BsAb), which are obtained by the noncovalent association of two hybrid single-chain fusion products consisting of the $\text{V_H}$ domain of specificity A connected by a short linker to the $\text{V_L}$ domain of another specificity, B (for review, see ref. 11 and Fig. 2b). Both $\text{V_H}$-to-$\text{V_L}$ and $\text{V_L}$-to-$\text{V_H}$ domain orientations in hybrid scFvs lead to formation of functional diabodies (12). The two antigen (Ag)-binding domains have been shown by crystallographic analysis to be on opposite sides of the diabody molecule, so that they are able to crosslink two cells (13, 14) (Fig. 2c). Diabodies are potentially less immunogenic than quadroma-derived BsAb, and can be easily produced in bacteria in high yields (15, 16).
Bispecific diabodies appeared to be more effective than quadroma-derived BsAb in mediating T-cell (17, 18) and NK-cell (19) cytotoxicity in vitro against tumor cells. In vivo, the antitumor activity of diabodies was similar to that of the quadroma-produced bispecific IgGs, although the latter ones had much longer retentions in blood circulation due to their larger size (16, 19). A synergistic antitumor effect in vitro and in animal models has been demonstrated for a combination of diabodies recruiting both human T cells and NK cells (20). Enhanced antitumor activity in vivo has also been shown for a combination of the NK-cell recruiting diabody and thalidomide in comparison with the diabody and chemotherapy alone (21). Bispecific diabodies can also be used for enhancement of the dendritic cell-induced T-cell reactivity (22).

In spite of these encouraging results, several drawbacks preclude the use of diabodies as therapeutic agents in clinical settings. First, the diabodies possess unfavorable pharmacokinetic properties due to their relatively small size (23). Second, the co-secretion of two hybrid scFvs may give rise to two types of dimer, active heterodimers and inactive homodimers, thus requiring additional laborious procedures for isolation of the active agent. A third problem is that the two chains of diabodies are held together by noncovalent associations of the V\_H and V\_L domains and, with time, the chains can diffuse away from one another. Inactivation of a functional bispecific diabody is initiated by a dissociation of the weaker V\_H/V\_L interface followed by domain swapping with
the formation of nonactive homodimers. The instability of one homodimer makes the process of diabody dissociation/reassociation irreversible, thus gradually decreasing the fraction of active molecules \((24)\). In addition, to ensure the assembly of a functional diabody, both hybrid scFvs must be expressed in the same cell in similar amounts. This latter requirement is difficult to uphold in eukaryotic expression systems, such as mammalian or yeast cells, which are often preferred because high yields of enriched product can be obtained. In contrast to native Abs, diabodies have only one binding domain for each specificity. However, bivalent binding is an important means of increasing the functional affinity and possibly the selectivity for particular cell types carrying densely clustered Ags.

To circumvent the drawbacks of diabodies, and to increase the valence, stability, and therapeutic potential of recombinant BsAbs, the author and colleagues generated single-chain molecules comprising four Ab-variable domains (\(V_H\) and \(V_L\)) of two different specificities, in an orientation that prevents Fv formation from the adjacent \(V_H\) and \(V_L\) domains \((23)\). These molecules can either form bivalent BsAbs by a diabody-like folding (sc-diabodies) or dimerize with the formation of tetravalent BsAbs with \(M_r\) 115 kDa (tandem diabodies \([\text{TandAb}]\); \textbf{Fig. 3}). The efficacy of formation, stability, and biological activity of the TandAb molecules are dependent both on the length and amino acid composition of the linkers separating individual \(V_H\) and \(V_L\) domains \((25)\) and on the strength of association of the complementary \(V_H\) and \(V_L\) domains located on different polypeptide chains. The TandAb is twice the size of a diabody, is able to

\[
\begin{align*}
\text{scDb, 50 kDa} & \quad \text{scDb, 50 kDa} \\
\text{TandAb, 115 kDa} & \quad \text{TandAb, 115 kDa}
\end{align*}
\]

\textbf{Fig. 3.} Single-chain, four-domain gene constructs for the production of dimeric or tetrameric bispecific molecules. Depending on the linker length, a single-chain diabody (scDb), or tetravalent tandem diabody (TandAb) can be formed. Ab-variable domains (\(V_H, V_L\)), peptide linkers (L), and antigen-binding sites (Ag) of specificities A and B are indicated. The TandAb orientation is shown according to a molecular model of the CD19 × CD3 TandAb \((23)\).
bind bivalently to both effector and target cells, and possesses improved pharmacological characteristics (e.g., longer half-life in circulation, greater stability, and enhanced biological activity both in vitro and in vivo \(23, 26\)). For example, a TandAb reactive with the B-cell markers CD19 and CD3 on T lymphocytes appeared to be much more potent than the diabody for inducing human T-cell proliferation in the presence of irradiated CD19⁺ B cells. In cytotoxicity assays, the TandAbs were able to retarget human T lymphocytes to malignant B cells, and the efficacy of TandAb-mediated cell lysis compared favorably with that obtained with a diabody of the same dual specificity \(23\). In vivo studies demonstrated that tetravalent TandAbs both were more stable and were retained longer in the blood of normal mice, compared with scFv and diabodies. In experiments with severe combined immunodeficient (SCID) mice bearing preestablished Burkitt’s lymphoma (5 mm in diameter), treatment with human peripheral blood lymphocytes (PBL), TandAb, and an anti-CD28 MAb resulted in the complete elimination of tumors in all animals within 10 days. In contrast, mice receiving human PBL in combination with the diabody alone or diabody plus anti-CD28 MAb showed only partial tumor regression \(26\). Moreover, the CD19 × CD3 TandAbs demonstrated very high efficacy in partnership with autologous T cells in eradicating malignant primary B cells from patients with B-cell chronic lymphocytic leukemia at very low effector-to-target ratios. In contrast, the structurally similar but bivalent diabody and single-chain diabody demonstrated practically no antitumor activity in an autologous system \(27\). The TandAb format could therefore prove to be particularly advantageous for cancer immunotherapy.

Secretion into the periplasm of bacteria often leads to improper folding and even aggregation of the single-chain antibodies comprising four and more immunoglobulin domains, such as scFv-scFv fusions, single-chain diabodies, or TandAbs \(24, 28, 29\). This is likely due to the low concentration of relevant chaperones. Therefore, mammalian cells (CHO, HEK-293, etc.) appeared to be better hosts for production of such complex molecules. For example, the author and colleagues found that TandAbs produced in CHO cells proved to be 5- to 15-fold more active in retargeting effector cell cytotoxicity than the molecules isolated from *Escherichia coli* (unpublished observation). However, folding of the recombinant BsAb in bacteria can significantly be improved by several means. For example, aggregation of the Ab fragments in the *E. coli* periplasm can be reduced by growing the induced cells under osmotic stress in the presence of certain non-metabolized additives, such as sucrose \(30\) or sorbitol and glycine betaine \(23\). Moreover, inducing synthesis of recombinant Ab fragments in bacteria under osmotic stress promotes the formation
of domain-swapped dimeric molecules, in particular, TandAbs (23). In addition, folding of secreted Ab fragments could be significantly improved by coexpression of bacterial periplasmic chaperones, such as Skp/OmpH/HlpA (31) and FkpA (32–34). Therefore, the author strongly recommends using a combination of Skp coexpression with induction of the protein synthesis under osmotic stress for the production of highly active TandAbs in bacteria, as described in the protocol below and in published work (23, 25).

Table 1

| Oligonucleotides for PCR amplification of DNA fragments used in assembly of bispecific CD19 × CD3 diabody and tandem diabody genes |
|---------------------------------------------------------------|
| **Construction of hybrid VHCD3-VLCD19 and VHCD19-VLCD3scFv genes** |
| **VH domains** |
| DP1 \( (n = 42) \) |
| 5’TCA CAC AGA ATT CTT AGA TCT ATT AAA GAG GAG AAA TTA ACC |
| \( \text{EcoRI} \quad \text{BglII} \) |
| DP2 \( (n = 40) \) |
| 5’AGC ACA CGA TAT CAC CGC CAA GCT TGG GTG TTG TTT TGG C |
| \( \text{EcoRV} \quad \text{HindIII} \) |
| **VL domains** |
| DP3 \( (n = 43) \) |
| 5’AGC ACA CAA GCT TGG CGG TGA TAT CTT GCT CAC CCA AAC TCC A |
| \( \text{HindIII} \quad \text{EcoRV} \) |
| DP4 \( (n = 57) \) |
| 5’AGC ACA CTC TAG AGA CAC ACA GAT CTT TAG TGA TGG TGA TGG |
| \( \text{XbaI} \quad \text{BglII} \quad \text{TGA TGT GAG TTT AGG} \) |
| **Construction of a gene encoding four domain fusion protein VHCD3-VLCD19-Linker-VHCD19-VLCD3** |
| **VHCD3-VLCD19-Linker** |
| Bi3sk \( (n = 33) \) |
| 5’CAG CCG GCC ATG GCG CAG GTG CAA CTG CAG CAG |
| \( \text{NcoI} \) |
| Li-2 \( (n = 57) \) |
| 5’TAT ATA CTG CAG CTG CAC CTG CGA CCC TGG GCC ACC ACC AGC GGC |
| \( \text{PvuII} \quad \text{CGC AGC ATC AGC CCG} \) |
This chapter describes the generation of genetic constructs encoding the bispecific diabody and TandAb, as well as protocols for bacterial expression and purification of active bispecific molecules. The generation of plasmids for high-level expression of bispecific diabody and TandAb in *E. coli* includes the following steps:

1. Construction of genes encoding hybrid scFvs consisting of the \(V_h\) domain from one Ab (\(V_h^A\)) connected by a 5–10 amino acid linker to the \(V_l\) domain of another Ab (\(V_l^B\)).

2. Construction of a dicistronic operon for coexpression and co-secretion of two hybrid scFvs, \(V_h^A\cdot V_l^B\) and \(V_h^B\cdot V_l^A\), with the formation of functional heterodimer (diabody) in the bacterial periplasm (Fig. 2b).

3. Joining the two dimerizing parts, \(V_h^A\cdot V_l^B\) and \(V_h^B\cdot V_l^A\), with a peptide linker of 10–20 amino acids in length.

As an example, the primers used for the generation of genetic constructs coding for a CD19 × CD3 diabody (18) and TandAb (23) are listed in Table 1.

### 2. Materials

#### 2.1. Gene Assembly by Polymerase Chain Reaction (PCR)

1. Thermocycler PTC 150–16 (MJ Research).
2. *Vent* DNA polymerase (New England Biolabs).
3. 10× *Vent* buffer (New England Biolabs).
4. Sterile deionized water.
5. 100 mM deoxyribonucleoside triphosphates (dNTPs) (New England Biolabs).
6. Bovine serum albumin (BSA), nonacetylated (10 mg/mL) (New England Biolabs).
7. \(\lambda\) BstEII DNA molecular weight marker (New England Biolabs).
8. Agarose (FMC BioProducts).
9. 1× Tris-acetate electrophoresis buffer (1× TAE buffer): Prepare a stock solution of 50× TAE, and dilute it 1:50 with \(H_2O\) before use.
10. 50× TAE buffer: 242 g/L Tris base, 57.1 mL/L glacial acetic acid, 100 mL/L of 0.5 M ethylenediamine tetraacetic acid (EDTA).
11. 0.5 M EDTA.
12. Ethidium bromide (10 mg/mL).

#### 2.2. Cloning into Expression Vector

1. Tabletop microcentrifuge.
2. QIAquick Gel Extraction kit (Qiagen).
3. QIAquick-spin PCR Purification kit (Qiagen).
4. AflII, BglII, EcoRI, EcoRV, HindIII, NcoI, NdeI, PvuII, and XbaI restriction endonucleases (New England Biolabs).
5. 10× restriction enzyme buffers (New England Biolabs).
6. Calf intestine alkaline phosphatase (CIP) (New England Biolabs).
7. T4 DNA ligase (Stratagene).
8. 10× T4 DNA ligase buffer (Stratagene).
9. 3 M sodium acetate, pH 4.8.
10. Glycogen, molecular biology grade (20 μg/mL) (Boehringer).
11. Absolute ethanol.
12. 80% (v/v) ethanol.

2.3. Preparation of Bacterial Culture

1. 85 mm Petri dishes.
2. Sterile glass Erlenmeyer flasks, 100, 1,000, and 5,000 mL.
3. Thermostatic shaker.
4. Centrifuge with a set of fixed-angle rotors (Kendro, Hanau, Germany).
5. Either E. coli K12 XL1-Blue (Stratagene) or E. coli RV308 (lac74galISII::OP308strA) (35) (ATCC 31608) prepared as competent cells (see Note 1).
6. 2YT medium: 16 g/L Bacto-tryptone, 10 g/L yeast extract, and 5 g/L NaCl, pH 7.5 (see Note 2).
7. 2YTGA: 2YT medium containing 0.1 g/L ampicillin and 2% (w/v) glucose.
8. 2YTGA agar plates. Media and agar plates are prepared according to standard protocols as described (36).
9. 2YTSA: 2YT medium containing 0.1 g/L ampicillin and 0.4 M sucrose (see Note 3).
10. YTBS: 2YT medium containing 0.1 g/L ampicillin, 1 M sorbitol, and 2.5 mM glycine betaine.
11. 100 mM solution of isopropyl-β-d-thiogalactopyranoside (IPTG). Store at −20°C.

