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Single-Domain Antibodies and Their Utility

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ABSTRACT

Engineered monoclonal antibody fragments have gained market attention due to their versatility and tailor-made potential and are now considered to be an important part of future immunobiotherapeutics. Single-domain antibodies (sdAbs), also known as nanobodies, are derived from VHHs [variable domains (V) of heavy-chain-only antibodies (HCAb)] of camelid heavy-chain antibodies. These nature-made sdAbs are well suited for various applications due to their favorable characteristics such as small size, ease of genetic manipulation, high affinity and solubility, overall stability, resistance to harsh conditions (e.g., low pH, high temperature), and low immunogenicity. Most importantly, sdAbs have the feature of penetrating into cavities and recognizing hidden epitopes normally inaccessible to conventional antibodies, mainly due to their protruding CDR3/H3 loops. In this unit, we will present and discuss comprehensive and step-by-step protocols routinely practiced in our laboratory for isolating sdAbs from immunized llamas (or other members of the *Camelidae* family) against target antigens using phage-display technology. Expression, purification, and characterization of the isolated sdAbs will then be described, followed by presentation of several examples of applications of sdAbs previously characterized in our laboratory and elsewhere. *Curr. Protoc. Immunol.* 103:2.17.1-2.17.57.

Keywords: single-domain antibody • heavy-chain antibodies • VHH • antibody fragment libraries dimeric VHH • pentabodies • multivalency

INTRODUCTION

Antibodies are becoming very useful both in research and in biotechnological applications, and these molecules have proven to be an excellent paradigm for the design of high-affinity, protein-based binding reagents. The discovery of mouse hybridoma technology to generate monoclonal antibodies (mAbs) opened a new era in antibody research. Subsequently, innovative recombinant DNA technologies, including chimerization and humanization, have enhanced the clinical efficiency of murine mAbs, and have led to regulatory approval as treatment for solid tumors and hematological malignancies, infectious disease, and inflammatory disease (Hudson and Souriau, 2003; Holliger and Hudson, 2005; Scott et al., 2012).

Antibodies have two distinct functions: binding to antigen, carried out by the antigen-binding fragments (Fabs), and interaction with the receptors on different cells, carried out by the fragment crystallizable (Fc) region. Minimizing the size of antigen-binding proteins to a single immunoglobulin domain with high affinity to a target antigen has been one of the major goals of antibody engineering (Revets et al., 2005). Smaller recombinant antibody fragments such as Fabs, single-chain Fvs (scFvs), and other engineered variants that are sometimes termed third-generation (3G) molecules (Nelson, 2010), such as diabodies, minibodies, and sdAbs, are emerging as credible alternatives. Genetic engineering methods have been successfully applied to improve several issues that are

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associated with unmodified IgG, such as size and immunogenicity. It has been clear that engineered antibodies have come of age as biopharmaceuticals.

STRATEGIC PLANNING

In this unit, we provide detailed protocols for the isolation and characterization of antigen-specific VHHs. Specifically, we describe the protocols for llama immunization and serum response monitoring (Basic Protocol 1 and Alternate Protocol 1), VHH phage-display library construction from heavy-chain repertoires (Basic Protocol 2), construction of non-immune naïve libraries (Alternate Protocol 2), rescue of phagemid library and panning (Basic Protocol 3), monitoring the progress of biopanning by phage ELISA and colony PCR (Basic Protocol 4), subcloning of VHH fragments in bacterial vectors (Basic Protocol 5), expression of single-domain antibodies in bacterial systems and purification of single-domain antibodies (Basic Protocols 6 and 7, respectively), and gel filtration/size-exclusion chromatography (Basic Protocol 8). Methods used to increase functional affinity and potency of VHHs (Basic Protocol 9), preparing VHH Fc fusion proteins (Basic Protocol 10), and characterizing antigen-specific VHHs (Basic Protocol 11) are also included. Finally, a set of Support Protocols are included for necessary ELISAs (Support Protocol 1), serum fractionation (Support Protocol 2), purification of DNA fragments from agarose gel (Support Protocol 3), preparation of electrocompetent bacterial cells (Support Protocol 4), SDS-PAGE and western blotting of samples (Support Protocol 5), pentamerization of VHHs (Support Protocol 6), and surface plasmon resonance and epitope mapping (Support Protocol 7). Each of these protocols involves some critical considerations, which are described in Critical Parameters in the Commentary. For immunization, it is important to pay attention to the route of immunization, nature and quality of antigen, and quantity of the antigen available. For library construction, it is very important to process the lymphocytes as soon as possible to purify RNA and make cDNA. If the lymphocytes are kept for too long, the quality of RNA and all further steps will be compromised. Another important parameter is the size and diversity of the library, as this will dictate the types of binders that can be isolated. During panning, consideration must be given as to whether the panning will be done in solution or on a coated surface. The amount of antigen to be used in each round of panning, the washing stringency, and subsequent enrichment in each round of panning are critical aspect to consider and observe. For better expression and purification, it is important to select an easy and convenient expression system in terms of vector and host. For certain types of antibody formats or applications, eukaryotic expression systems are preferred or advantageous. Antibody purity is a critical factor for accurate binding and functional studies, and therefore proper signal sequences for directing antibodies to the bacterial periplasm or other cell compartments, as well as appropriate tag sequences at the N- or C-terminals, are key parameters to be considered and planned in advance.

BASIC PROTOCOL 1

IMMUNIZATION OF LLAMAS

Immunization is a critical step in the isolation of high-affinity antibodies. It is common practice in many research laboratories to use emulsions of purified immunogen and Freund's complete/incomplete adjuvants. The adjuvant forms a stable emulsion with the immunogen and results in its sustained presentation to the animal immune system, thereby stimulating and enhancing the immune response to the immunogen. Other complex materials such as fractionated membranes or even cell lines expressing antigen of interest can be also used for immunization (Baral et al., 2011). As immunization plans, short and long llama immunization schedules (see Table 2.17.1) are provided here and both have worked well in our laboratory. The short immunization protocol works well for soluble and accessible protein antigens, and saves considerable time on the immunization. However, for less immunogenic antigens including haptens, it is recommended to follow the long immunization protocol.

Table 2.17.1 Long and Short Immunization Schedules

Time	Activity
Long immunization schedule	
Day 1	Pre-immune bleed
Day 1	Immunization (CFA as an adjuvant)
Day 22	Boost no. 1 (IFA as an adjuvant)
Day 29	Test bleed 1
Day 36	Boost no. 2 (IFA as an adjuvant)
Day 43	Test bleed 2
Day 50	Boost no. 3 (IFA as an adjuvant) and test bleed 2
Day 57	Test bleed 3
Day 64	Boost no. 4 (no adjuvant)
Day 71	Production bleed
Short immunization schedule	
Day 0	Pre-immune bleed
Day 0	Immunization (CFA as an adjuvant)
Day 21	Boost no. 1 (IFA as an adjuvant)
Day 28	Boost no. 2 (IFA as an adjuvant)
Day 35	Test bleed
Day 35	Boost no. 3 (no adjuvant)
Day 42	Production bleed

Subcutaneous injection in the lower back or intramuscular injection at the lower rump are regular routes of immunization. We routinely use llamas (*Lama glama*) for immunization due to their availability; however, other camelid members such as alpacas, camels, or dromedaries can be used for this purpose should they be available.

Materials

Llama (*Lama glama)* (Cedarlane Laboratories)
Antigen (1 mg required)
Phosphate-buffered saline (PBS; see recipe)
Freund's complete and incomplete adjuvant (Sigma; also see *UNIT 2.4*)
1- to 2-ml syringes with 21-G, 1- to 1.5-in. long needles for llama immunization and 10- to 15-ml Vacutainer for blood collection
Heparin-coated tubes (Becton Dickinson)
Additional reagents and equipment for emulsifying antigen using Freund's adjuvant (*UNIT 2.4*), isolation of lymphocytes (*UNIT 7.1*), ELISA (Support Protocol 1), and fractionation of sera (Support Protocol 2)

1. On Day 1, conduct a pre-immune bleed (10 to 15 ml) and then immunize the animal with 100 to 200 µg of each antigen (if multiple antigens are used). Dilute the antigen in PBS to 1 ml total and filter sterilize. Mix the antigen solution well with 1 ml of complete Freund's adjuvant (CFA) to make a total immunization volume of 2 ml (see *UNIT 2.4*).

   Intramuscular injections (i.m.) at multiple sites in the lower rump on either side of the back leg are used for the initial and subsequent immunization.

   UNIT 2.4 provides guidance on how to emulsify antigen using Freund's adjuvant.

All immunizations are performed at Cedarlane Facility in Burlington, Ontario, Canada (http://www.cedarlane.ca) and are based on the protocol provided here and following the guidelines set by the Canadian Council on Animal Care (CCAC). If feasible, use two llamas for immunization, as the quality of the immune response is dependent on the individuals. Access to alpacas is also possible at Cedarlane.

2. On Days 22, 36, and 50, immunize with 100 µg of the same antigens (diluted in PBS to 1 ml total and filter-sterilized), mixed well with 1 ml of incomplete Freund's adjuvant (IFA; also see *UNIT 2.4*).

3. On Day 64, immunize with 100 µg of each antigen (diluted in PBS to 1 ml total) with no adjuvant.

4. Collect blood (10 to 15 ml) into heparin-coated tubes on Days 29, 43, 57 as test bleed, and collect 50 ml of blood on Day 71 as production bleed. Place the blood immediately on ice.

   It is important to have a pre-immune bleed on Day 1 (see step 1), which will be used as a non-immunized control for a subsequent ELISA (Support Protocol 1).

5. Store the collected blood from each bleed overnight at 4°C. Prepare the serum the next day by centrifuging 10 min at 2700 × *g*, 4°C, and store and the sera at 4°C.

6. Isolate the lymphocytes (*UNIT 7.1*) from blood collected on Day 71 and use for phage-display library construction (Basic Protocol 2).

7. Following immunizations, analyze llama to monitor antigen-specific heavy-chain antibody responses by ELISA (Support Protocols 1 and 2).

8. Fractionate serum from production bleed (Support Protocol 2).

ALTERNATE PROTOCOL 1

IMMUNIZATION OF LLAMAS USING SHORT IMMUNIZATION PROTOCOL

The short immunization protocol was adapted in our laboratory for easy-to-access and soluble protein antigens. It has the advantage of saving immunization time, requiring lower amounts of antigen, and requiring fewer boosting steps for the animals.

For materials, see Basic Protocol 1.

1. On Day 1, conduct a pre-immune bleed (10 to 15 ml), and then immunize the animal with 200 µg of each antigen (if multiple antigens are used). Dilute the antigen in PBS to 1 ml total and filter sterilize. Mix the antigen solution well with 1 ml of complete Freund's adjuvant (CFA) to make a total immunization volume of 2 ml (see *UNIT 2.4*).

   Intramuscular injections (i.m.) at multiple sites in the lower rump on either side of the back leg are used for the initial and subsequent immunization.

   UNIT 2.4 provides guidance on how to emulsify antigen using Freund's adjuvant.

   All immunizations are performed at Cedarlane Facility in Burlington, Ontario, Canada (http://www.cedarlane.ca) and are based on the protocol provided here and following the guidelines set by the Canadian Council on Animal Care (CCAC). If feasible, use two llamas for immunization, as the quality of the immune response is dependent on the individuals. Access to alpacas is also possible at Cedarlane.

2. On Days 21 and 28, immunize with 100 µg of the same antigens (diluted in PBS to 1 ml total and filter sterilized), mixed well with 1 ml of incomplete Freund's adjuvant (IFA; also see *UNIT 2.4*).

3. On Day 35, immunize with 100 µg of each antigen (diluted in PBS to 1 ml total) with no adjuvant.

4. Collect blood (10 to 15 ml) into heparin-coated tubes on Day 35 and 50 ml of blood on Day 42 (production bleed). Immediately place on ice.

   It is important to have a pre-immune bleed on Day 1, which will be used as a non-immunized control for a subsequent ELISA.

5. Store the collected blood from each bleed overnight at 4°C. Prepare the serum the next day by centrifuging 10 min at 2700 × *g*, 4°C, and store and the serum at 4°C.

6. Isolate the lymphocytes (*UNIT 7.1*) from blood collected on Day 42 and use for phage-display library construction.

7. Following immunizations, analyze llama sera to monitor antigen-specific heavy-chain antibody responses by ELISA (Support Protocols 1 and 2).

8. Fractionate serum from production bleed (Support Protocol 2).

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

SUPPORT PROTOCOL 1

The enzyme-linked immunosorbent assay is a routine and widely practiced assay for analyzing the specific interaction between antigens and antibodies in a relatively short period of time. In order to correctly interpret the ELISA results, it is strongly recommended to use appropriate negative/blank and positive controls. It is also recommended to examine the stability of the protein antigen before adding it to the ELISA well, in order to determine the appropriate coating conditions. For most proteins, coating at 4°C is recommended.

Materials

Antigen at 5 to 10 µg/ml
5 µg/ml BSA in PBS (filter sterilize and store at 4°C)
PBS (see recipe)
PBST: PBS (see recipe) containing 0.05% (v/v) Tween 20
Blocking buffer A: 3% (w/v) BSA in PBS-T
Goat anti-llama IgG and swine anti-goat IgG labeled with horseradish peroxidase (HRP) (Cedarlane)
HRP substrate solutions for ELISA (GE Healthcare)
1 M H_3PO_4

96-well MaxiSorp microtiter plates (VWR)
Microtiter plate reader (Biochrom)

Additional reagents and equipment for ELISA (*UNIT 2.2*)

1. For ELISA, coat 96-well microtiter plates with 100 µl of the antigen at 5 to 10 µg/ml and BSA at 5 µg/ml as a control for overnight at 4°C (*UNIT 2.2*).

   UNIT 2.2 includes detailed protocols for ELISA.

   The amount of antigen used is 1 to 5 µg/well in a volume of 100 µl in PBS.

2. The next day, wash three times with PBST, then block the wells with 200 µl/well of blocking buffer A for 2 hr at 37°C.

3. Wash the plate and add serial dilutions of pre-immune total serum (collected on Day 1), post-immune total serum from various bleeds (collected on Days 22, 43, 57, and 71), and fractionated serum (IgG3, IgG1 and IgG2a/b/c fractions) from Day 57 and Day 71 bleeds. Make the dilutions in PBS and use 100 µl per well. Incubate for 1.5 hr at room temperature.

   The last well should contain PBS only as a control.

4. Wash the wells five times, each time with 300 µl PBST, and add 100 µl/well of goat anti–llama IgG-HRP (diluted 1:10,000 in PBS). Incubate 1 hr at 37°C.

   Alternatively, goat anti–llama IgG (diluted 1:1,000 in PBS) can be used. Incubate 1 hr at 37°C and then, after washing the plates, add 100 µl/well of swine anti-goat IgG-HRP (diluted 1:3,000 in PBS) for 1 hr at 37°C.

5. Wash the wells again as described in step 4, add HRP substrate (100 µl/well), and incubate at room temperature for 5 to 10 min. Stop the reaction with 1 M H_3PO_4 (100 µl/well) and read at 450 nm with a microtiter plate reader.

   If a positive immune response is detected from the IgG2a/b/c fraction, but not from the IgG3 fraction, make sure that the response in the IgG2a/b/c fraction is not from contaminating IgM.

SUPPORT PROTOCOL 2

LLAMA SERUM FRACTIONATION TO PREPARE POOLED IgG2a, IgG2b, AND IgG2c ISOTYPES

This protocol fractionates llama sera using protein G and an ÄKTA FPLC purification system followed by the use of a protein A column to prepare the heavy-chain IgG2 fraction, which consists of IgG2a, IgG2b, and IgG2c isotypes.

Below is a procedure to fractionate the sera prepared from blood collected on Day 57 and Day 71 of Basic Protocol 1 (Day 42 of Alternate Protocol 1) to separate conventional IgG and HCAbs (Hamers-Casterman et al., 1993; Doyle et al., 2008). A schematic drawing of the serum fraction workflow is presented in Figure 2.17.1.

Materials

Sera prepared from blood collected on Day 57 and Day 71 of Basic Protocol 1 or day 42 of Alternate Protocol 1

20 mM NaPi buffer: 8.46 ml of 1 M NaH_2PO_4, 11.54 ml of 1 M Na_2HPO_4, diluted in 1 liter of distilled, deionized H_2O (pH 7.0)

100 mM citrate buffer: 3.11 g citric acid, 1.53 g sodium citrate, pH 3.5, per 200 ml of distilled, deionized H_2O—adjust to pH 3.5 with 1 M NaOH, filter sterilize, and store at 4°C.