2.4. Isolation of Recombinant Product from Soluble Periplasmic Fraction and Culture Medium

1. Ammonium sulfate powder.
2. Magnetic stirrer.
3. Dialysis tubing with a 12-14 kDa cutoff.
4. 200 mM Tris-HCl, 20% sucrose, and 1 mM EDTA, pH 8.0.
5. 50 mM Tris-HCl and 1 M NaCl, pH 7.0.
6. 50 mM Tris-HCl, 1 M NaCl, and 50 mM imidazole, pH 7.0.
7. 50 mM Tris-HCl, 1 M NaCl, and 250 mM imidazole, pH 7.0.
8. C16/20 column (GE Healthcare).
9. Chelating Sepharose Fast Flow (GE Healthcare).
10. 0.1 M CuSO₄.

2.5. Purification of BsAb Fragments and Analysis of Molecular Forms

1. Mono S HR5/5 column (GE Healthcare).
2. Mono Q HR5/5 column (GE Healthcare).
3. Superdex 200 10/300 GL column (GE Healthcare).
4. 50 mM imidazole-HCl, pH 6.4. Filter (0.2 µm) and store at 4°C.
5. 50 mM imidazole-HCl and 1 M NaCl, pH 6.4. Filter (0.2 µm) and store at 4°C.
6. 20 mM Tris-HCl, pH 8.0. Filter (0.2 µm) and store at 4°C.
7. 20 mM Tris-HCl and 1 M NaCl, pH 8.0. Filter (0.2 µm) and store at 4°C.
8. Phosphate-buffered saline (PBS): 15 mM Na phosphate and 0.15 M NaCl, pH 7.4. Filter (0.2 µm), and store at 4°C.
9. PBSI: PBS containing 50 mM imidazole, pH 7.4. Filter (0.2 µm), and store at 4°C.
10. Biomax-10 Ultrafree-15 centrifugal filter device (Millipore).
11. PD-10 prepacked disposable columns containing Sephadex G-25 (GE Healthcare).
12. High- and low-molecular weight gel-filtration calibration kits (GE Healthcare).
13. Bio-Rad protein assay kit (Bio-Rad).
14. 20% human serum albumin.

3. Methods

3.1. Generation of Plasmids for Expression of BsAbs

3.1.1. PCR Amplification

1. Perform PCR amplifications in 50 µL reaction mix containing 50 ng plasmid DNA as template, 25 pmol of each primer, 300 µM dNTPs, 5 µL of 10× PCR buffer, 5 µg BSA, and 1 U Vent DNA polymerase.
2. Run 15–20 PCR cycles on a thermocycler. Cycles comprise 95°C for 1 min (denaturation), 57°C for 2 min (annealing), and 75°C for 2 min (extension). At the beginning of the first cycle, incubate for 3 min at 95°C, and, at the end of the last cycle, incubate for 5 min at 75°C.
3. The author and colleagues have described the construction of a CD19 × CD3 diabody (16,18) that serves here as an
example of the general approach. Primers DP1 and DP2 (Table 1) were used to amplify the V\textsubscript{H} domain from pHOG-\alphaCD19, which encodes an scFv reactive with human CD19 \cite{18}. This is shown schematically in Fig. 2a. The primers introduce EcoRI and BglII sites upstream of the ribosome-binding site and a short linker downstream from V\textsubscript{H}\textsuperscript{CD19} to HindIII and EcoRV sites. Amplification from the same template with DP3 and DP4 (Table1) recovered the V\textsubscript{L}\textsuperscript{CD19} domain and detection/purification tags, fitting the product with a linker sequence and restriction sites (HindIII/EcoRV [upstream] and BglII/XbaI [downstream]).

4. For TandAb construction \cite{23}, the hybrid scFv 3–19 gene encoded by pHOG3–19 (see Subheading 3.1.2, step 6) was amplified with primers, Bi3sk and Li-2 (Table 1). This introduces a short rigid linker downstream of the V\textsubscript{L} domain and restriction sites (NcoI upstream of V\textsubscript{H}\textsuperscript{CD3}, PvuII downstream of the linker).

5. Analyze the amplified DNA fragments by electrophoresis on a 1.5% agarose gel prestained with ethidium bromide.

3.1.2. Cloning into Expression Vector (see Note 4)

1. Digest 10 \textmu g of appropriate vector with suitable restriction endonuclease, in presence of alkaline phosphatase (CIP). Incubate for at least 2 h at the optimal temperature as recommended by the supplier.

2. Purify the PCR fragments and the linearized vector by agarose gel electrophoresis, and then extract the DNA from gel fragments using a QIAquick gel extraction kit.

3. Digest the isolated PCR fragments with restriction endonucleases suitable for cloning into the vector of choice.

4. Remove stuffer fragments, and purify the digested PCR products using the QIAquick-spin PCR purification kit.

5. Ligate the vector, and insert using a molar ratio of between 1:1 and 1:3. The reaction mixture should consist of 50 ng DNA, 1 U of T4 DNA ligase, ligation buffer, and water to a final volume of 10–20 \textmu L. Incubate overnight at 16°C.

6. In our construction of the CD19 × CD3 diabody, the DP1/DP2 amplicon (see Subheading 3.1.1, step 3), was cut with EcoRI/EcoRV and ligated into pHOG-dmOKT3, a plasmid carrying an anti-CD3 scFv \cite{37}, after digestion of the vector with the same endonucleases. This substituted the V\textsubscript{H}\textsuperscript{CD3} domain with V\textsubscript{H}\textsuperscript{CD19}, fusing it to V\textsubscript{L}\textsuperscript{CD3} through the linker encoded on primer DP2. The construct was designated pHOG19-3. The DP3/DP4 product was cut with HindIII/XbaI and ligated into pHOG-dmOKT3, thereby fusing V\textsubscript{L}\textsuperscript{CD19} to V\textsubscript{H}\textsuperscript{CD3} through the linker encoded on DP3, creating pHOG3-19. The plasmid encoding the BsDb was
finally generated by excising the \( \text{V}_{\text{H}}^{\text{CD19}}.\text{V}_{\text{L}}^{\text{CD3}} \text{scFv} \) cassette from pHOG19-3 and ligating it into pHOG3-19 (I8). This construct is shown schematically in Fig. 2b.

7. The CD19 × CD3 TandAb was generated by cutting the Bi3sk/Li-2 product \( \text{Neol/PvuII} \) and ligating it into pHOG19-3, shown schematically in Fig. 3.

8. Precipitate the DNA by adding one-tenth volume of 3 M sodium acetate, 20 \( \mu \)g glycogen, and 2.5 volumes of absolute ethanol. Incubate for at least 3 h at \(-20^\circ\text{C}\). Recover the precipitate by centrifugation for 15 min at 10,000 \( \times g \) in a minicentrifuge. Wash the pellet four times with 500 \( \mu \)L of 80% ethanol, followed by centrifugation for 10 min at 10,000 \( \times g \). Allow the pellet to dry at room temperature. Dissolve the dry pellet in 5 \( \mu \)L water.

9. Use the products of one ligation reaction for the electroporation of 40 \( \mu \)L of electrocompetent \( E. \text{coli} \) cells (see Note 1), according to the supplier’s protocol. Plate the bacteria on 2YT agar plates containing 0.1 g/L ampicillin and 2% (w/v) glucose. Incubate overnight at 37°C.

10. Test individual colonies for the presence of the desired insert by plasmid minipreps (see Note 5).

3.2. Preparation of Bacterial Culture

1. Inoculate a few milliliters of 2YTGA (see Note 2) with an individual bacterial colony, and let it grow overnight at 37°C (\( E. \text{coli} \) XL1-Blue) or 26°C (RV308).

2. Dilute the overnight bacterial culture 40-fold with fresh 2YTGA and incubate at 37°C (XL1-Blue) or at 26°C (RV308) with vigorous shaking (180–220 rpm), until the optical density at 600 nm reaches 0.8–0.9.

3. Harvest bacteria by centrifugation at 1,500 \( \times g \) for 10 min at 20°C.

4. Resuspend the pelleted bacteria in the same volume of either fresh 2YTSA or YTBS medium (see Note 6). Add IPTG to a final concentration of 0.2 mM (see Note 7) and incubate the bacterial culture for 14–16 h with shaking at room temperature (22–24°C).

5. Collect the cells by centrifugation at 6,200 \( \times g \) for 20 min and either discard the culture supernatant (RV308) or retain it and keep on ice (XL1-Blue) (see Note 8).

3.3. Isolation of Recombinant Product from Soluble Periplasmic Fraction and Culture Medium (see Note 4)

1. Resuspend the pelleted bacteria to 5% of the initial culture volume using ice-cold 200 mM Tris-HCl, 20% sucrose, 1 mM EDTA, pH 8.0, and incubate on ice for 1 h with occasional stirring.

2. Centrifuge the cell suspension at 30,000 \( \times g \) for 40 min at 4°C and carefully collect the supernatant. This comprises
the soluble periplasmic extract. When using RV308, go to **step 5**. When using XL1-Blue, combine the culture supernatant (**see Subheading 3.2, step 5**) and the soluble periplasmic extract.

3. Concentrate the bispecific recombinant product by ammonium sulfate precipitation (**see Note 9**). Place the beaker with the culture supernatant and the soluble periplasmic extract on a magnetic stirrer. Slowly add ammonium sulfate powder to a final concentration of 70% saturation (472 g/L). Continue stirring for at least another 2 h at 4°C.

4. Collect the protein precipitate by centrifugation (30,000 × g, 4°C, 30 min) and dissolve it to one-tenth the initial volume using 50 mM Tris-HCl, 1 M NaCl, pH 7.0.

5. Thoroughly dialyze the concentrated protein against 50 mM Tris-HCl, 1 M NaCl, pH 7.0, at 4°C. Clarify the dialyzed material by centrifugation (30,000 × g, 4°C, 60 min).

6. For immobilized metal-affinity chromatography (IMAC), prepare a column of Chelating Sepharose (1–2 mL resin/L flask culture), and wash with five bed volumes of water. Charge the column with Cu²⁺ by loading 0.7 bed volumes of 0.1 M CuSO₄ (**see Note 10**), wash out the excess of ions with ten bed volumes of water, then equilibrate with three volumes of 50 mM Tris-HCl, 1 M NaCl, pH 7.0 (**see Note 11**).

7. Pass the soluble periplasmic proteins through the Chelating Sepharose column, either by gravity-flow or by using a peristaltic pump. Wash the column with ten bed volumes of the starting buffer (50 mM Tris-HCl, 1 M NaCl, pH 7.0), followed by the buffer containing 50 mM imidazole (**see Note 12**) until the absorbance (280 nm) of the effluent is minimal (20–30 column volumes). Perform all chromatography steps at 4°C.

8. Elute bound Ab fragments with the starting buffer containing 250 mM imidazole (**see Note 13**).

9. Analyze the purity of eluted material by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (**38**).

1. If SDS-PAGE indicates that the IMAC has yielded a homogeneous BsAb preparation, go to **step 6** (**see Note 14**). Otherwise, calculate the isoelectric point (pI) of the bispecific product on the basis of its amino acid composition (**see Note 15**).

2. Subject the protein material eluted from the IMAC column to buffer exchange, either into 50 mM imidazole-HCl, pH 6.0–7.0, or 20 mM Tris-HCl, pH 8.0–8.5, using pre-packed PD-10 columns (**see Note 16**). Remove any turbidity from the protein solution by centrifugation (30,000 × g, 4°C, 30 min).
3. Load the protein solution, either on a Mono Q or Mono S column, equilibrated either with 20 mM Tris-HCl (pH 8.0–8.5) or 50 mM imidazole-HCl (pH 6.0–7.0), respectively. Wash the column with at least ten column volumes of the starting buffer.

4. Elute the bound material using a linear gradient of 0–1 M NaCl in the starting buffer and collect 0.5 mL fractions.

5. Perform SDS-PAGE analysis with the eluted fractions.

6. Pool those fractions that contain the pure recombinant Ab. Determine the protein concentration (see Note 17).

7. Perform a buffer exchange into PBSI, pH 7.0–7.4 (see Note 18), and concentrate the purified Ab preparations up to 1.0–2.0 mg/mL using Ultrafree-15 centrifugal filter units.

8. Equilibrate a Superdex 200 column with PBSI buffer and calibrate the column using high- and low-molecular weight gel-filtration calibration kits.

9. For analytical size-exclusion chromatography, apply 50 µL of concentrated preparation of bispecific product to a Superdex 10/300 GL column. Perform gel filtration at 4°C, monitor the UV absorption of effluent at 280 nm, and, if necessary, collect 0.5 mL fractions.

10. For long-term storage, stabilize purified Ab fragments by adding human serum albumin to a final concentration of 10 mg/mL. Store the sample at −80°C (see Note 19).