1 M Tris·Cl, pH 8.8 (*APPENDIX 2A*)

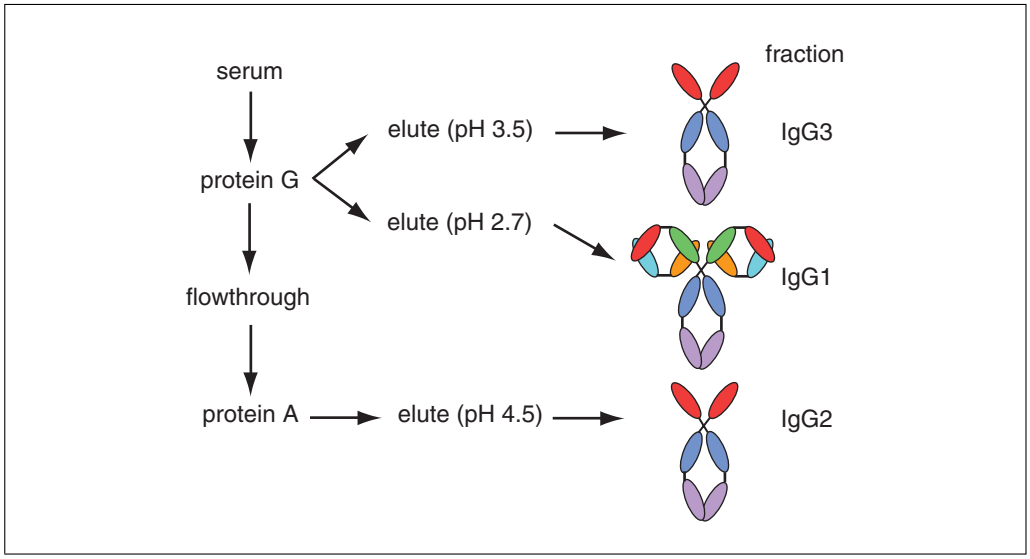

Figure 2.17.1 Schematic drawing of fractionation of llama serum to separate HCAb (IgG 2 and IgG3) and conventional Ab (IgG1).

100 mM glycine buffer: 1.5 g glycine, pH 2.7, per 200 ml of distilled H_2O—adjust to pH 2.7 with 3 M HCl, filter sterilize, and store at 4°C.

100 mM sodium acetate buffer: 2.7 g sodium acetate, pH 4.5, per 200 ml of distilled H_2O—adjust to pH 4.5 with acetic acid, filter sterilize, and store at 4°C.

Phosphate-buffered saline (PBS; see recipe)

Dialysis tubing with MWCO 8000 (Biodesign Inc., *http://biodesignofny.com*

1-ml HiTrap Protein G HP and HiTrap Protein A HP columns (GE Healthcare)

ÄKTA FPLC purification system (GE Healthcare)

pH paper

Additional reagents and equipment for dialysis (*APPENDIX 3H*), SDS-PAGE (Support Protocol 5; also see *UNIT 8.4*), and ELISA (Support Protocol 1)

1. Dialyze 5 ml of llama serum overnight against 500 ml NaPi buffer using an 8000 MWCO membrane (*APPENDIX 3H*).

2. Centrifuge 5 min at 3600 × *g*, 4°C, using a bench-top centrifuge with a swinging-bucket rotor and pass supernatant through a 0.22-µm filter.

3. Load 1 to 2 ml of the dialyzed llama serum onto a 1-ml HiTrap Protein G HP column that has been equilibrated with 10 ml NaPi buffer, using ÄKTA FPLC purification system.

   The column buffers and Milli-Q water should be degassed prior to column chromatography.

4. Collect the flowthrough, wash the protein G column with 10 ml NaPi buffer, and elute the heavy-chain IgG3 fraction with 1 to 2 ml of citrate buffer (pH 3.5). Immediately neutralize the eluted fraction by adding 0.5 to 1 ml 1 M Tris·Cl (pH 8.8).

   It is recommended to check the pH of the eluate by pH paper in order to add the exact amount of Tris·Cl buffer.

5. Perform a second elution with 2 to 4 ml of glycine buffer (pH 2.7) to elute the conventional IgG1 fraction. Neutralize the fraction by adding 1 to 2 ml 1 M Tris·Cl (pH 8.8).

6. Next, load the flow-through from the protein G column onto a 1 ml HiTrap Protein A HP column equilibrated with 10 ml NaPi buffer. After loading, wash the protein A column with 10 ml of NaPi buffer and apply 1 to 2 ml of sodium acetate buffer (pH 4.5) to elute the heavy-chain IgG2 fraction, which consists of IgG2a, IgG2b, and IgG2c isotypes. Neutralize the fractions by adding 0.1 to 0.2 ml 1 M Tris·Cl (pH 8.8).

7. Analyze the eluted fractions on an SDS-PAGE gel under non-reducing and reducing conditions (Support Protocol 5; also see *UNIT 8.4*).

   By running an SDS-PAGE gel, successful separation of heavy-chain IgG and conventional IgG antibodies from the llama sera can be confirmed. The expected molecular weights of conventional IgGs, IgG heavy chains, and IgG light chains are approximately 150 kDa, 55 kDa, and 25 kDa, respectively. The expected molecular weight of heavy-chain IgGs and the heavy chains of HCAb are approximately 85 kDa and 42 kDa, respectively.

8. Test the separated fractions by ELISA (Support Protocol 1) against the immunogen. If a positive response is found within the heavy-chain IgG fractions, proceed with VHH phage-display library construction (Basic Protocol 2).

9. Dialyze the correct fractions against PBS (using 2 to 10 ml of fraction and 2 liters of PBS with an 8000 MWCO dialysis membrane) and store them at 4°C for further analysis by ELISA.

   ELISA is performed on total and fractionated sera to determine if there was an antigen specific heavy-chain antibody immune response.

CONSTRUCTION OF VHH LIBRARY

In the first steps of this protocol, peripheral blood lymphocytes (PBLs) are isolated from the blood. The storage period and storage conditions for the blood are very critical for isolation of good-quality RNA and cDNA synthesis. It is recommended to perform the PBL purification, RNA isolation, and cDNA synthesis as quickly as possible. In this step, PBLs are isolated from the blood obtained on Day 71 (or 42 in the short immunization protocol) post-immunization and used as a source of mRNA for library construction. By reverse transcription, cDNA is synthesized using the mRNA as template and oligo(dT) or random hexamers as primers. The cDNA is used as template in PCR with a combination of IgG-specific 5′-end primers (VH/VHH framework 1 region) and 3′-end primers (constant region 2; CH2). This PCR will amplify both VH and VHH from IgG1, IgG2, and IgG3 genes. The first PCR normally yields a DNA band of about 800 to 900 bp corresponding to VH-CH1-Hinge and almost the first half of the CH2 region (called VH-CH2) of conventional antibodies, and another DNA band of about 550 to 650 bp corresponding to the VHH-Hinge and almost the first half of the CH2 region (called VHH-CH2) of heavy-chain antibodies (Fig. 2.17.2). The VHH region of heavy-chain antibodies, which is a pool from IgG2 and IgG3 genes, is amplified in a second PCR using VHH-specific primers anchored with *Sfi*I restriction sites. The VHH fragments are cloned into the pMED1 phagemid vector (Arbabi Ghahroudi et al., 2009a; Fig. 2.17.3A) between the two *Sfi*I restriction sites, located at the 3′-end of the *pelB* leader sequence and 5′-end of the gene III coding sequence. This allows VHHs to be expressed as fusions to gene III, transported to the *E. coli* periplasmic space, and displayed on the surface of phage when it is super-infected by a suitable helper phage such as M13KO7. In principle, any other phagemid vector with similar features such as pHEN1(Clackson et al., 1991) or pCANTAB 5E (GE Healthcare) can be used. In constructing immune VHH libraries, a library size of 5×10^6 to 5×10^7 individual clones with unique sequences should fairly represent the lymphocyte population in llama blood (approximately $1–5 \times 10^6$/ml blood; De Genst et al., 2006a) used for the extraction of RNA and cDNA synthesis. Generally, a sampling size of 50 to 100 colonies from the library provides a good estimate of its

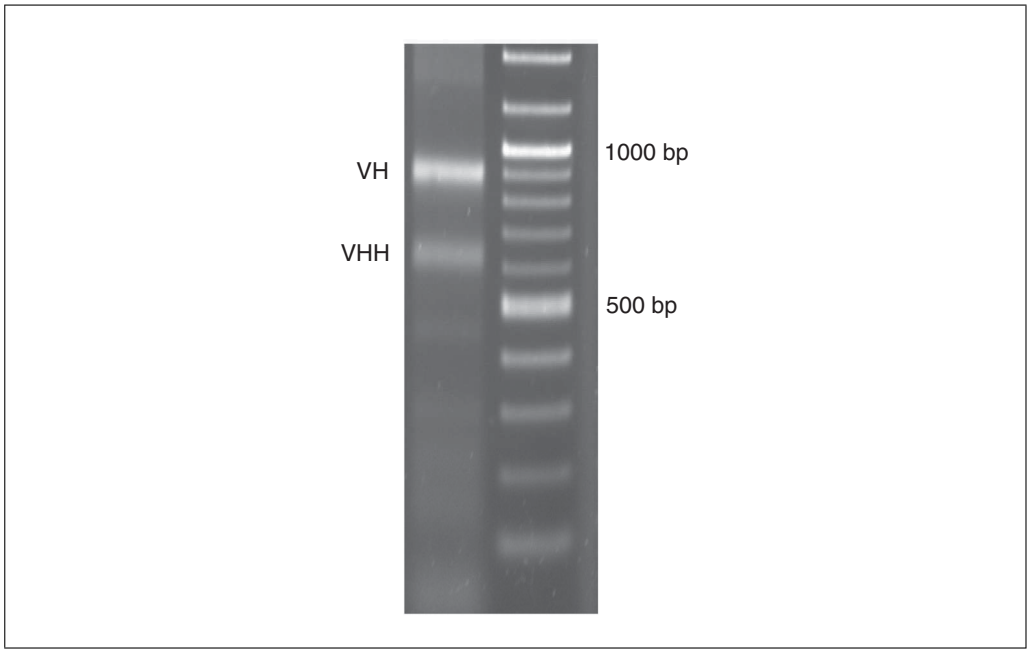

Figure 2.17.2 A DNA agarose gel after first PCR of library construction showing VH and VHH band (left lane) and molecular ladder (right lane).

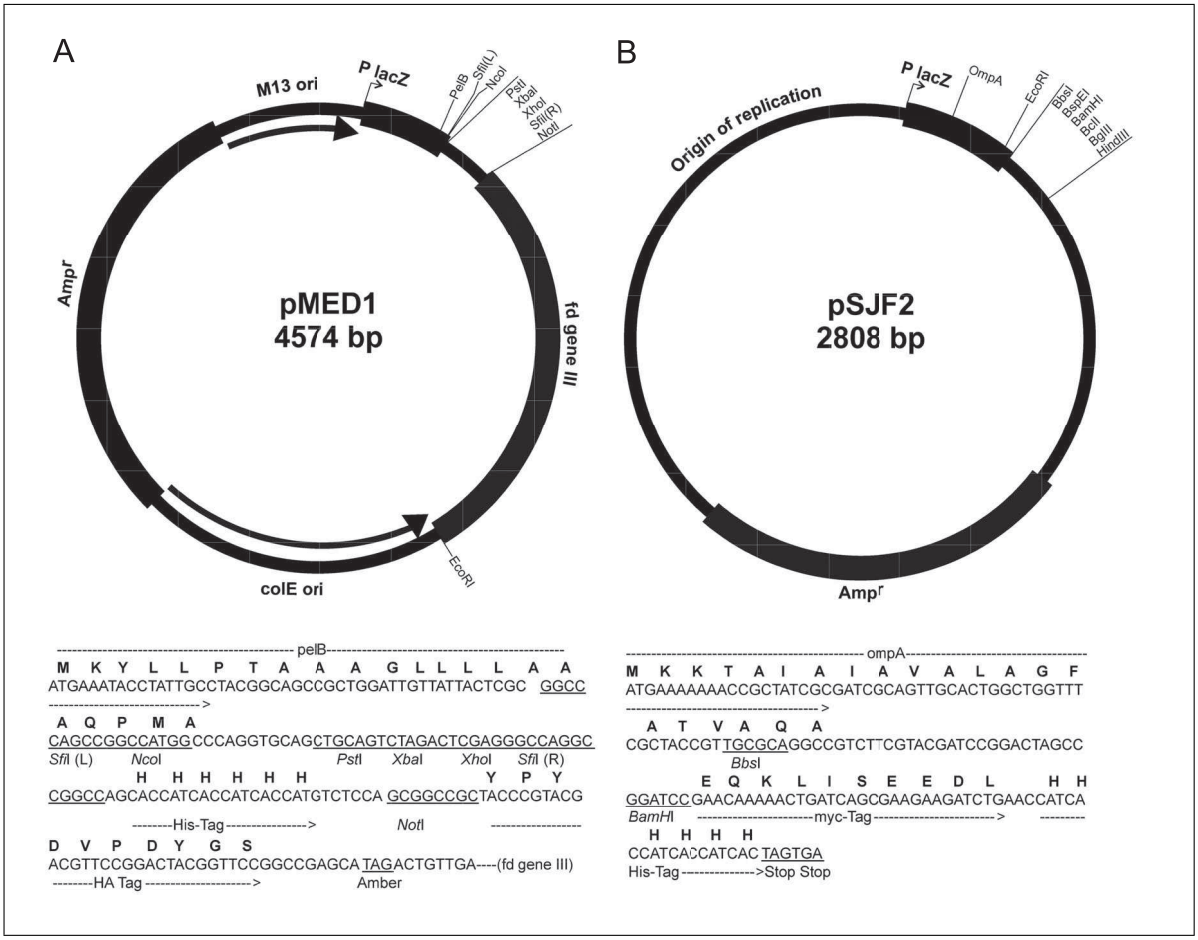

Figure 2.17.3 Schematic drawing of phagemid vector pMED1 and expression vector (pSJF2) with their respective multiple cloning sites.

complexity, which is expressed as the number clones with full inserts, each having a unique sequence. Finally, the library is grown in the presence of 2% to 4% glucose for a number of hours before making a glycerol stock of the bacterial cells and freezing the aliquots at – 80°C.

Materials

Llama blood drawn on Day 71 of Basic Protocol 1 (or Day 42 of Alternate Protocol 1)
RPMI 1640 medium (e.g., Life Technologies)
Lymphoprep Tube (Cedarlane)
Trizol (Life Technologies)
First-Strand cDNA Synthesis kit (GE Healthcare)
10 pmol/µl primers: CH2FORTA4, CH2B3-F, MJ1, MJ2, MJ3, MJ7, MJ8, PN2, M13RP (see Table 2.17.2)—primers were purchased from Integrated DNA Technology (IDT)
5 U/µl *Taq* DNA polymerase and 10× PCR buffer
dNTPs: 10 mM each of dTTP, dATP, dCTP and dGTP (New England Biolabs)
QIAquick Gel Extraction and QIAquick PCR purification kits (Qiagen)
pMED1 phagemid vector (Arbabi Ghahroudi et al., 2009a; the vector is available from the authors upon request)
*Sfi*I, *Xho*I, and *Pst*I restriction endonucleases and their respective 10× buffers
LigaFast Rapid DNA Ligation System (Promega)

Electrocompetent TG1 *E. coli* cells (see Support Protocol 4)
SOC medium (see recipe)
2xYT medium (see recipe)
2xYT-Amp plates: 2×YT plates (see recipe) containing 100 µg/ml ampicillin2xYT-Amp-Glu medium: 2xYT medium (see recipe) containing 100 µg/ml ampicillin and 2% (w/v) glucose
70% (v/v) glycerol, autoclave to sterilize

ND-1000 spectrophotometer (Thermo Scientific) or similar instrument
Electroporation cuvettes (BioRad)
MicroPulser electroporator (BioRad) or equivalent electroporation device
37°C shaking water bath or incubator
Refrigerated centrifuge

Additional reagents and equipment for measuring nucleic acid concentration (*APPENDIX 3L* and Gallagher and Desjardins, 2008), counting cells (*APPENDIX 3A*), agarose gel electrophoresis (see Support Protocol 3 and *UNIT 10.4*), and DNA sequencing (*UNIT 10.25*)

PBL isolation, RNA extraction, and cDNA synthesis

1. Take 10 ml of llama blood drawn on Day 71 of Basic Protocol 1 (or Day 42 of Alternate Protocol 1) and dilute 1:1 in RPMI medium or physiological saline solution.
2. Slowly add the 20 ml diluted blood to the tube containing 10 ml Lymphoprep.
3. Centrifuge the tubes for 15 min at 800 × *g*, 18° to 22°C.
4. Collect the mononuclear cells that have formed interface in between two layers using a Pasteur pipet.