4. Notes

1. Both XL1-Blue and RV308 are suitable hosts for expression of BsAb fragments in shake-flask bacterial cultures. XL1-Blue has the following advantages: electrocompetent bacterial cells are commercially available (Stratagene) and standard DNA isolation protocols yield DNA preparations of sufficient purity for restriction analysis and sequencing. However, RV308 is a more robust, fast-growing strain, suitable for high-cell density fermentation (39). Moreover, unlike XL1-Blue, the author and colleagues have not observed leakage of Ab fragments into the culture medium. Note that, unlike XL1-Blue, RV308 does not contain lacIq gene for over-expression of the lac repressor. Therefore, if expression of the gene of interest is controlled by a lac operator, the expression vector must also carry a lacI gene to ensure robust, IPTG-dependent regulation of Ab expression.
2. Luria–Bertani (LB) broth can also be used. However, we observed that the simple substitution of LB for the richer 2YT medium gave an essential increase in the yield of soluble bispecific molecules.

3. 2YTSA medium is prepared directly before use by dissolving sucrose at 137 g/L in sterile 2YT medium containing 0.1 g/L ampicillin.

4. The protocols were established for vectors pHOG21 (30) and pSKK (23, 40), which were designed for periplasmic expression of a single recombinant product, and for their derivatives, pKID (18, 20) and pSKID2 (16), respectively, which allowed coexpression of two-hybrid scFvs. Note that these vectors add to the expressed inserts C-terminal His-tags for IMAC isolation of the recombinant proteins (see Subheading 3.3). Alternative methods of purification must be sought for those vector systems that do not confer this property.

5. All DNA manipulations and transformation experiments are performed according to standard cloning protocols (36).

6. The change of medium and induction of protein synthesis in bacteria under osmotic stress significantly increases the yield of BsAb fragments, since these conditions favor domain swapping and promote the formation of dimers (23).

7. This concentration of IPTG was found to be optimal for vectors containing an scFv gene under the control of wild-type lac promoter/operator, such as pHOG21 (30) or pSKK (23, 40). Nevertheless, performing small-scale experiments to optimize the induction conditions is recommended for each vector.

8. For XL1-Blue, a significant portion of Ab fragments is found in the culture medium, either because of leakage through the bacterial outer membrane or because of partial cell lysis. Therefore, supernatant should also be used as a starting material for isolation of recombinant protein.

9. Ammonium sulfate precipitation is especially recommended for concentrating bispecific diabodies. This procedure was shown to be ineffective for precipitating monospecific scFvs (18).

10. IMAC can be also performed on Ni2+-charged Chelating Sepharose or Ni-NTA-Superflow resin (Qiagen). However, the use of Cu2+ instead of Ni2+ is recommended for isolation of Ab fragments for clinical applications (41).

11. Tris-HCl buffer is usually not recommended for IMAC because of the presence of amines interacting with immobilized metal ions. However, we have found that such conditions do not influence the absorption of strong binders containing six histidines, while preventing nonspecific interactions of some E. coli proteins with the Chelating Sepharose.
12. Unlike Chelating Sepharose, the Ni-NTA columns should not be washed with buffers containing imidazole at concentrations higher than 20 mM.

13. To avoid the unnecessary dilution of eluted scFvs, collect 0.5 to 1.0 mL fractions, and monitor the UV absorbance at 280.

14. The purity of the Ab fragments eluted from the IMAC column depends on the expression level of the particular recombinant protein. As a rule, the yield and purity of bispecific diabody are higher than those of TandAb.

15. The isoelectric point of the protein can be calculated using a number of computer programs, e.g., Protein Calculator v3.3, freely available over the internet (http://www.scripps.edu/~cdputnam/protcalc.html). The calculated pI value hints at the ion exchange matrix and buffer system that should be used.

16. For bispecific molecules with pI values below 7.0, the author recommends using an anion exchanger, such as a Mono Q with a linear 0–1 M NaCl gradient in 20 mM Tris–HCl, pH 8.0. For proteins with pI values higher than 7.0, cation-exchange chromatography on a Mono S column with linear 0–1 M NaCl gradient in 50 mM imidazole–HCl buffer (pH 6.0–7.0) can be recommended. Moreover, the author has found that, by exchanging the buffer for 50 mM imidazole–HCl, pH 6.4–6.7, after IMAC, most of the contaminating bacterial proteins precipitate, while the recombinant Ab fragments remain soluble (23).

17. For determination of protein concentrations, the author recommends using a Bradford dye-binding assay because it is easy to use, sensitive, and fast (42).

18. The author recommends PBSI buffer because PBS alone appears to destabilize some Ab fragments. It was determined empirically that PBS with 50 mM imidazole, pH 7.0–7.5, is a suitable buffer for various Ab fragments kept at relatively high concentrations (2–3 mg/mL). Moreover, this buffer does not interfere with Ag binding, and does not show any toxic effects after incubation with cultured cells, or after injection into mice (intravenous injection of 200 μL) (23).

19. Alternatively, recombinant protein can be stabilized by adding BSA or fetal calf serum. Human serum albumin is recommended for Ab fragments developed for clinical applications. The recombinant Abs stabilized by albumin can be stored at −80°C for years without loss of activity. These preparations may be used for a number of biological assays, such as ELISA, flow cytometry, and analyzes of antitumor activity, both in vitro and in vivo.
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Intrabody Expression in Eukaryotic Cells

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Summary

We describe procedures for intracellular expression of scFv in eukaryotic cells. Starting from a scFv gene cloned in a phage-display vector, we describe the cloning step into a mammalian expression vector, the transient transfection of a HeLa cell line, and the monitoring of intrabody expression by immunofluorescence staining and FACS analysis.

Key words: Antibody fragment, scFv, Intrabody, Intracellular immunization, Disulfide bond

1. Introduction

Single-chain Fv fragments (scFv) are 28 kDa immunoglobulin-derived molecules composed of the variable region of a heavy and a light chain, joined together by a flexible linker. scFvs are expressed from a single recombinant gene and retain the specificity and the binding affinity of the parental antibodies.

Intracellular antibodies, or intrabodies, are scFvs that are ectopically expressed within the cytosol of the cell. This offer intrabodies the opportunity to interact with their cognate antigen inside the cell. This interaction may result in the modulation or inhibition of the functions of the antigen, either by direct interference or by diverting it from its normal intracellular location (1). In addition, by adding to the scFv a targeting signal, such as a nuclear localization signal or a retention signal for the endoplasmic reticulum (ER), expression of the scFv can be restricted to a specific location within the cell.

One of the main limitations to the application of intrabodies is that many scFvs are not able to fold under the reducing conditions
that pertain in the cell cytosol and nucleus. This is due to the limited stability of scFvs when conserved disulfide bonds present in the heavy and light chain variable domains remain in a reduced state. The main consequence is the formation of insoluble aggregates, rendering antibodies nonfunctional (Fig. 1). Even when aggregated, some intrabodies may retain the capacity to sequester the target antigen, preventing it from reaching its intended cellular location and thereby inhibiting its function (2–3). Even if the intrabody is expressed as soluble protein in the cell cytoplasm, its inhibitory potential may be limited by the level of expression with respect to the target molecule, and by its half-life. These considerations have prompted the development of stable frameworks (4) upon which scFvs can be constructed that fold correctly and that are formed in quantities sufficient to be active as intracellular antibodies.

Intrabody construction requires a panel of antibodies against the antigen of interest. These can be derived by ribosome display, by a yeast two-hybrid system, or even by classic hybridoma technology. Antibodies selected from the panel must be tested in vivo for their correct expression and ability to inhibit their target. Selection of scFvs from an optimized library will greatly increase the success rate of this experiment (5–8).

**Fig. 1.** Expression of scFvs in transfected HeLa cells. Analysis of HeLa cells 48 h after transfection with scFvs cloned into a pCMV/myc/cyto vector. Left panels: use of anti-myc reagents to detect expression patterns of a soluble intrabody that does not recognize a cellular target (a), a soluble intrabody directed against tubulin (b), and an aggregating intrabody (c). Right panels: Hoechst staining of nuclear DNA in cells transfected with designated scFvs.
In this chapter, we describe procedures for intracellular expression of scFv in eukaryotic cells. We also give details for the subcloning of a selected scFv sequence into a mammalian expression vector, the transient transfection of the HeLa cell line, and the monitoring of intrabody expression by immunofluorescence staining and fluorescence-activated cell sorting (FACS) analysis.

## 2. Materials

1. pCMV/myc/cyto (Invitrogen) plasmid DNA (see Notes 1 and 2).
2. Plasmid DNA encoding a candidate scFv in pCANTAB6 or an equivalent vector.
3. restriction enzymes and buffers: NeoI, NotI (New England Biolabs).
4. Kit for purification of DNA fragments from agarose gels.
5. T4 DNA ligase.
6. Competent *Escherichia coli* cells.
7. Luria–Bertani (LB) liquid and solid media: 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl, pH adjusted to 7.0 with 5 M NaOH. For solid medium, add 15 g/L of agar. Autoclave. Allow the solution to cool to 60°C or less before adding ampicillin to a final concentration of 100 μg/mL.
8. Kit for large-scale preparation of plasmid DNA.
9. Dulbecco’s modified Eagles’ medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS). Store at 4°C.
10. Trypsin/EDTA.
11. 6 well and 12 well tissue culture plates.
12. 12 mm-diameter glass coverslips. Store in 95% ethanol and air dry before use.
13. 150 mM NaCl. Sterilize by filtration through a 0.22 μm filter or by autoclaving.
14. jetPEI transfection reagent (Polyplus Transfection).
15. Phosphate-buffered saline (PBS): 8 g/L NaCl, 0.2 g/L KCl, 0.61 g/L Na₂HPO₄, and 0.2 g/L KH₂PO₄, pH 7.4. Sterilize by autoclaving.
16. Cold absolute methanol. Store at −20°C.
17. 1% (w/v) bovine serum albumin (BSA) in PBS.
18. 9E10 mouse anti-c-myc monoclonal antibody (mAb) (see Notes 1 and 14).
19. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody.

20. Cell permeant, fluorescent DNA stain (e.g., DAPI, Hoechst 33342, or similar) at 1 mg/mL in 150 mM NaCl.

21. Mowiol coverslip mounting solution: add 6 g glycerol and 2.4 g Mowiol 4–88 (Sigma) to 6 mL distilled water and leave at room temperature for 2 h. Add 12 mL of 0.2 M Tris-HCl, pH 8.5, and incubate at approximately 55°C until Mowiol has dissolved. Clarify by centrifugation at 5,000 × g for 15 min. Store in 1 mL aliquots at −20°C.

22. FACS buffer: PBS, 0.1% (w/v) BSA, and 0.01% (w/v) sodium azide. Store at 4°C.

23. 3.7% (w/v) formaldehyde diluted in FACS buffer.

24. 0.5% (w/v) saponin diluted in FACS buffer.

3. Methods

3.1. Subcloning of scFv into Eukaryotic Expression Vector pCMV/myc/cyto (see Note 3)

The following protocol assumes that a scFv clone of interest has been first isolated from a library constructed in a pCANTAB- or pHEN-derived phagemid vector (8).

1. The coding sequence of the candidate scFv is obtained by digesting the selected pCANTAB6 plasmid with NeoI and NotI (see Note 4) in a reaction mix comprising 1 μg DNA, 5 μL of 10× NEBuffer 3, 0.5 μL 100x BSA, 1 μL NeoI (10 U/μL), 1 μL NotI (10 U/μL), and water to 50 μL. Incubate for 3 h at 37°C.

2. Also digest 1 μg of the expression plasmid pCMV/myc/cyto with the same enzymes (see Note 5).

3. Purify the 750 bp scFv fragment and the expression vector fragment plasmid on an agarose gel using a commercial DNA purification kit.

4. Set up the ligation reaction with an excess of insert (1:3 vector:insert molar ratio). Mix 100 ng digested and purified vector, 40 ng digested and purified scFv gene, 2 μL of 10× ligase buffer, 0.4 μL T4 DNA ligase (5 Weiss U/μL), and water to 20 μL. Incubate for 1 h at room temperature (see Note 6).

5. Inactivate the T4 DNA ligase by heating for 10 min at 65°C.

6. Transform competent E. coli cells with 10 μL of the ligation reaction, plate on LB agar containing 100 μg/mL ampicillin, and incubate overnight at 37°C.
7. Test individual colonies for the presence of the insert either by isolation of plasmid DNA preparation followed by digestion with \textit{Neol} and \textit{NotI}, or by polymerase chain reaction (PCR), using as a template crude extracts from single bacterial colonies. Primers used for the amplification are: pCMV-Forward: 5' CGCAAATGGGCGGTAGGCGTG 3' BGH-Reverse: 5' TAGAAGGCACAGTCGAGG 3'

8. Perform a large-scale plasmid DNA extraction of a positive clone (see Note 7).

The human HeLa cell line is grown in DMEM supplemented with 10% heat-inactivated FCS at 37°C in a 5% CO$_2$ humidified atmosphere. Cells should be seeded 24 h before transfection.

1. Aspirate and discard culture medium and rinse the cells with trypsin/EDTA.

2. Add enough trypsin/EDTA to cover the cell monolayer and incubate at 37°C for approximately 4 min until cells become round and start to float.