   Alternatively, the entire contents of the tube above the plastic insert can be removed by decanting.

5. Dilute the harvested fraction with RPMI medium to reduce the density and pellet the cells by centrifugation for 10 min at 250 × *g*, 18° to 22°C.
6. After counting the cells (*APPENDIX 3A*), either freeze them at −80°C or continue to purify RNA with the Trizol method.
7. Take 5×10^7 cells and isolate total RNA by using the Trizol reagent according to the manufacturer's instructions. Measure the RNA concentration and purity by measuring the optical density at 260 and 280 nm (OD_{260} and OD_{280}; *APPENDIX 3L*).
8. Use a total of 3 to 5 µg of RNA in 20 µl of distilled, deionized water to synthesize cDNA in a total reaction volume of 33 µl, using the First-Strand cDNA Synthesis kit and oligo(dT) or random hexamers, according to the manufacturer's instructions.

   Prepare the primer solutions in autoclaved distilled water and always store at −20°C to prevent degradation. We typically prepare a 100× (100 pmol/µl) stock solution in TE buffer and 10× working solutions (in sterile water) in 100-µl aliquots.

   It may sometimes be necessary to optimize the amount of input RNA, but generally 3 to 5 µg total RNA per cDNA synthesis reaction results in a good yield of DNA by RT-PCR.

   Alternatively, mRNA can be isolated from the total RNA obtained from the lysed lymphocytes using, for instance, the Dynabeads mRNA Direct kit (Invitrogen), and following the manufacturer's recommendations. This can eventually lead to a higher efficiency of cDNA synthesis.

Table 2.17.2 Primers Used in Working with Single-Domain Antibodies

Primer no.	Primer name	Primer sequence
First PCR VH/VHH5′ end primers		
1	MJ1	GCC CAG CCG GCC ATG GCC SMK GTG CAG CTG GTG GAK TCT GGG GGA
2	MJ2	GCC CAG CCG GCC ATG GCC CAG GTA AAG CTG GAG GAG TCT GGG GGA
3	MJ3	GCC CAG CCG GCC ATG GCC CAG GCT CAG GTA CAG CTG GTG GAG TCT
First PCR VH/VHH3′ end primers		
4	CH2FORTA4	CGC CAT CAA GGT ACC AGT TGA
5	CH2B3-F	CH2B3-F
Second PCR VH/VHH5′ end primers		
6	MJ7	CAT GTG TAG ACT CGC GGC CCA GCC GGC CAT GGC C
Second PCR VH/VHH3′ end primers		
7	MJ8	CAT GTG TAG ATT CCT GGC CGG CCT GGC CTG AGG AGA CGG TGA CCTGG
Subcloning VHH5′ end primers		
8	BbsI1-VHH	TAT GAA GAC ACC AGG CCC AGG TAA AGC TGG AGG AGT CT
9	BbsI2-VHH	TAT GAA GAC ACC AGG CCC AGG TGC AGC TGG TGG AGT CT
Subcloning VHH3′ end primers		
10	BamHI-VHH	TTG TTC GGA TCC TGA GGA GAC GGT GAC CTG
Colony-PCR, sequencing5′ end primers		
11	M13RP	CAG GAA ACA GCT ATG AC
Colony-PCR, sequencing 3′ end primers		
12	M13FP	GTA AAA CGA CGG CCA GT
13	PN2	CCC TCA TAG TTA GCG TAA CGA TCT
Primers for dimeric VHH		
14	DIMBACK1	GGT GGC AGT GGC GGT GGA GGT GGC GGA GGC AGC GGA GGC GGT GGC AGT CAG GTG CAG CTG GAG GAG TCT
15	DIMFOR1	TCC ACC GCC ACT GCC ACC GCT ACC TCC GCC ACC AGA ACC TCC ACC GCC TGA GGA GAC GGT GAC CTG GGT
Primers for mouse Fc fusion		
16	FC-FW	GGT GTA CAG TGT XXXXXX (Xs are the nucleotides at the 5′ of the VHH gene)
17	FC-RE	AAT GGG CCC GCT GGG CTC AAG TTT TTT GTC CAC CGT CAT
18	pTT5-mFC7	AGC TGT TGG GGT GAG TAC TCC
19	pTT5-mFC8	GGA TGG TCC ACC CT CGA GGT TAG G
Primer for pentamerization		
20	VT-BbsI-f	TAA TAA GAA GAC ACC AGG CCG ATG TGCA GCT GCA GGC GTC TG
21	VT-ApaI-r	ATT ATT ATG GGC CCT GAG GAG ACG GTG ACC TGG GTC

PCR amplification of VHH repertoire and cloning of VHH fragments into a phagemid vector for library construction

9. Perform test PCRs (*UNIT 10.20*) using various amounts of the cDNA ranging in volume from 0.5 µl to 5 µl using an equimolar mix of framework 1–specific primers MJ1, MJ2, and MJ3 with either the CH2FORTA4 or CH2B3-F primer. Set up the PCR reaction as follows:

   5 µl 10× PCR buffer
   0.5 µl 10 pmol/µl MJ1–3 primer mix (10 pmol/µl each primer)
   0.5 µl 10 pmol/µl CH2FORTA4 or CH2B3-F primer
   1 µl 10 mM dNTPs
   0.5 to 5 µl cDNA
   0.5 µl *Taq* DNA polymerase
   Deionized, distilled H_2O to 50 µl.

   Perform a PCR (*UNIT 10.20*) using the following thermal cycling program:

1 cycle:	3 min	94°C	(initial denaturation)
30 cycles:	1 min	94°C	(denaturation)
	30 sec	55°C	(annealing)
	30 sec	72°C	(extension)
1 cycle:	7 min	72°C	(final extension).

10. Analyze 5 µl of the PCR reaction on a 1% agarose gel (Support Protocol 3 and *UNIT 10.4*). Identify the cDNA volume that gives the best amplification of the VHH genes. Perform PCR (*UNIT 10.20*) with the remaining cDNA under the same conditions. Gel-purify the VHH bands from a 1% agarose gel (Support Protocol 3 and *UNIT 10.4*) using the QIAquick Gel Extraction kit. Pool the DNA and measure the concentration (*APPENDIX 3L*).

    Three bands are obtained following RT-PCR: one with a size of ~850 bp, which corresponds to VH-CH2 of conventional antibodies, and two close bands with sizes in the range of 550 to 650 bp, which correspond to the VHH-CH2 of heavy-chain antibodies. We frequently observe that the CH2B3-F primer gives a banding pattern that consists of an intense VHH-CH2 band and a faint VH-CH2 band. The aim of optimizing the PCR reaction is to increase the intensity of the VHH-CH2 bands relative to the VH-CH2 band. However, differential intensities of the two VHH-CH2 bands with respect to each other are routinely observed on agarose gels. Ideally, the PCR may be optimized in six separate reactions, each utilizing a unique primer pair, but we have found this is not necessary for VHH libraries from immunized llamas.

11. Re-amplify the purified product (using 10 to 20 ng per reaction tube) in a second PCR (*UNIT 10.4*) under the same conditions as above, using sense (fragment 1)- and framework 4–specific primers MJ7 and MJ8, respectively. Perform a total of 20 PCR reactions. Analyze a small amount of the PCR reaction on a 1% agarose gel (Support Protocol 3 and *UNIT 10.4*)—a band around 400 to 450 bp corresponding to the VHH fragment is expected. Desalt the PCR products with the QIAquick PCR Purification kit and determine the DNA concentration (*APPENDIX 3L*).