3. Neutralize the trypsin with a two-fold excess of supplemented culture medium.

4. Centrifuge the cells at 300 $\times$ $g$ and resuspend the pellet in fresh medium.

5. Count the cells and plate 4 $\times$ 10$^5$ cells/well in 6 well plates containing 12 mm diameter glass coverslips (see Notes 9 and 10).

6. On the day of transfection, check that the cells have reached 50–60% confluence. Replace the culture medium with 2 mL fresh DMEM with 10% FCS.

7. For each well of cells that are to be transfected, set up two tubes. In the first, dilute 3 $\mu$g of DNA into 50 $\mu$L of 150 mM NaCl; in the second, mix 6 $\mu$L of jetPEI solution into 50 $\mu$L of 150 mM NaCl. Vortex the tubes and then add 50 $\mu$L of the jetPEI solution to 50 $\mu$L of the DNA solution. Vortex again, and incubate for 30 min at room temperature.

8. Add the 100 $\mu$L transfection mixture dropwise to a well of the culture plate and distribute by gently swirling the plate.

9. Incubate the cells for 5 h at 37°C, then replace the medium with 3 mL of fresh culture medium.

3.3. Immunofluorescence Microscopy

1. Twenty-four to 48 h after transfection, recover the coverslips with sterile forceps and place each one, cells uppermost, in a well of a 12 well culture plate.

2. Wash the cells once with PBS.

3. Immerse the coverslips in cold absolute methanol (~1 mL), then incubate for 10 min at −20°C (see Notes 11 and 12).
4. Gradually rehydrate the cells by adding PBS in aliquots of approximately 500 μL (see Note 13). Finally, wash cells once more in PBS.

5. Cover the cells with 100 μL of 1% BSA in PBS to minimize nonspecific adsorption of the antibodies to the coverslips. Incubate for 30 min at room temperature.

6. Remove the blocking solution by aspiration and incubate the cells with 100 μL of the 9E10 anti-c-myc primary antibody solution (1:10 dilution in 1% BSA in PBS) for 1 h at room temperature (see Note 14).

7. Wash the cells three times with PBS over the course of 5 min.

8. Incubate the cells with 100 μL of a FITC-labeled anti-mouse antibody solution (1:2,500 in 1% BSA in PBS) for 30 min at room temperature in the dark.

9. To visualize the DNA, add cell-permeant DNA stain dye to the secondary antibody solution to a final concentration of 5 μg/mL. Incubate for 10 min at room temperature in the dark.

10. Wash the cells three times with PBS over the course of 5 min.

11. Place 5 μL of Mowiol mounting medium on the surface of a clean glass microscope slide. With forceps, invert the coverslip and place it cell-side down onto the Mowiol medium. Leave the slide for 1 h at room temperature in darkness before examining under a fluorescent microscope.

3.4. FACS Analysis of Intrabody Expression Level

1. Harvest the transfected cells as described above. Count and transfer 5 × 10⁵ cells per sample into a FACS tube. Pellet the cells by centrifugation for 5 min at 300 × g.

2. Discard the supernatant and resuspend the pellet in 1 mL of 0.1% BSA in PBS. Centrifuge the cells as before.

3. Gently resuspend the pellet in 200 μL of 0.1% BSA in PBS containing 3.7% formaldehyde. Incubate for 10 min at room temperature.

4. Wash the cells with 1 mL of 0.1% BSA in PBS.

5. Resuspend the cells in 200 μL of 0.1% BSA in PBS containing 0.5% saponin. Incubate for 10 min at room temperature.

6. Wash the cells once and add 200 μL of the 9E10 antibody solution (1:10 diluted in PBS containing 0.5% saponin and 0.1% BSA). Incubate for 30 min at 4°C (see Note 14).

7. Wash the cells twice with 1 mL of 0.1% BSA in PBS.

8. Add 200 μL of the FITC-labeled anti-mouse antibody solution (1:2,500 diluted in PBS containing 0.5% saponin and 0.1% BSA). Incubate for 30 min at 4°C in the dark.
9. Add 1 mL of 0.1% BSA in PBS, centrifuge, and resuspend the cells in 0.5 mL FACS buffer. Analyze immediately or store at 4°C in the dark.

4. Notes

1. The plasmid used here for scFv expression in eukaryotic cells is the 5.9 kb pCMV/myc/cyto vector from Invitrogen, designed for cytoplasmic expression. It includes the cytomegalovirus (CMV) promoter for constitutive expression of the protein. The expressed scFv will be also be tagged at its C terminus with the ε-myc epitope recognized by mAb 9E10 (see Note 14). An alternative vector with the stronger EF-1α promoter is also available from Invitrogen.

2. To target your recombinant scFv to a specific intracellular location in mammalian cells (nucleus, mitochondria, ER), vectors containing a targeting signal are also available from Invitrogen (for example pCMV/myc/nuc, pCMV/myc/mit, and pCMV/myc/ER vectors).

3. The NcoI site contains the ATG initiation codon. If the ε-myc detection epitope tag is to be successfully retained, make sure that the appropriate reading frame exists downstream from the NotI site. The reading frame should be successfully retained if the scFv is cloned from pCANTAB or pHEN vectors.

4. The scFv sequence can also be obtained by PCR using appropriate oligonucleotides. Design primers to introduce a NcoI site at the 5′ end and a NotI site at the 3′ end of the gene in the correct reading frame (see Note 3). Digest the PCR product, and purify on silica-based columns prior to ligation.

5. Recircularization of partially digested plasmid can be avoided by dephosphorylation of the 5′ termini with calf intestinal alkaline phosphatase (CIP). Add 0.5 U CIP/µg plasmid directly to the digestion reaction and incubate for 1 h at 37°C.

6. Incubation overnight at 16–20°C is also possible.

7. It is necessary to streak the positive clone at least once on a selective LB plate before moving on to large-scale plasmid isolation to minimize the risk of contamination with other clones. Choose a single, well-isolated colony for the large-scale DNA preparation. It is good practice to sequence the insert to make sure that no mutation has appeared during the cloning procedure.

8. Our protocol uses jetPEI, but other reagents (e.g., Lipofectamine [Invitrogen]) or transfection methods (e.g., electroporation) can be employed.
9. Cells can also be grown and transfected on glass chamber slides. Adapt the transfection conditions according to the manufacturer’s instructions.

10. HeLa cells are adherent. If you wish to perform immunofluorescence staining on nonadherent cells, coat coverslips with poly-l-lysine (0.01% [w/v] solution) for 10 min at room temperature. Remove the poly-l-lysine solution and let the coverslips air dry.

11. To keep the temperature as low as possible, do not use a pipette to transfer methanol. Instead, pour cold methanol directly from the bottle onto the cells and incubate immediately at −20°C.

12. Methanol fixes the cells by dehydration and it precipitates proteins and permeabilizes the cells at the same time. Use methanol fixation for the detection of nuclear and cytoskeletal proteins. An alternative method is to fix the cells in formaldehyde. Formaldehyde cross-links proteins and permits detection of soluble proteins. Incubate the cells for 20 min at room temperature in 3.7% formaldehyde in PBS, then permeabilize the cells for 5 min at room temperature with 0.2% (v/v) Triton X-100 in PBS.

13. Add 500 μL of PBS, mix, then remove 500 μL before adding a fresh 500 μL aliquot of PBS. Repeat three times: the final methanol concentration will be approximately 30%. Alternatively, aspirate the methanol and let the coverslips air dry. The coverslips can be stored at −20°C for months in this state or incubated in PBS for 5 min to rehydrate the cells for further analysis.

14. 9E10 hybridoma is available from ATCC and ECACC. If using culture supernatant from the hybridoma cell line 9E10, an appropriate dilution must be determined by testing 1:10, 1:20, and 1:50 dilutions. Purified 9E10, conjugated or not, can also be purchased from several suppliers (e.g., Santa-Cruz, Abcam, or Sigma) and is generally used at a concentration of 1 μg/mL for immunofluorescence experiments and at 1 μg/106 cells for FACS analysis.

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High-Level Periplasmic Expression and Purification of scFvs

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Summary

The isolation of recombinant antibodies by phage display naturally leads to experiments to evaluate their biological and immunological properties. Although crude preparations may have their value in initial studies, the need often exists for highly purified protein that can be tested in vivo. This chapter describes methods to generate high yields of scFv from bacterial cultures and to purify protein to the degree of homogeneity required for the most exacting analysis.

**Key words:** scFv, Bacterial expression, Purification

1. Introduction

Over recent decades, some of the limitations of monoclonal antibodies (MAbs) as therapeutic agents have been addressed by genetic engineering. Such an approach is particularly suitable because of the domain structure of the antibody (Ab) molecule, where functional domains carrying antigen (Ag)-binding activities (Fabs or Fvs) or effector functions (Fcs) can be exchanged between Abs. Furthermore, genetically truncated versions of an Ab may be produced, ranging in size from the smallest Ag-binding unit or Fv, to Fab’ and F(ab’)2 constructs. To stabilize the association of the recombinant V H and V L domains, they are joined with a short peptide linker to form constructs that are termed single-chain Fv fragments (scFv) (1, 2). These small scFvs are particularly interesting for clinical applications (3–5). They are only half the size of Fabs, and thus have lower retention...
times in nontarget tissues, more rapid blood clearance, and better tumor penetration (6). They are also less immunogenic, and are amenable to fusions with proteins and peptides (7–10).

Unlike glycosylated full-length IgGs, scFvs can be easily produced in bacterial cells as functional Ag-binding molecules. There are two basic strategies for obtaining recombinant Ab fragments from *Escherichia coli*. The first is to produce Ab proteins as cytoplasmic inclusion bodies, followed by refolding in vitro. In this case, the protein is expressed without a signal sequence, under a strong promoter. The inclusion bodies contain the recombinant protein in a nonnative and inactive conformation. To obtain functional Abs, the recombinant polypeptide chains have to be dissolved and folded using laborious and time-consuming refolding procedures (for a review see ref. 11). The second approach for obtaining functional Ab fragments is to imitate the situation in the eukaryotic cell for secreting a correctly folded Ab. Rapid growth in the field of Ab engineering occurred after it was shown that functional Ab fragments could be secreted into the periplasmic space of *E. coli* cells by fusing a bacterial signal peptide to the Ab N terminus and even escape from this location into the culture medium (12, 13). The scFvs are usually correctly processed in the periplasm; they contain intramolecular disulfide bonds and are soluble. However, the high-level expression of a recombinant protein with a bacterial signal peptide in *E. coli* often results in the accumulation of insoluble Ab fragments after transport to the periplasm (14, 15).

It is now recognized that aggregation in vivo is not a function of the solubility and stability of the native state of the protein, but of those of its folding intermediates in their particular environment (16, 17). The degree of successful folding in the periplasm appears to depend to a large extent on the primary sequence of the variable domains (18). Nevertheless, modifying the bacterial growth and induction conditions can increase the proportion of correctly folded soluble scFvs. For example, lowering the bacterial growth temperature has been shown to decrease periplasmic aggregation and to increase the yield of soluble Ab protein (17, 19).

Alternatively, the aggregation of recombinant Ab fragments in the *E. coli* periplasm can be reduced by growing the induced cells under osmotic stress in the presence of certain nonme-tabolized additives, such as sucrose (20) or sorbitol and glycine betaine (21). For example, addition of 0.4 M sucrose to the growth medium gives a 15- to 25-fold increase in the yield of soluble scFvs for bacterial shake-tube cultures and an 80- to 150-fold increase for shake-flask cultures (20). Moreover, inducing synthesis of recombinant Ab fragments in bacteria under osmotic stress promotes the formation of domain-swapped scFv dimers, or so-called “diabodies” (21). In addition, folding of secreted Ab fragments could be significantly improved by coexpression
of bacterial periplasmic chaperones, such as Skp/OmpH/HlpA (22) and FkpA (23–25). In this chapter, protocols for growing and inducing bacterial cells with or without nonmetabolized additives, as well as for the purification of active scFvs from soluble periplasmic extracts, are described.

The purification scheme includes immobilized metal-affinity chromatography (IMAC) as the main step for separating recombinant Abs from bacterial proteins. In contrast to methods based on Ag-affinity chromatography, this does not depend on the specificity of the particular scFv. The procedure is useful for any Ab fragment (scFv, diabody, Fab, etc.) that is secreted into the periplasm, and that has five, six, or eight histidine (His) residues as a C-terminal tag. If the His-tagged protein is highly over-expressed in E. coli, one-step IMAC purification can result in sufficiently pure material for most applications (26, 27). However, if the protein of interest is present only as a small fraction, several contaminating bacterial proteins can bind to the IMAC column under the purification conditions and coelute (for a list of His-rich E. coli proteins, see ref. 28). For further purification of Ab fragments from IMAC-eluted material, Ag-affinity chromatography (14), thiophilic adsorption chromatography (29, 30), or immunoaffinity purification, using immobilized anti-His-tag-MAbs (28), have been used. A simple alternative procedure is presented here, based on the separation of proteins by ion-exchange chromatography. This purification technique has been tested for a number of scFvs and seems to be generally applicable.

2. Materials

2.1. Preparation of scFv Expression Cultures

1. Either E. coli XL1-Blue (Stratagene) or RV308 (Δlac74galISII::OP308strA) (31) competent cells (see Note 1).
2. scFv Ab in an appropriate vector for His-tagged periplasmic expression.
3. 2YT medium: 16 g/L Bacto-tryptone, 10 g/L Bacto-yeast extract, and 5 g/L NaCl, pH 7.5 (see Note 2).
4. 2YTGA: 2YT medium containing 0.1 g/L ampicillin and 2% (w/v) glucose; 2YTGA agar plates.
5. 2YTSA: 2YT medium containing 0.1 g/L ampicillin and 0.4 M sucrose (see Note 3).
6. 2YTBS: 2YT medium containing 0.1 g/L ampicillin, 1 M sorbitol, and 2.5 mM glycine betaine.
7. 100 mM solution of isopropyl-β-D-thiogalactopyranoside (IPTG). Store at −20°C.
2.2. Isolation of scFvs

1. 200 mM Tris–HCl, 20% sucrose, and 1 mM ethylene diamine tetraacetic acid (EDTA), pH 8.0.
2. All-glass bacteria filter of pore size 10–16 μm (porosity 4) (Schott Glaswerke); Membrex TF filters of pore size 0.2 μm (MembraPure).
3. Amicon High-Performance Stirred Ultrafiltration Cell (Millipore).
4. Amicon YM10 membranes with a 10 kDa cutoff (Millipore).
5. 50 mM Tris–HCl and 1 M NaCl, pH 7.0.
6. Chelating Sepharose Fast Flow (GE Healthcare).
7. C16/20 column (GE Healthcare).
8. 0.1 M CuSO₄.
9. 50 mM Tris–HCl, 1 M NaCl, and 50 mM imidazole, pH 7.0.
10. 50 mM Tris–HCl, 1 M NaCl, and 250 mM imidazole, pH 7.0.

2.3. Purification of scFvs and Analysis of Molecular Forms

1. PD-10 prepacked disposable columns containing Sephadex G-25 (GE Healthcare).
2. 20 mM Tris–HCl, pH 8.0; or 50 mM imidazole–HCl, pH 6.4. Filter (0.2 μm), and store at 4°C.
3. Mono Q HR 5/5 column or Mono S HR 5/5 column (GE Healthcare).
4. 20 mM Tris–HCl and 1 M NaCl, pH 8.0; or 50 mM imidazole–HCl and 1 M NaCl, pH 6.4. Filter (0.2 μm), and store at 4°C.
5. Phosphate-buffered saline (PBS): 15 mM Na phosphate and 0.15 M NaCl, pH 7.4. Filter (0.2 μm), and store at 4°C.
6. PBSI: PBS containing 50 mM imidazole, pH 7.4. Filter (0.2 μm), and store at 4°C.
7. Biomax-10 Ultrafree-15 Centrifugal Filter Device (Millipore).
8. Superdex 75 10/300 GL or Superdex 200 10/300 GL column (GE Healthcare).
9. High and low molecular weight gel-filtration calibration kits (GE Healthcare).
10. Bio-Rad protein assay kit (Bio-Rad).
11. 20% human serum albumin.

3. Methods

3.1. Expression of scFvs

1. Plate the scFv expression clone on 2YTGA agar and inoculate a single, freshly grown colony into 10 mL 2YTGA. Grow overnight at 37°C (E. coli XL1-Blue) or at 26°C (RV308).
2. Dilute the overnight bacterial culture 1:40× in fresh 2YTGA, and incubate at 37°C (XL1-Blue) or at 26°C (RV308), with vigorous shaking (180–220 rpm) until the optical density at 600 nm reaches 0.8–0.9.

3. Induce the expression of the scFvs by adding IPTG to a final concentration of 0.2 mM (see Note 4). Alternatively, harvest the bacteria by centrifugation at 1,500 × g for 10 min at 20°C (see Note 5), resuspend the pellet in the same volume of either fresh 2YTSA or 2YTBS medium, and add IPTG to a final concentration of 0.2 mM.

4. Incubate the culture for 14–16 h with shaking at room temperature (22–24°C).

5. Collect the cells by centrifugation at 6,200 × g for 20 min and retain the bacterial cell pellets. If using RV308, discard the culture supernatant; for XL1-Blue, retain the supernatant, and keep it on ice (see Note 6).

1. Resuspend the pelleted bacteria in 5% of the initial volume using ice-cold 200 mM Tris–HCl, 20% sucrose, and 1 mM EDTA, pH 8.0, and incubate on ice for 1 h with occasional stirring.

2. Centrifuge the cell suspension at 30,000 × g for 40 min at 4°C and then carefully collect the supernatant (soluble periplasmic extract). If using RV308, go directly to step 4.

3. If using XL1-Blue, combine the culture supernatant and the soluble periplasmic extract, and clarify by an additional centrifugation (30,000 × g, 4°C, 1 h). Pass through a glass filter of pore size 10–16 μm, then through a filter of pore size of 0.2 μm. Reduce the volume by 10× by concentrating the solution, using an Amicon ultrafiltration cell and YM 10 membrane (see Note 7).

4. Thoroughly dialyze the extracts against 50 mM Tris–HCl and 1 M NaCl, pH 7.0, at 4°C. Clarify the dialyzed extracts by centrifugation (30,000 × g, 4°C, 1 h).

5. For IMAC, prepare a column of Chelating Sepharose (1–2 mL of resin/L flask culture), and wash with five bed volumes of water.

6. Charge the column with Cu²⁺ by loading with 0.7 bed volumes of 0.1 M CuSO₄ (see Note 8), then remove the excess ions by washing the column with ten bed volumes of water. Finally, equilibrate the column with three bed volumes of 50 mM Tris–HCl and 1 M NaCl, pH 7.0 (see Note 9).

7. Pass the soluble periplasmic proteins over the column, either by gravity flow or by using a peristaltic pump. Wash the column with ten bed volumes of starting buffer (50 mM Tris-HCl and 1 M NaCl, pH 7.0), followed by starting buffer containing 50 mM imidazole (see Note 10), until the absorbance (280 nm) of the effluent is minimal (20–30 column volumes). Perform all chromatography steps at 4°C.
8. Elute the bound scFvs using starting buffer containing 250 mM imidazole (see Note 11).

9. Assess the purity of the eluted material by analysis on 12% reducing sodium dodecyl sulfate (SDS)-polyacrylamide gels (PAGE).

1. If the IMAC procedure yields a homogeneous scFv preparation, go directly to step 6 (see Note 12). Otherwise, calculate the isoelectric point (pI) of the scFv on the basis of the amino acid composition of the Ab fragment (see Note 13).

2. Taking the protein material eluted from the IMAC column, exchange the buffer to either 20 mM Tris–HCl, pH 8.0–8.5, or 50 mM imidazole–HCl, pH 6.0–7.0, using prepacked PD-10 columns (see Note 14). Remove the turbidity of protein solution by centrifugation (30,000 × g, 4°C, 30 min).

3. Load the protein solution on either a Mono Q or Mono S column, equilibrated either with 20 mM Tris-HCl, pH 8.0–8.5, or 50 mM imidazole–HCl, pH 6.0–7.0, respectively. Wash the column with a least ten column volumes of starting buffer.

4. Elute the bound material using a linear 0–1 M NaCl gradient in the starting buffer, collecting 0.5 mL fractions.

5. Perform SDS–PAGE analysis with the eluted fractions to assess recovery and purity.

6. Pool the fractions containing pure scFvs and determine the protein concentration (see Note 15).

7. Perform a buffer exchange into PBSI, pH 7.0–7.4 (see Note 16), then concentrate the purified Ab preparation up to 1.0–2.0 mg/mL, using Ultrafree-15 centrifugal filter units.

8. Equilibrate a Superdex column with PBSI buffer and calibrate the column, using high molecular weight (HMW) and low molecular weight (LMW) gel-filtration calibration kits (see Note 17).

9. For analytical size-exclusion chromatography, apply 50 μL of concentrated scFv preparation to a Superdex 10/300 GL column. Perform the gel filtration at 4°C, monitoring the UV absorption of effluent at 280 nm, and, if necessary, collect 0.5 mL fractions.

10. For long-term storage, stabilize the purified scFv by adding human serum albumin to a final concentration of 10 mg/mL. Store the sample at −80°C (see Note 18).
4. Notes

1. The *E. coli* strains XL1-Blue and RV308 are suitable hosts for expression of Ab fragments in bacterial shake-flask cultures. XL1-Blue has the following advantages: electrocompetent bacterial cells are commercially available (Stratagene) and standard DNA isolation protocols yield DNA preparations of high purity for restriction analysis and sequencing. However, RV308 is a more robust, fast-growing strain suitable for high-cell density fermentation (31). Moreover, unlike XL1-Blue, no leakage of Ab fragments into the culture medium has been observed for RV308.

2. Luria–Bertani (LB) broth can also be used for the culture of the bacteria. However, we have observed that the simple substitution of LB broth for the somewhat richer 2YT medium gives a four-fold increase in the yield of soluble scFv.

3. 2YTSA medium is prepared directly before use, by dissolving 137 g of sucrose powder in 1 L sterile 2YT medium containing 0.1 g/L ampicillin.

4. This concentration of IPTG was empirically determined to be optimal for vectors containing an scFv gene under the control of a wild-type lac promoter/operator systems, such as pHOG21 (20) or pSKK (21, 32). Nevertheless, performing small-scale experiments to optimize the induction conditions is recommended for each vector.

5. Direct induction without medium change is recommended for production of scFv in predominantly monomeric form. However, the change of medium and induction of scFv synthesis in bacteria under osmotic stress significantly increases the production yields of Ab fragments, although these conditions promote the formation of domain-swapped dimers (21).

6. For XL1-Blue, a significant fraction of the scFv is found in the culture medium, most probably due to leakage through the outer membrane or partial cell lysis. Therefore, the supernatant should also be used as a starting material for the scFv isolation.

7. Other devices suitable for concentrating protein solutions with a cutoff of 10–12 kDa may also be used.

8. IMAC can also be performed on Ni$^{2+}$-charged Chelating Sepharose or Ni-NTA-Superflow resin (Qiagen). However, the use of Cu$^{2+}$, instead of Ni$^{2+}$, is recommended for isolation of Ab fragments for clinical applications (26).
9. Tris buffers are usually not recommended for IMAC, due to the presence of amines interacting with immobilized metal ions. However, the author has found that such conditions do not influence the absorption of strong binders containing six His, and prevent nonspecific interactions of some E. coli proteins with the Chelating Sepharose.

10. Unlike Chelating Sepharose, the Ni-NTA columns should not be washed with buffers containing imidazole at concentrations higher than 20 mM.

11. To avoid the unnecessary dilution of eluted scFv, collect 0.5- to 1.0 mL fractions, and monitor the UV absorbance at 280.

12. The purity of the scFvs eluted from the IMAC column depends on the expression level of a particular Ab fragment.

13. The pI of a protein can be calculated using a number of computer programs, e.g., Protein Calculator v3.3, freely available over the internet (http://www.scripps.edu/~cdputnam/protcalc.html). The calculated pI value gives an estimate of which ion exchange matrix and buffer system should be used.

14. For scFvs with pI values below 7.0, we recommend using an anion exchanger, such as a Mono Q with a linear 0–1 M NaCl gradient in 20 mM Tris-HCl, pH 8.0. For proteins with pI values higher than 7.0, cation-exchange chromatography on a Mono S column with a linear 0–1 M NaCl gradient in 50 mM imidazole-HCl buffer, pH 6.0–7.0, is recommended. Moreover, we have found that, by exchanging the buffer after IMAC for 50 mM imidazole-HCl, pH 6.4–6.7, most of the contaminating bacterial proteins precipitated, but the recombinant Ab fragments remained soluble (21).

15. For determination of protein concentrations, we recommend using a Bradford dye-binding assay because it is easy to use, sensitive, and fast (33).

16. We recommend using PBSI buffer because PBS alone appears to be unfavorable for the stability of some Ab fragments. The presence of imidazole was found to stabilize the scFvs. PBS with 50 mM imidazole, pH 7.0–7.5, was determined empirically to be a suitable buffer for a variety of Ab fragments kept at relatively high concentrations (2–3 mg/mL). Moreover, this buffer does not interfere with Ag binding, and does not show any toxic effects after incubation with cultured cells, or after injection into mice (intravenous injection of 200 μL) (21).

17. Size-exclusion chromatography on a Superdex 75 column separates scFv monomers (Mₚ 25–30 kDa) from dimers (diabody, Mₚ 50–60 kDa) (14). HMW forms will be eluted from this column in the void volume. In contrast, Superdex 200 determines whether the Ab preparation contains
trimers (triabody, $M_r$ 90 kDa) and tetrmers (tetrabody, $M_r$ 120 kDa) (32, 34). Accordingly, the Superdex 200 column should be calibrated with both the HMW and LMW gel-filtration calibration kits. In contrast, the LMW gel-filtration calibration kit is sufficient for calibrating the Superdex 75 column.

18. Alternatively, scFvs can be stabilized by adding bovine serum albumin or fetal calf serum (35). Human serum albumin is recommended for Ab fragments developed for clinical applications. The recombinant Abs stabilized by albumin can be stored at ~80°C for years without loss of activity. These preparations may be used for a number of biological assays, such as enzyme-linked immunosorbent assay, flow cytometry, and analyzes of antitumor activity both in vitro and in vivo.