    We always elute the DNA with sterile distilled water instead of the manufacturer's recommended elution buffer. This will ensure its compatibility with the subsequent reactions.

12. Digest the PCR products with *Sfi*I (5 U/µg DNA) overnight at 50°C and subsequently analyze a few microliters on a 1% agarose gel (Support Protocol 3 and *UNIT 10.4*) to ensure that it is of the proper size. Re-purify the digested DNA with a QIAquick

PCR Purification kit and measure its concentration using an ND-1000 or similar microvolume spectrophotometer (Gallagher and Desjardins, 2008).

In contrast to a conventional spectrophotometer, the ND-1000 spectrophotometer (or instrument with similar technology) measures absorbance at very low volumes (1 µl) and without the use of cuvettes.

13. Digest 30 µg of pMED1 phagemid vector with *Sfi*I (5 U/µg DNA) for 5 hr at 50°C. The next day, add 1 µl (10 U) of each of *Xho*I and *Pst*I enzymes for an additional 2 hr at 37°C to reduce self-ligation of pMED1. Examine the digested pMED1 on a 1% agarose gel (Support Protocol 3 and *UNIT 10.4*) using undigested vector as a control to ensure that the vector is completely linearized. Purify the digested vector with a QIAquick PCR Purification kit and measure its concentration using an ND-1000 or similar microvolume spectrophotometer (Gallagher and Desjardins, 2008).

14. Use the LigaFast Rapid DNA Ligation System to ligate the *Sfi*I-digested VHH DNA with *Sfi*I-digested pMED1 vector as follows:

    20 µg digested vector (from step 13)
    3.5 µg digested VHH insert (from step 12)
    10 µl T4 DNA ligase buffer
    8 µl T4 DNA ligase
    Sterile distilled, deionized H_2O to 100 µl.

    Incubate at room temperature for 1 hr.

    With a ligation of this magnitude, library sizes of around 10^8 should be obtained. When larger-size libraries are required, as in the case of synthetic and naïve libraries, it is advisable to first identify the ligation conditions that will give the biggest library size. This can be done by performing small-scale ligations with different total DNA and molar ratios of insert to vector. Moreover, the scale of the ligation and the number of transformations needs to be significantly increased. In the case of naïve libraries, use of blood taken from several animals will increase the number of antibody-displaying B cells and the library diversity.

15. Purify the ligated materials with a QIAquick PCR Purification kit using two spin columns and elute the DNA in a final volume of 35 µl of sterile distilled, deionized water per column. Pool the eluted material and measure its concentration using an ND-1000 or similar microvolume spectrophotometer (Gallagher and Desjardins, 2008).

16. Transform 50 µl of electrocompetent TG1 cells with 3 µl of the purified ligated material using a MicroPulser electroporator or equivalent instrument. Transfer the electroporated cells into a tube containing 1 ml of SOC medium and incubate for 1 hr at 37°C with shaking at 180 rpm. Repeat the transformation for the remaining of the ligated material.

17. Pool the transformed cells, take a small aliquot and carry out 10^3-, 10^4-, and 10^5-fold dilutions in 2xYT medium. Spread 100 µl of the diluted cells on 2xYT-Amp plates and incubate overnight at 32°C. In the morning, use the plates to determine the functional size of the library as described below.

18. Amplify the library by transferring the transformed cells into 500 ml of 2xYT-Amp-Glu and incubating 3 hr at 37°C with shaking at 220 rpm.

19. Centrifuge the cells 20 min at 5000 × *g*, 4°C. Discard the supernatant and resuspend the cells in 3 to 5 ml of 2xYT-Amp-Glu medium. Make dilutions of the cells in 2xYT, measure the OD_{600}, and use this value to calculate the cell density (number of cells/ml) in the stock solution (see, e.g., *UNIT 10.3*). Add sterile 70% glycerol to

the cell stock, make several aliquots of 10^{10} bacterial cells/vial, and freeze the cells at −80°C.

One may additionally store the constructed library as a purified library vector (plasmid) preparation.

Determine library size and complexity

20. Perform colony PCR on the colonies from the titer plates (step 17) in a total volume of 15 µl. Prepare a master mix for 50 PCR reactions as follows:

    80 µl 10× PCR buffer
    16 µl 10 mM dNTPs
    8 µl 10 pmol/µl PN2 primer
    8 µl 10 pmol/µl M13RP primer
    8 µl 5 U/µl *Taq* DNA polymerase
    680 µl distilled, deionized H_2O.

    Aliquot 15-µl volumes from the master mix into 50 PCR tubes. Touch single colonies from the titer plates (step 17) with a sterile toothpick or 10-µl (P-10) pipet tip and swirl in the PCR tubes. Place the reaction tubes in a thermal cycler and perform a PCR with the following program:

1 cycle:	5 min	94°C	(initial denaturation)
30 cycles:	30 sec	94°C	(denaturation)
	30 sec	55°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	7 min	72°C	(final extension).

21. Analyze a few microliters of each PCR reaction on a 1% agarose gel (Support Protocol 3 and *UNIT 10.4*) to identify the clones with full inserts (~600 bp band). Use the insert-positive percentage to calculate the library size with insert.

22. Use 0.5 µl of the PCR mixture and M13RP as primer to sequence the VHH gene (*UNIT 10.25*). Analyze the sequences to evaluate the diversity of the library. Determine the functional library size by multiplying the percentage of the clones with unique VHH sequences by the total library size.

    With typical PCR yields, a 0.5-µl aliquot can be used directly for DNA sequencing that will give clean sequencing profiles. In instances where the sequencing profile is not readable, purify the PCR product with a QIAquick PCR purification kit before proceeding with DNA sequencing.

    Normally, one sequencing primer provides enough sequence-read coverage, and thus it is not necessary to use both reverse and forward primers for DNA sequencing.

    VHHs can be distinguished from contaminating VHs by the nature of the amino acids at positions 37, 44, 45, and 47 (Kabat numbering; Kabat et al., 1991). VHHs characteristically have Phe or Tyr; Glu or Gln; Arg or Cys; and Gly, Ser, Leu, or Phe at positions 37, 44, 45 and 47, respectively, whereas VHs have Val, Gly, Leu, and Trp at these four positions.

SUPPORT PROTOCOL 3

PURIFICATION OF DNA FRAGMENTS FROM AGAROSE GELS

Materials

Agarose
I× TAE buffer (prepare from 50× stock; see recipe)
DNA sample (Basic Protocol 2)
Loading dye (*UNIT 10.4*)
Ethidium bromide staining solution (*UNIT 10.4*)

UV transilluminator (see *UNIT 10.4*)
Gel Extraction Kit (Qiagen)

Additional reagents and equipment for agarose gel electrophoresis (*UNIT 10.4*) and measurement of DNA concentration (*APPENDIX 3L*)

Prepare agarose gel

1. Weigh 0.5 g agarose and add 50 ml 1× TAE buffer (for 1% gel).
2. Heat for 1 min 20 sec.
3. Let agarose solution cool before pouring into gel casting plates (see *UNIT 10.4*). Also be sure the plates are secured tightly.
4. Pour the gel and insert the comb without forming any air bubbles; let the gel solidify.

Purify DNA fragments from agarose gels

5. Load a total of 10 µl of DNA sample (2 µl loading dye plus 8 µl DNA sample).
6. Let the DNA run for 30 to 45 min from negative to positive (*UNIT 10.4*).
7. Stain the DNA with ethidium bromide (EtBr) by slightly covering the gel with EtBr solution for 15 to 20 min.

   Our lab has recently switched to GelRed (Biotium Inc.), which has quite similar sensitivity in visualizing the DNA. Additionally, it is claimed by the company to be an environmentally safe reagent.

8. Visualize the DNA on a UV transilluminator.
9. Using a sharp blade, excise the DNA band of interest and purify the DNA from the gel by using a Gel Extraction Kit (Qiagen).
10. Measure the DNA concentration (*APPENDIX 3L*) and keep in −20°C until further use.

    Use caution when working with the ethidium bromide, as it is a carcinogen. Alternatives to ethidium bromide that are more environmentally friendly are also available.

    The percentage of gel can be different based on the size of DNA to be run. A 1% gel is good for DNA sizes from 500 bp up to 10 kb.