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Expression of Single-Chain Fv Fragments in \textit{E. coli} Cytoplasm

Laurence Guglielmi and Pierre Martineau

Summary

The most frequently used approach to produce single-chain Fv fragments (scFv) and Fab in \textit{Escherichia coli} is to express them in the periplasm of the bacteria. We present here an alternative procedure that uses cytoplasmic expression of soluble active scFv. This can be accomplished by using either specially engineered \textit{E. coli} strains or hyperstable scFvs.

\textbf{Key words:} Antibody fragment, scFv, Fab, Cytoplasmic expression, Purification, Disulfide bond, \textit{Escherichia coli}

1. Introduction

\textit{Escherichia coli} is the host of choice for laboratory production of antibody fragments. Recent advances have shown that it is possible to produce full-length antibody molecules in \textit{E. coli} (1), but the most frequently used formats remain single-chain Fv fragments (scFv) or Fab, a situation that is unlikely to change in the near future. These fragments retain the whole antigen-binding site and usually bind to target molecules with affinities comparable to that of the parental antibody molecule.

When expressed in B cells, antibodies are secreted molecules that are glycosylated and possess disulfide bonds. Glycosylation is required for effector functions and glycosylation sites are mainly located in the Fc fragment. Since this part of the antibody is absent in both scFv and Fab molecules, the lack of posttranslational glycosylation in \textit{E. coli} is not an obstacle to the production of these
recombinant antibodies in this prokaryotic host. In full-length antibodies, disulfide bonds exist within and between domains of the folded structure. Inter-domain disulfide bonds link together the heavy and light chains and are present in Fab but not in scFv proteins; intra-domain disulfide bonds are a hallmark of the so called “immunoglobulin fold,” and two bonds exist in scFv proteins whereas Fabs carry four bonds.

The intra-domain disulfide bond is crucial for the stability of the antibody molecule. Indeed, reduction of the two disulfide bonds of an scFv results in a decrease in thermodynamic stability of approximately 5 kcal/mol, which is close to the intrinsic stability of many antibody domains (2). This explains why the first demonstrations of successful expression of active antibody fragments in *E. coli* utilized the properties of the periplasm where disulfide formation is promoted by the *dsb* machinery (3). This is still the most frequently used approach to the large-scale production of scFv and Fab in *E. coli* (4).

An alternative approach to the production of antibody fragments in *E. coli* is to express them in the cytoplasm. It has been known for years that much higher levels of expression can be obtained in this compartment, albeit usually as aggregated and/or inactive molecules. Explanations include the absence of disulfide bond formation in the reducing environment of the cytoplasm, resulting in aggregation or rapid degradation by the cell. There are however other factors that may influence the production of antibody fragments in the cytoplasm, such as the kinetic competition between folding, aggregation, and degradation (5,6).

In recent years, two approaches have been developed to improve the production of active antibody fragments in the cytoplasm of *E. coli*: the use of mutant strains that can promote disulfide bond formation in the cytoplasm, and the selection and engineering of hyperstable antibody fragments. Of course, both approaches can be combined by expressing hyperstable antibody fragments in such mutant *E. coli* strains.

In *E. coli*, the reducing environment of the cytoplasmic compartment is maintained by the thioredoxin and the glutathione pathways. Using elegant genetic selections, Jon Beckwith and collaborators (3) selected a triple-mutant strain containing knockouts in the two pathways (*trxB and gor*) and a compensatory point mutation in the *ahpC* gene that allowed near-normal growth of the bacteria. This *E. coli* strain allows the expression of scFv and Fab fragments at levels comparable to that obtained in the periplasm.

Here, we describe a step-by-step procedure to test the expression of a candidate antibody fragment in the cytoplasm of *E. coli*, and then to purify it. We usually use scFv, but the approach could also be used for Fab fragments. It is worth noting that this approach will be particularly successful if the intrinsic stability of
the scFv lies in the upper range of values obtained for scFv molecules. This should be the case if the scFv has been selected from optimized libraries \(7–10\) or if the VH is from human families 1, 3, or 5 \(11\).

2. Materials

1. scFv cloned in a pET-derived vector (see Note 1).
2. BL21(DE3), BL21(DE3)pLys, Origami (DE3), Origami (DE3)pLysS (see Note 2).
3. LBG plates: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0. Add 15 g/L agar and autoclave. Allow the solution to cool to 60°C or lower, add 50 mL of a filter-sterilized 40\% (w/v) solution of glucose, and suitable antibiotics (100 \(\mu\)g/mL ampicillin and eventually 25 \(\mu\)g/mL of chloramphenicol), then pour the plates.
4. Ampicillin stock: Dissolve 1 g of ampicillin (sodium salt) in 10 mL of ultrapure water. Filter-sterilize (0.22 \(\mu\)m) and store in 1 mL aliquots at \(-20^\circ\text{C}\) (see Note 3).
5. Chloramphenicol stock: 25 mg/mL in 95\% (v/v) ethanol. Store at \(-20^\circ\text{C}\).
6. 17 mm × 100 mm 14 mL sterile culture tubes.
7. ZY: 10 g/L N-Z-amine AS (or any tryptic digest of casein), 5 g/L yeast extract. Autoclave.
8. 1 M MgSO\(_4\). Autoclave.
9. 50× 5052: 250 g/L glycerol, 25 g/L glucose, 100 g/L \(\alpha\)-lactose. Autoclave (see Note 4).
10. 20× NPS: Add in sequence to dissolve 66 g/L \((\text{NH}_4\)\)_2\text{SO}_4, 136 g/L \(\text{KH}_2\text{PO}_4\), and 142 g/L \(\text{Na}_2\text{HPO}_4\). Autoclave. The final concentrations in 1× NPS should be 100 mM \(\text{PO}_4^{3-}\), 25 mM \(\text{SO}_4^{2-}\), 50 mM \(\text{NH}_4^+\), 100 mM \(\text{Na}^+\), and 50 mM \(\text{K}^+\), with a pH of approximately 6.75.
11. ZYP-5052: 930 mL/L ZY, 1 mL/L of 1 M MgSO\(_4\), 20 mL/L of 50× 5052, and 50 mL/L of 20× NPS. Prepare the day of use using the stock solutions (see Note 5).
12. TE: 50 mM Tris-\(\text{HCl}\), 1 mM EDTA, pH 8. Store at \(4^\circ\text{C}\).
13. 10 mg/mL lysozyme solution in TE (see Note 6).
14. 5 M NaCl.
15. 10\% Nonidet P-40 (w/v). Store at room temperature.
16. Benzonase (see Note 7).
17. 1 M imidazole (see Note 8).
18. Nickel affinity gel (see Note 9).
19. 5x PBN: 29.82 g/L Na₃HPO₄, 5.52 g/L NaH₂PO₄·H₂O, 147 g/L NaCl. Autoclave. Store at room temperature. Final concentrations in 1x PBN should be 50 mM PO₄³⁻ and 0.5 M NaCl, pH 7.5.
20. WB: 4 mL of 5x PBN, 0.4 mL of 1 M imidazole, and water to a final volume of 20 mL.
21. EB: 3 mL of 5x PBN, 4.5 mL of 1 M imidazole, and water to a final volume of 15 mL.
22. 10x PBS: 6.1 g/L Na₃HPO₄, 2 g/L KH₂PO₄, 80 g/L NaCl, 2 g/L KCl. Autoclave.
23. 8 M guanidine hydrochloride (GndHCl) (see Note 10).
24. DB: 100 μL of 10x PBS, 500 μL of 8 M GndHCl, and water to 1 mL.
25. 100 mM dithiothreitol (DTT) made freshly on the day of use.
26. Desalting spin columns for 100 μL volume samples (Pierce).
27. 2x DTNB: 0.8 mg/mL 5,5'-dithio-bis(2-nitrobenzoic acid) in DB (see Note 11).

3. Methods

3.1. Small-Scale Expression

1. Clone your scFv in a pET23d(+) vector (see Note 1). Transform into E. coli BL21(DE3), BL21(DE3)pLysS, Origami(DE3), and Origami(DE3)pLysS (see Notes 2 and 12).
2. Pick single colonies from each transformation into sterile 17 mm × 100 mm culture tubes containing 1 mL ZYP-5052 and appropriate antibiotics (see Note 12).
3. Grow with vigorous agitation at 37°C until the absorbance at 600 nm reaches 0.4 (see Note 13).
4. Reduce the temperature to 24°C and grow overnight with vigorous agitation (see Note 14).
5. Transfer the cultures into 1.5 mL microfuge tubes and centrifuge for 5 min at 5,000 × g at 4°C. Remove the supernatant by aspiration and proceed with Subheading 3.2 (see Note 15).
3.2. Small-Scale Cell Lysis

All the steps must be done at 4°C, either on ice or in a cold-room.

1. Freeze and thaw the pellet twice in a dry ice/ethanol bath (see Note 16).
2. Resuspend the pellet carefully in 100 μL of cold TE by pipetting up and down or by vortexing.
3. Add 10 μL of a 3 mg/mL solution of lysozyme (see Notes 6 and 17).
4. Incubate for 1 h on ice.
5. Add 7 μL of 5 M NaCl. Mix gently.
6. Add 7.5 μL of 10% Nonidet P-40. Mix gently.
7. Add Benzonase to a final concentration of 10 U/mL and incubate for 10–15 min at room temperature (see Notes 7 and 18).
8. Centrifuge for 15 min at 13,000 × g at 4°C.
9. Transfer the supernatant to a clean tube (see Note 19).
10. Analyze the soluble fractions to determine the best expression system (see Note 20).

3.3. Large-Scale Purification

All the steps must be done at 4°C, either on ice or in a cold-room.

1. Pick a colony of the clone chosen for expression into a sterile 17 mm × 100 mm culture tube containing 2 mL of ZYP-5052 and the appropriate antibiotics. Grow at 37°C with vigorous shaking until the absorbance at 600 nm reaches 0.5 (see Note 12 and 13).
2. Transfer the culture to a 200 mL flask containing 40 mL of ZYP-5052 and appropriate antibiotics. Grow at 37°C with vigorous shaking until the absorbance at 600 nm reaches 0.5 (see Notes 12 and 13).
3. Transfer the culture to a 5 L flask containing 1 L of ZYP-5052 and appropriate antibiotics and grow overnight at 24°C with vigorous shaking (see Notes 12–14 and 21).
4. Cool the culture on ice for 15 min, centrifuge at 3,000 × g for 15 min at 4°C, and then discard the supernatant (see Note 15).
5. Freeze in a dry ice/ethanol bath then thaw in a water bath at room temperature. Yields are improved by repeating this freezing/thawing step (see Note 16).
6. Resuspend the pellet in 20 mL of cold TE.
7. Add 600 μL of a 10 mg/mL lysozyme solution in TE and incubate for 1 h on ice (see Note 17).
8. Add 1.4 mL of 5 M NaCl and mix gently by inverting the tube approximately five times (see Note 22).
9. Add 1.5 mL of 10% Nonidet P-40 and mix gently by inverting the tube approximately five times.
10. Add 115 μL of 1 M MgSO₄ and Benzonase to a final concentration of 50 U/mL. Incubate for 10–15 min at room temperature (see Note 18).
11. Centrifuge for 30 min at 16,000 × g at 4°C.
12. Filter using a 0.45 μm filter mounted on a 20 mL syringe (see Note 23).
13. Add 460 μL of a 1 M imidazole solution.
14. Apply onto an equilibrated, 2 mL metal-chelate affinity column (see Note 9).
15. Wash with 10 mL of WB.
16. Elute the bound proteins with 10 mL of EB, collecting 1 mL fractions.
17. Measure the absorbance at 280 nm of the fractions, using EB as a blank (see Note 24), and pool the best fractions.
18. Desalt against PBS either by dialysis or gel exclusion chromatography.
19. Measure the protein concentration and store in aliquots at −20 or −80°C (see Notes 25 and 26).

3.4. Estimation of Disulfide Bonds
(see Note 27)

1. Dissolve approximately 50 μg of the purified scFv in 150 μL of DB. This sample is designated Sox.
2. Transfer 75 μL of this solution to a fresh tube and add 0.75 μL of 100 mM DTT (see Note 28).
3. Incubate both tubes for 1 h at room temperature (see Note 29).
4. Equilibrate a spin column with DB and apply the 75 μL sample from step 2 (see Note 30). Recover the 75 μL to a fresh tube. This sample is designated Sred.
5. Add 60 μL of 2× DTNB to 60 μL samples of Sox and Sred and incubate at room temperature for approximately 5 min.
6. Measure the absorbance of both samples at 412 nm in 100 μL cuvettes against a solution prepared from equal volumes of 2× DTNB and DB (see Note 31).
7. Calculate the percentage of oxidized scFv in the purified protein preparation using the formula: 100% − 100 × \frac{A_{412nm}[S_{ox}]}{A_{412nm}[S_{red}]}$. Most preparations should give a value between 50 and 100%.
4. Notes

1. scFv isolated from phage libraries can usually be cloned as an *NcoI-NotI* fragment into vectors such as pET23d(+) from Novagen. The resulting scFv should be tagged at its C terminus with a purification tag comprising six histidine residues that can be used for purification as in Subheading 3.3. A modified vector that adds an extra c-myc tag for easy detection using the 9E10 antibody is available on request (pET23NN; (6)).