PREPARATION OF ELECTROCOMPETENT CELLS

SUPPORT PROTOCOL 4

Materials

TG1 cells (Stratagene)
LB medium (see recipe)
10% (v/v) glycerol

37°C shaking water bath or incubator
Spectrophotometer for measuring OD_{600}
0.5-ml microcentrifuge tubes, autoclaved

NOTE: It is crucial to work under strict sterile conditions because we do not use any antibiotics. All work should be carried out on ice or in a cold room.

1. Pick a single colony of TG1 cells from a freshly prepared plate and inoculate into 15 ml of LB medium. Incubate at 37°C overnight to prepare the pre-culture.
2. Inoculate 10 ml of pre-culture into 1 liter of LB medium in a 4-liter flask and incubate at 37°C with shaking at 200 rpm.

3. Measure OD_{600} every 45 min, and once the OD_{600} reaches 0.4, put the flask in an ice-water bath for 1 hr.
4. Pellet the cells by centrifuging 20 min at 3500 × *g*, 4°C.
5. Discard the supernatant and resuspend the pellet in 1 liter ice-cold water.
6. Pellet the cells by centrifuging 20 min at 3500 × *g*, 4°C.
7. Discard the supernatant and resuspend the pellet in 500 ml ice-cold water.
8. Pellet the cells by centrifuging 20 min at 3500 × *g*, 4°C.
9. Discard the supernatant and resuspend the pellet in 2.5 ml ice-cold 10% glycerol.
10. Divide into 50-µl aliquots in 0.5-ml microcentrifuge tubes, freeze, and store at −80°C until further use.

ALTERNATE PROTOCOL 2

CONSTRUCTION OF NON-IMMUNE (NAÏVE) LIBRARY

It is not always feasible to construct an immune library, especially if the target antigen is nonimmunogenic, scarce, or unavailable. Alternatively, nonimmune or naïve libraries can be constructed following the same protocol described in Basic Protocol 2, except that a larger-size library with higher complexity is required. Therefore, it is recommended to use the lymphocytes isolated from a number of different animals to increase the diversity of the repertoire. Here, the major limitations are the efficiency of transformation in *E. coli* and the number of lymphocytes that can be isolated. Successful panning of llama naïve libraries has been achieved where VHH antibodies with micromolar to nanomolar affinities against mammalian peptides and protein antigens were isolated (Muruganandam et al., 2002; Groot et al., 2006; Monegal et al., 2009). A naïve library avoids the lengthy and laborious immunization step and the use of large amounts of antigen for immunization. Moreover, the single-pot library can be maintained indefinitely and panned against virtually any target antigen, including cell surface target antigens that are unknown. The main concern with a naïve library is the affinity of the binders, as they have not gone through an in vivo affinity maturation step induced by repeated immunization. However, genetic engineering techniques in combination with phage-display technology provide opportunities for affinity maturation of the selected low-affinity VHHs. The affinity maturation can be achieved by random or site directed mutagenesis of CDR regions of the antibodies. The mutagenized libraries are then re-panned against the same target antigen under more stringent conditions in order to isolate VHH clones with higher affinities (Yau et al., 2005).

Additional Materials *(also see Basic Protocol 2)*

Blood (50 ml) from at least five non-immunized llamas (or other camelid species if available)
Fd-tet-M vector (Zacher et al., 1980; Kumaran et al., 2012)

The VHHs are displayed on the surface of a phage vector, which provides multivalent display of VHH antibodies. This will help to isolate binders, which are not expected to be of high affinity. Therefore, avidity rather than affinity plays the major role in the antibody:antigen interaction.

Due to the nature of a naïve library, the panning conditions need to be optimized in order to discriminate between specific binders to the target of interest versus unwanted binders to blocking buffers, plastic surface, etc. It should be emphasized that there is an equal opportunity to find binders to almost any protein, nonprotein material, and the surface used during the panning. Therefore, pre-absorption against undesired materials

and surfaces is a key element in the success of panning experiments. Moreover, it is strongly recommended to alternate blocking buffers and panning surfaces to avoid the enrichment of undesired phage clones.

Extra panning rounds are required to enrich for binders to difficult target antigens such as conserved mammalian antigens or cell surface antigens. On the contrary, panning against infectious reagents to which llama may have been exposed could yield robust VHH binders. In our experience, several VHH binders with picomolar affinities have been isolated from a *Camelidae* naïve library (unpub. observ.).

RESCUE OF PHAGEMID LIBRARY AND PANNING

BASIC PROTOCOL 3

Once a desired library is achieved in a phagemid format, it is essential to rescue the library as phage particles. In this format, antibodies will be expressed on the tip of the phage as fusion proteins to the gIII protein (gp3). Since a phagemid vector carries only the gene III and the intergenic region (IG) of a filamentous phage, all the genes that are required for the phage assembly and production are provided by a helper phage such as M13KO7 or VCSM13 (for a review, see Carmen and Jermutus, 2002). The helper phage with a kanamycin-resistance gene, however, is packaged less efficiently due to its mutated IG region, and, therefore, more than 95% of the phage progeny are of phagemid origin. Despite this, the VHH-gp3 fusion display rate on the recombinant phage remains very low (1% to 10%) due to the limited number of gp3 on the phage (about five per phage particle) and low expression of VHH-gp3 fusions, which is under the control of the *lacZ* promoter (Lowman, 1997). This will ensure mono-display and, subsequently, monovalent binding of the VHH antibodies to their target antigens during the panning and selection process. The nature of the rescued phages is generally examined by plating on both ampicillin and kanamycin plates. During panning, the phage-displayed antibodies on the tip of phage will bind to the antigen, and after washing off the non-bound phages, the bound phages can be eluted at low or high pH and high salt concentration, with free target antigen or by using another competitive binder to the target antigen.

Materials

VHH library glycerol stock (Basic Protocol 2)
M13KO7 helper phage (New England Biolabs)
2xYT-Amp-Glu medium: 2xYT medium (see recipe) containing 100 µg/ml ampicillin and 2% (w/v) glucose
2xYT-Amp-Kan medium: 2xYT medium (see recipe) containing 100 µg/ml ampicillin and 50 µg/ml kanamycin
PEG/NaCl (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), sterile
Frozen stock of *E. coli* TG1 cells (Stratagene)
M9 minimal medium plate (see recipe)
2xYT medium (see recipe)
2xYT-Amp plates: 2×YT plates (see recipe) containing 100 µg/ml ampicillin
Antigens (100 to 200 µg required)
Buffer B: PBS plus 1% (w/v) casein (autoclave to sterilize)
PBST: PBS (see recipe) containing 0.05% (v/v) Tween 20
0.1 M triethylamine, prepared fresh daily
1 M Tris·Cl, pH 7.4 (*APPENDIX 2A*)
Kanamycin: 50 mg/ml, filter sterilize and store at −20°C in 1-ml aliquots

Spectrophotometer
37°C shaking water bath or incubator
Refrigerated centrifuge
0.2-µm filter units

15-ml conical centrifuge tubes (e.g., BD Falcon)
37°C shaking water bath or incubator
32°C incubator
96-well MaxiSorp microtiter plates (VWR)

Prepare phages

1. To rescue the library in the phage format for panning, thaw 1 to 2 ml of frozen library cells (approximately 5×10^{10} cells) and grow in 200 ml of 2xYT-Amp-Glu medium at 37°C with shaking at 220 rpm to an OD_{600} of 0.5 (2 to 3 hr). Infect the cells with a 20-fold excess of M13KO7 helper phage (1×10^{12} pfu) for 1 hr at 37°C. Pellet the infected cells by centrifuging 10 min at 5000 × *g*, 4°C. Resuspend the pellets in 200 ml of 2xYT-Amp-Kan medium and grow overnight at 37°C with shaking at 250 rpm.

2. To purify the phage, pellet the overnight culture (15 min at 10,000 × *g*, 4°C), filter the supernatant through a 0.2-µm filter unit, and add 1/5 the volume of PEG/NaCl to the filtrate. Incubate for 1 hr on ice, centrifuge 15 min at 10,000 × *g*, 4°C, and discard the supernatant. Resuspend the pelleted phage in 1.5 ml of sterile PBS, determine the titer, and store at −80°C. Use the purified phage as the input phage for round 1 of library screening (steps 9 to 18).