2. The *E. coli* strains are available from Novagen. If testing four strains is not feasible, use BL21(DE3) and Origami(DE3) only. The protocol described here uses an autoinducible medium developed by Studier (12) and requires a *lac*+ strain. It is thus not possible to use Origami2 strains with this protocol since they are *lac*.

3. Anhydrous ampicillin must be dissolved in 0.3 M NaOH.

4. Lactose is slow to dissolve. Heating the solution in a microwave may help.

5. Mix in the indicated order to avoid precipitation.

6. Make a fresh solution the day of use. Chicken egg lysozyme from Fluka (>100,000 U/mg) is used in our laboratory. Alternatively, phage lysozymes such as rLysozyme (Novagen) or Ready-Lyse (Epicentre Biotechnologies) can be used.

7. Benzonase is a nuclease that digests both DNA and RNA and is available from Merck and Sigma. An acceptable substitute is DNaseI prepared to a final concentration of 10 μg/mL in the presence of 5 mM Mg2+.

8. Many batches of imidazole contain impurities that absorb at 280 nm. When purchasing, select a grade that has been tested for low UV absorbance (Merck, Sigma).

9. In our laboratory, we use HIS-Select Nickel Affinity Gel (Sigma). The binding capacity is approximately 20 mg protein/mL of packed gel. The required volume of gel is packed to an appropriately sized Econo-Pac chromatography column (BioRad). Resin from other suppliers should give equivalent results. If the color of the column turns from blue to white after applying the sample, remove the EDTA by dialysis against PBS, regenerate the column (see Note 26), then reapply the dialyzed sample.

10. GndHCl is highly hygroscopic so it is not possible to prepare the solution by weighing. Either buy it directly in solution
(Pierce) or use a refractometer to measure the precise concentration (13).

11. The solution should be of a light green-yellow color. It can be stored at 4°C and used until the color deepens.

12. For *E. coli* BL21(DE3) and Origami(DE3), use media containing ampicillin as the expression plasmid should carry this marker. If the bacterial strain also contains the pLys plasmid, use media containing ampicillin and chloramphenicol. To make a glycerol stock of your strain, grow a clone in 1 mL of liquid medium until the absorbance at 600 nm reaches 0.8, add sterile glycerol to a final concentration of 8–10% (w/v), then freeze at −70°C. You can directly start a culture from this glycerol stock by scraping the surface with a sterile toothpick or a micropipette tip.

13. To get efficient induction, it is very important to oxygenate the culture efficiently. The shaking rate should be adjusted accordingly. For large cultures, baffled flasks are very efficient but normal flasks usually work well.

14. To maintain the temperature at 24°C, a refrigerated shaker is required. If it is not available, flasks can be shaken at room temperature or at the lowest possible temperature that the machine can maintain (usually approximately 27°C).

15. It is possible to pause at this step and freeze the bacterial pellets at −20°C for later processing.

16. If dry ice is not available, the pellet can be frozen at −20°C. Thaw the pellet in a water bath at room temperature for 5 min.

17. This step can be omitted if the bacterial strain contains the pLys plasmid since the T7 lysozyme expressed from the plasmid will be sufficient for efficient lysis.

18. It is also possible to keep the tube on ice, but longer incubation times will be necessary. Alternatively, for large-scale purifications, draw the lysate through a narrow-gauge syringe needle several times to reduce the viscosity.

19. The pellet can be stored for analysis of the insoluble fraction.

20. The method of analysis will depend on the scFv under investigation. Ideally, a method should be chosen that will assess the activity of the scFv (e.g., antigen-specific enzyme-linked immunosorbent assay [ELISA]). Tags that enable immunochemical detection (e.g., c-myc) or purification can be exploited in analysis using specific antibody reagents. Failing this, levels of soluble protein can be compared by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), Coomassie staining and Western blotting. If possible,
include as a control the same scFv produced in the periplasm of *E. coli* (4). If the results are comparable, we usually prefer BL21(DE3), with or without pLys, since it is deficient in lon and ompT proteases and grows better than the *trxB gor ahpC* mutant strains.

21. The final absorbance at 600 nm should be approximately 5.

22. Efficient lysis of high-density cultures of *E. coli* is difficult. The protocol given here enables efficient lysis without special equipment. If a French press is available, it can be used and steps 8–9 can then be omitted. If a sonicator is used, test the effect of 1 min total sonication time at a moderate power on ice using short pulses. Aside from cell lysis, this will shear the DNA and hence use of Benzonase at step 10 may not be necessary. It remains important to add the MgSO₄ for the later purification steps.

23. If the extract is still not clear after step 11, centrifuge a second time at 20,000 × g for 30 min before the filtration. If clogging occurs, change the filter during filtration to avoid excessive pressure.

24. Alternatively, other protein determination methods can be used (e.g., Bradford protein assay).

25. A 1 mg/mL scFv solution has an absorbance of approximately 1.5 (see Note 24). Store aliquots at −20°C or −80°C. Avoid repeated freezing and thawing. It is better to store purified scFv at a concentration of at least 1 mg/mL, but if this is not possible, the sample can be concentrated, or a carrier protein such as bovine serum albumin can be added (at 10 mg/mL) before freezing.

26. In our experience, columns can be reused at least ten times with the following regeneration method. Wash successively with 5 mL water, 10 mL of 6 M GndHCl, 5 mL water, 10 mL of 100 mM EDTA, 5 mL water, 5 mL of 10 mg/mL NiSO₄, and 5 mL water. Equilibrate the column with 10 mL of EB for immediate use or 30% ethanol prior to storage at 4°C.

27. Most of the scFv is expressed as reduced protein in the cell cytoplasm, even in *trxB gor ahpC* mutant strains. However, the protein will oxidize during extraction if the protein is correctly folded and the fraction of oxidized cysteine residues, as measured in this protocol, is thus a good indicator of native protein. It is however not always the case as, for instance, in the case of the scFvs based on the scFv13R4 framework that contain only one disulfide bond when purified from the cytoplasm (10). Of course, a precise determination of the active fraction using a functional test like ELISA or surface plasmon resonance could be performed and would be more relevant.
28. Most protocols use 10–100 mM DTT. However, 1 mM DTT provides a 100-fold molar excess and is easier to remove completely in step 4.

29. This incubation step can be increased to overnight at room temperature.

30. Aside from speed, one advantage of the spin column is that the sample is not diluted. The spin column can be replaced by a gravity column or dialysis against a few hundred milliliters of DB for several hours with two changes of buffer. If the dialysis conditions are changed or an unfamiliar brand of spin column is used, it is important to check that DTT is fully removed by performing the experiment without protein and verifying that the absorbance at 412 nm recorded at step 6 is close to zero. If dialysis or a gravity column is used, record the sample volume after desalting and add the dilution factor in the formula given in step 7.

31. Disposable 100 μL plastic cuvettes can be used (BrandTech). UV-transparent cuvettes are not required, but it seems that small-volume cuvettes are only commercially available in quartz and UV-transparent plastic.

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High-Level Expression of a Phage Display-Derived scFv in *Pichia pastoris*

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Summary

Numerous techniques are available for investigating protein-ligand interactions. The phage display technique is one such method routinely used to identify antibody-antigen interactions and has the benefit of being easily adaptable to high-throughput screening platforms. Once identified, antigen-binding domains on fragment antibodies or single-chain fragment antibodies (scFv) can be expressed and purified for further studies. In this chapter, we describe a method for high-level expression of a phage display-derived scFv in *Pichia pastoris*.

The phage display-derived antibody A33scFv recognizes a cell surface glycoprotein (designated A33) expressed in colon cancer that serves as a target antigen for radioimmunoimaging and/or immunotherapy of human colon cancer. The expression and purification of A33scFv was optimized for the methylotrophic yeast *P. pastoris*. *P. pastoris* with a MutS phenotype was selected to express A33scFv under regulation of the methanol-inducible *AOX1* promoter. Here we describe a large-scale fed-batch fermentation process with an efficient online closed-loop methanol control for the production of the recombinant protein. Purification of A33scFv from clarified culture medium was done using a two-step chromatographic procedure using anion exchange and hydrophobic interaction chromatography, resulting in a final product with more than 90% purity. This chapter provides protocols that can be used as a base for process development of recombinant protein expression in *P. pastoris* and purification of these proteins for use in further functionality studies and in diagnostic and therapeutic applications.

**Key words:** *Pichia pastoris*, scFv, Protein purification, Anion-exchange chromatography, Hydrophobic interaction chromatography, Fermentation, Methanol control

1. Introduction

The use of phage display technology has greatly simplified the isolation of completely human antibodies with high affinity and specificity, bypassing the need for chimeric, humanized, or hybridoma-derived antibodies, and thereby eliminating human anti-mouse
antibody responses in human patients. Antibodies isolated using phage display are either single-chain variable fragments (scFvs) or fragment antibodies (Fabs), with the former being the preferred molecule used in this technique (1). scFvs are one of the most useful antibody fragments and comprise $V_H$ and $V_L$ domains joined by a short peptide linker, forming a monovalent binding protein. Once an scFv has been isolated from a phage display library, it can be used as is, or engineered into other formats (Fv, Fab, [Fab']2, IgG, or fused to other proteins) (2).

Because of their small molecular size (~30 kDa), scFvs clear more rapidly from the blood and are considered to have better tumor-penetrating properties than full-length antibodies. They may also elicit little or no immune response after administration due to their short duration in the circulatory system. The advantages of scFvs over full-length antibodies make them an attractive antibody format for tumor targeting and they are being exploited for radioimmunoimaging (3), specific delivery of cytotoxic agents (4), and intracellular immunization (intrabody) applications (5).

The scFv discovery platform used to identify novel antigen binders involves a three-part process: discovery, characterization, and production. The discovery and characterization components have been established using phage display, but, in order to fully utilize the capabilities of scFvs for practical applications including clinical trials, efforts are now focused on developing the production component to obtain purified active scFvs. Much effort has been focused on Escherichia coli systems, but expression at high levels may lead to the formation of inclusion bodies, requiring harsh denaturation steps followed by time consuming and inefficient refolding steps. Therefore, use of the methylotrophic yeast Pichia pastoris as a recombinant protein expression system has become very popular, and high levels of soluble protein that can be purified directly from the culture supernatant have been reported (6,7). This organism also has the advantage of having neither endogenous sources of endotoxin as found in E. coli nor potentially oncogenic or viral nucleic acids, as are sometimes found in mammalian cells (8).

The most commonly used approach for heterologous protein expression in P. pastoris has been to express the gene of interest under the control of the $AOX1$ promoter ($P_{AOX1}$). A characteristic of $P_{AOX1}$ is that it is strongly repressed in cells grown on glycerol, but is induced more than 1,000-fold when cells are shifted to a medium containing methanol as the sole carbon source (9). Genes of interest are integrated into the P. pastoris genome via homologous recombination resulting in stable clones with no need for phenotypic selection (e.g., antibiotics) during culture.

In this chapter, we describe the high-level expression of the phage display-derived scFv A33scFv in P. pastoris using an optimized fermentation process (6). In addition, purification of this recombinant protein using a two-step chromatographic procedure
yielding a homogeneous preparation is also reported. The fermentation process is divided into two phases: batch and induction (Fig. 1). During the batch phase, cells are fed glycerol as the sole carbon and energy source. This results in the accumulation of biomass without expression of the recombinant protein. After the initial carbon source has been exhausted, the second phase is implemented, where cells are induced to produce the desired protein by a continuous, tightly controlled methanol feed. During the induction phase, biomass continues to accumulate, and, depending on the methanol utilization phenotype (Mut), this can happen at a high rate (Mut+) or at a slower rate (MutS). Since methanol level control during this second phase is crucial, we also describe the use of a simple and efficient online closed-loop methanol control system that does not require prior knowledge of P. pastoris growth rates during the induction phase.

2. Materials

2.1. Strain

The strain used was P. pastoris GS115 (his4) (Invitrogen) transformed with a pPIC9K expression plasmid (Invitrogen) containing the scFv open reading frame. This strain has a MutS phenotype.
2.2. Fermentor
Inoculum Medium and Material

1. Minimal Glycerol Medium (MG): 1.34% (w/v) Yeast Nitrogen Base without amino acids, 4 × 10^{-5} % (w/v) biotin, and 1% (v/v) glycerol (see Note 1). For plates, add 2% (w/v) agar.

2. 1 L baffled Erlenmeyer flask.

2.3. Fermentation Medium and Material

1. Basal Salts Medium: 0.23 g/L CaSO₄·2H₂O, 4.55 g/L K₂SO₄, 3.73 g/L MgSO₄·7H₂O, 1.03 g/L KOH, 6.68 mL/L H₃PO₄, 5% (v/v) glycerol, and 0.5 mL/L Antifoam 204 (Sigma) (see Note 2).

2. PTM1 Trace Salts: 6 g/L CuSO₄·5H₂O, 0.08 g/L NaI, 3 g/L MnSO₄, 0.2 g/L Na₂MoO₄·2H₂O, 0.02 g/L boric acid, 0.5 g/L CoCl₂, 20 g/L ZnCl₂, 65 g/L FeSO₄·7H₂O, 0.2 g/L biotin, and 5 mL/L sulfuric acid.

3. Base Feed: 15% (v/v) (NH₄)OH (see Note 3).

4. Methanol Feed: 100% methanol containing 10 mL/L PTM1 trace salts solution.

5. New Brunswick BioFlo3000 controller with a 2.5 L vessel (New Brunswick Scientific) interfaced with AFS-Biocommand Bioprocessing software version 2.6 (New Brunswick Scientific) for data acquisition and supervisory control.

6. Methanol Sensor Model 2.1 and probe unit (Raven Biotech).

7. PID (proportional-integral-derivative) controller model Cni852-C4EI (Omega Engineering).

8. Masterflex variable speed pump model U-77521-50 with an Easy Load II pump head (Cole-Parmer Instruments).

9. Dissolved oxygen probe InPro6110/220 (Mettler-Toledo).

10. Accumet pH electrode (Fisher Scientific).

2.4. Protein Purification and Analysis

1. ÄKTA Explorer interfaced with Unicorn v4.12 (GE Healthcare Life Sciences), or equivalent.

2. 500 mL conical bottom centrifuge tubes (Corning), or equivalent, and centrifuge.

3. 1 L disposable vacuum filter units, 0.2 µm PES membrane (Corning) or equivalent.

4. Anion exchange: XK 50/20 column with 200 mL packed bed of Q Sepharose HP resin (GE Healthcare Life Sciences).

5. Anion-exchange running buffer: 20 mM ethanolamine, pH 10.0.

6. Anion-exchange elution buffer: 20 mM ethanolamine, 1 M NaCl, pH 10.0.

7. Hydrophobic interaction: XK 50/20 column with 200 mL packed bed of Phenyl Sepharose HP (GE Healthcare Life Sciences).
8. Hydrophobic interaction running buffer: 20 mM Tris–HCl, 0.8 M (NH₄)₂SO₄, pH 7.5.
9. Hydrophobic interaction elution buffer: 20 mM Tris-HCl, pH 7.5.
10. Minimate tangential flow filtration (TFF) system with Omega membrane and 3 kDa molecular weight cut-off (Pall Corporation).
11. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) equipment and supplies.
12. Immunoblot equipment and supplies.
13. scFv detection reagents: rProtein L-HRP (Actigen) and colorimetric Opti-4CN detection reagent (Bio-Rad) (see Note 4).

3. Methods

3.1. A33scFv Expression in Fermentor Cultures

3.1.1. Fermentation

P. pastoris strain carrying the expression cassette with A33scFv was plated on MG medium and incubated at 30°C for 48 h. A single colony was used to inoculate 100 mL of MG medium in a 1 L baffled Erlenmeyer flask. The inoculum culture was incubated at 30°C and 200 rpm until an OD₆₀₀ of between 4 and 10 was reached. A starting volume of 1.1 L of basal salts medium was sterilized inside the reactor and PTM1 trace salt solution was aseptically added at 4.35 mL/L after sterilization. Prior to inoculation, the pH of the fermentation medium was adjusted to 3.0 using the 15% ammonium hydroxide feed solution and the temperature was set to 25°C. All of the inoculum culture was used to inoculate the fermentor. Dissolved oxygen (DO) was maintained at 40% of saturation and was controlled by a DO cascade of agitation starting at 300 rpm (maximum of 1,000 rpm) followed by supplementing with pure oxygen as needed.

This initial batch phase was continued until the primary carbon source (glycerol) was exhausted, indicated by a sharp rise in DO (DO spike). The culture was then induced with a methanol feed initiated automatically and methanol was kept at 1 g/L. To initiate the methanol feed and maintain a constant concentration of methanol during the induction phase, an online methanol sensor under a closed-loop PID control scheme was employed (Fig. 2). This configuration allows for a versatile setup, where Mut⁺ and Mut⁻ phenotypes can be cultured without a priori knowledge of the culture growth parameters. PID parameters were tuned by using Root Locus analysis on a simple mathematical model of the fermentation system (see Note 5).
To calibrate the methanol sensor for PID control, two methanol concentrations were used that flanked the desired set point (see Note 6). For a set point of 1 g/L of methanol, set point values of 0.75 and 1.25 g/L were chosen. For the first calibration point, the methanol concentration was adjusted to 0.75 g/L by adding 100% methanol aseptically to the fermentation medium during the glycerol batch phase. After methanol sensor stabilization, the value, given in millivolts, was noted. For the second calibration point, 100% methanol was added to a final concentration of 1.25 g/L and the millivolt value noted after stabilization of the methanol sensor. These calibration values were then used as the minimum and maximum methanol concentrations in the PID controller. By using this approach, methanol is stably maintained at 1 g/L throughout the induction phase. The optimum harvest time for A33scFv is 72 h from time of induction, where degradation of the protein is not visible (Fig. 3). This variable has to be considered carefully and determined empirically, since recombinant protein stability during the induction phase might vary from protein to protein (see Note 7). In addition, the use of Mut⁺ or Mut⁶ strains also need to be considered since induction times and product yields might vary greatly.

3.2. A33scFv Purification

The A33scFv is purified using a simple two-step chromatographic procedure, from which a highly pure preparation can be obtained. In the first step, A33scFv is captured on an anion-exchange resin (Q Sepharose HP) and eluted using a step-gradient...
elution profile. The second and final purification step consists of capturing A33scFv using a hydrophobic interaction resin (Phenyl Sepharose HP). All chromatographic steps were carried out on an ÄKTA Explorer interfaced with Unicorn v4.12. Depending on the purification scale, linear flow rates (in centimeters per hour) should be kept constant for each different size column to ensure reproducibility of purification profiles.

1. Harvest cells at \(4,000 \times g\) for 25 min and collect the supernatant.
2. Filter the supernatant using a 0.2 \(\mu\)m disposable vacuum filter to further remove any remaining cells and insoluble salts (see Note 8).
3. Using a Minimate TFF cassette with a 3 kDa molecular weight cut-off, or equivalent device, concentrate the cleared and filtered supernatant two-fold and begin buffer exchange against at least 5 volumes of 20 mM ethanolamine, pH 10.0. Concentration of the supernatant prior to the buffer exchange not only reduces the volume of buffer that needs to be used, but also reduces the final sample volume, which decreases the column loading time on the first chromatographic step.

**3.2.2. Anion-Exchange Chromatography**

1. Equilibrate a Q Sepharose HP column with 5 column volumes (CVs) of anion-exchange running buffer (20 mM ethanolamine, pH 10.0) at a linear flow rate of 150 cm/h (see **Note 9**).

2. Load 200 mL of cleared and buffer-exchanged culture supernatant at 150 cm/h. After sample loading, wash the column with 4 CVs of anion-exchange running buffer at 150 cm/h or until the Abs$_{280nm}$ returns to the buffer baseline.

3. To elute the A33scFv, first change the original flow direction from a “down flow” to an “up flow.” We have found this to be useful for this particular protein because it increases the resolution and decreases broadening of peaks during the elution. If the equipment being used does not have bidirectional column valves, simply switch the lines connected to the column, being careful not to introduce air bubbles. Begin the elution with a linear gradient from 0 to 6% of anion-exchange elution buffer (20 mM ethanolamine, 1 M NaCl, pH 10.0) over 1 CV with a linear flow rate of 75 cm/h. After this initial step, maintain at 6% of elution buffer for 5 CVs. Most of the A33scFv will be eluted in the first three CVs of this step. To remove and clean the resin of remaining unspecific proteins for the next run, increase the gradient from 6 to 100% of elution buffer over 1 CV and maintain for 2 CVs (**Fig. 4**).

4. Analyze fractions by SDS-PAGE and/or immunoblotting and pool those fractions containing the protein of interest.

**3.2.3. Hydrophobic Interaction Chromatography**

1. Pool the fractions containing the A33scFv and adjust to a final concentration of 0.8 M ammonium sulfate with a saturated solution of filter-sterilized ammonium sulfate under constant mixing. By adding a saturated ammonium sulfate solution, as opposed to ammonium sulfate crystals, the formation of microenvironments with high salt concentrations are greatly reduced, thereby minimizing the potential for protein precipitation.

2. Equilibrate the Phenyl Sepharose HP column with 5 CVs of hydrophobic interaction running buffer (20 mM Tris-HCl, 0.8 M $\left[\text{NH}_4\right]_2\text{SO}_4$, pH 7.5) at a linear flow rate of 150 cm/h.

3. Load the total volume of adjusted/pooled fractions at 75 cm/h and wash the column with 4 CVs of hydrophobic
interaction running buffer or until the $\text{Abs}_{280\text{nm}}$ returns to the buffer baseline.

4. Elute the recombinant protein using a decreasing linear gradient of 100 to 0% of hydrophobic interaction elution buffer (20 mM Tris-HCl, pH 7.5) over 20 CVs. Due to the hydrophobic nature of the A33scFv, it will elute toward the end of this chromatographic step (Fig. 5). If a less hydrophobic protein is being purified and is eluted at the beginning of the chromatography, an increase in ammonium sulfate concentration in the sample solution and running buffer will cause increased binding of the protein to the resin. This in turn will lead to increased resolution and better separation.

5. Analyze the fractions by SDS-PAGE and/or immunoblotting and pool those containing the protein of interest. Depending on the purpose of this recombinant protein, fractions can be pooled and buffer exchanged against appropriate buffer using a Minimate TFF cassette with a 3 kDa molecular weight cut-off as described in Subheading 3.2.1, step 3. The final protein concentration should not be higher than 0.8 mg/mL because this will cause single-chain aggregation and precipitation.
3.3. Considerations for Scale-Up and Transfer to GMP Production

There is a growing interest in the scale-up of scFv production, under Good Manufacturing Practices (GMP) conditions, for use in preclinical or clinical studies. In our pilot-scale GMP facility, we have addressed some issues regarding the use of *P. pastoris* as the expression host for A33scFv with great success.

1. Use of animal-free medium is a clear necessity as dictated by FDA standards (10). We have found the use of soybean-derived peptone (Difco) a suitable substitute for animal-derived peptone.

2. The fermentation process was scaled-up to a BioFlo 4500 controller with a 30 L vessel (New Brunswick Scientific) and identical methanol controller set up with similar results.

3. The initial TFF diafiltration of the culture supernatant was carried out using a 0.5 m² cassette, Omega membrane medium channel screen of 3 kDa molecular weight cut-off (Pall).

4. The chromatography steps were carried out with BPG 100/50 columns on an ÄKTA purifier 100 (GE Healthcare Life Sciences).
4. Notes

1. MG medium should be filter sterilized since biotin is heat labile. When making plates, autoclave water and agar, cool to approximately 55°C, and add the remaining reagents.

2. One modification to our initial fermentation medium was to reduce the concentration of basal salts to one-fourth of the original recipe (Pichia Expression Kit, Version M, Invitrogen). Consistent with the findings of Brady et al. (11), this reduced basal salts medium had no adverse effect on cell growth rate or biomass yield during either batch or induction phases. Two main advantages of reducing the concentration of basal salts in the fermentation medium are: (1) decreased salt precipitation during preparation of cleared supernatant for downstream purification, and (2) elimination of a lipid-like substance in the medium, which can also interfere with downstream purification.

3. Sterile ammonium hydroxide solution is made by first autoclaving distilled water and then adding the required amount of concentrated ammonium hydroxide aseptically.

4. rProtein L-HRP is an immunoglobulin kappa light chain-binding protein conjugated with horseradish peroxidase (HRP). It has a high affinity for the $V_L$ portion of the scFv.

5. It is not in the scope of this chapter to outline the full model development for the PID parameters and methanol control system, however, this detailed information can be found in work by Pla et al. (12).

6. It is very important that the methanol sensor calibration be done early during the glycerol batch phase. P. pastoris cells will preferentially use glycerol during this phase and the methanol added will not be used as an additional carbon source, nor induce protein production. If calibration is performed late in the batch phase, cells may be encountering carbon limitation and have begun to metabolize methanol. This makes the calibration impossible.

7. Parameters such as pH, temperature, methanol concentration, and length of induction have to be determined empirically for different recombinant proteins. Although this is usually done in the initial screening stages using small-scale experiments, these parameters should also be tested in different fermentation experiments, as this will be the actual production setup.

8. Filtration of the cleared supernatant is an important step to further remove any particulates that could lower the buffer exchange efficiency during TFF. We have found that further centrifugation of the cleared supernatant prior to filtration
further removes heavier particulates remaining in the supernatant after the initial cell harvest. Also, the supernatant will have a greenish tint, which is a combination of trace salts and flavin-bound proteins naturally produced by *P. pastoris*.

9. Although it is common practice to refer to volumetric flow rates (i.e., milliliters per minute) during chromatographic procedures, linear flow rates (centimeters per hour) are more precise when scaling up or down between different size columns. To calculate the linear flow rate based on volumetric flow rates, this simple equation is used:

\[
\text{linear flow rate (cm/h)} = \frac{\text{volumetric flow rate (mL/min)} \times 60}{\text{column cross section area (cm}^2\text{)}}.
\]

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