Determine phage titer

3. To make a stock plate of *E. coli* TG1 cells, streak out a frozen stock of TG1 cells (as purchased from Stratagene) on an M9 minimal medium plate. Incubate at 37°C for at least 24 hr. Seal the plate with Parafilm and store at 4°C for up to a month.

   It is recommended to grow the TG1 cells on minimal medium to ensure that the F pilus, which mediates phage infection, is maintained on the cells. Thiamine is added to the medium since TG1 cells are auxotrophic for thiamine.

4. Prepare exponentially growing TG1 cells by inoculating 2 to 3 ml of 2xYT medium in a sterile 15-ml Falcon tube with a single colony from a stock plate of *E. coli* TG1 cells.

5. Incubate at 37°C in a rotary shaker with shaking at 220 rpm. Remove aliquots from the culture flask at different time intervals and measure the OD_{600} in a spectrophotometer in disposable cuvettes using 2xYT medium as the blank. Stop the incubation at $OD_{600} = 0.4$ to 0.5 (typically after 2 to 3 hr).

6. To determine the titer of the phage, make 10^6, 10^8, 10^{10}, and 10^{12} serial dilutions of phage in PBS, and mix 10 µl of each dilution with 100 µl of the exponentially-growing TG1 cells.

7. Incubate the cells at room temperature for 15 min and subsequently plate them on 2xYT-Amp plates. In the morning, count the colonies and determine the titer.

   Phage titers are typically 1×10^{13} to 5×10^{13} colony-forming units/ml.

8. Make serial dilutions (10^{-2} to 10^{-6}) of the infected cells in 2xYT medium in 500-µl volumes. Spread 100 µl of each dilution on 2xYT-Amp plates. Also plate 100 µl of the uninfected cells as a negative control. Incubate at 32°C overnight. Keep the plates Parafilm-sealed and stored at 4°C for clonal analysis (colony-PCR, sequencing, and phage ELISA; see Basic Protocol 4).

Biopanning of the phage library

In this step, the library is screened to isolate phage displaying VHHs with specificity for the antigen of interest. After two to three rounds of panning, a phage ELISA is

performed to identify individual phage displaying VHHs that bind to the antigen of interest.

9. For panning, coat a well in 96-well microtiter plate with 20 µg of the antigen in a volume of 100 µl in PBS overnight at 4°C.
10. On the next day, remove the contents of the coated wells, wash with 300 µl of PBS, and block for 2 hr at 37°C with 200 µl/well of buffer B. Start preparing 10 ml of exponentially growing TG1 cells in a sterile 50-ml conical centrifuge tube (see step 4).
11. Remove the blocking buffer and add $\sim 10^{12}$ input phage in 100 µl of PBS to the blocked well for 2 hr at 37°C. Remove unbound phage, wash the well ten times with PBST (300 µl per wash), and elute the bound phage by incubating with 0.1 M triethylamine (100 µl/well) for exactly 10 min at room temperature. Pipet the elution solution up and down several times in the well, remove the contents, and neutralize with 50 µl of 1 M Tris·Cl, pH 7.4, in a separate tube. Keep the tube on ice.
12. Keep 100 µl of the exponentially growing TG1 cells as a negative control.
13. Add 100 µl of the eluted phage to the remaining TG1 culture and let the phage infect the TG1 cells by incubating the mixture at 37°C for 15 min without shaking, then for 1 hr with shaking at 220 rpm (store the remaining phage at −80°C for future reference).
14. Add 10^{11} pfu of M13KO7 helper phage to the infected bacteria (~10 ml) to super-infect them. Let the super-infection take place by incubating at 37°C for 15 min without shaking. Subsequently, add kanamycin at a final concentration at 50 µg/ml and incubate overnight at 37°C with shaking at 250 rpm.
15. The next day, purify the phage in a final volume of 200 µl of PBS and determine the phage titer (see steps 2 to 8 of this protocol). Use the purified phage as the input phage for the next round of panning (Basic Protocol 3, step 2).
16. To assess the progress of the panning, perform colony PCR and DNA sequencing of the colonies (see Basic Protocol 2) on the titer plate.

    We routinely sequence 15 to 20 clones in each of the first two rounds, 25 clones in the third round, and 50 to 100 clones in the fourth round.
17. Repeat the panning for three more rounds using the amplified phage from the previous round as the input phage for the next round and reduce the amount of coated antigen by 5 µg for each subsequent round.
18. After four rounds of panning, perform a phage ELISA (Basic Protocol 4) to identify the clones that are harboring antigen-binding VHHs.

    The plate from round four should be labeled as reference titer plate for future use.

MONITORING THE PROGRESS OF BIOPANNING BY PHAGE ELISA

BASIC PROTOCOL 4

The progress of panning can be monitored by colony PCR (see Basic Protocols 2 and 3) by analyzing the number of clones with VHH inserts and their sequences, or by phage ELISA analysis of enrichment for binders from the titer plate. Amplified phage after each round of panning or phage prepared from randomly selected colonies after each round of panning, in particular the last round, can be used in an standard ELISA format to evaluate their binding to the antigen of interest. For detection, anti-M13 antibodies conjugated to horseradish peroxidase are used. The results of phage ELISA, although quite specific and reliable, should be interpreted with caution since ELISA signals are

greatly amplified due to interaction of the secondary antibodies with highly abundant copies of the phage major coat protein (gp8).

Materials

2xYT-Amp-Glu medium: 2xYT medium (see recipe) containing 100 µg/ml ampicillin and 2% (w/v) glucose
TG1 cells containing the VHH phagemid (previously screened from the titer plate by colony PCR and DNA sequencing; Basic Protocol 3)
M13KO7 helper phage (New England Biolabs)
2xYT-Amp-Kan medium: 2xYT medium (see recipe) containing 100 µg/ml ampicillin and 50 µg/ml kanamycin
Antigens
Phosphate-buffered saline (PBS; see recipe)
Buffer B: PBS (see recipe) plus 1% (w/v) casein (autoclave to sterilize)
PBST: PBS (see recipe) plus 0.05% (v/v) Tween 20
Anti-M13 IgG conjugated to HRP (GE Healthcare)
HRP substrate solutions for ELISA (GE Healthcare)
1 M H_3PO_4
15-ml conical centrifuge tubes
Spectrophotometer
96-well MaxiSorp microtiter plates (VWR)
Microtiter plate reader (Biochrom, *http://www.biochrom.co.uk/*)
Additional reagents and equipment for preparing phage (Basic Protocol 3, steps 1 to 2)

1. Aliquot 1 ml of 2xYT-Amp-Glu into a sterile 15-ml conical centrifuge tube. Add a small colony of TG1 cells containing the VHH phagemid (that was previously screened by colony PCR and DNA sequencing). Grow the cell culture at 37°C with shaking at 250 rpm until the OD_{600} of the culture reaches 0.5. Next, infect the cells with 10^{10} pfu of M13KO7 helper phage, add kanamycin, and then grow overnight as described in Basic Protocol 3, step 1.

2. Coat wells of a 96-well microtiter plate with the antigen at a concentration of 5 to 10 µg/ml and in a volume of 100 µl in PBS overnight at 4°C. The next day, remove the contents of the coated wells, wash with 300 µl of PBS, and block for 2 hr at 37°C with 200 µl/well of buffer B.

3. While blocking, microcentrifuge the overnight cultures 20 min at 10,000 × *g*, 4°C. Carefully remove the supernatant, which contains the VHH-displaying phage particles, and decant into a new microcentrifuge tube. Keep on ice.

4. Remove the blocking agent from the microtiter plate, wash each well five times, each time with 300 µl PBST and pat dry. Add 100 µl/well of phage supernatants to the appropriate wells. Include duplicate negative control wells with 10^9 M13KO7 helper phage replacing the phage supernatant. Incubate the wells for 1.5 hr at room temperature.

   Additionally, one may use amplified phage (Basic Protocol 3, step 15; 10^9 pfu/ml) after each round of panning as well as phage rescued from the original library (Basic Protocol 3, step 2).

5. Remove unbound phage particles and wash wells six times, each time with 300 µl PBST. Add 100 µl/well of anti-M13 IgG-HRP conjugate (previously diluted 1:5000 in PBS) and incubate for 1 hr at room temperature.

6. Discard the unbound anti-M13 IgG-HRP, wash six times, each time with 300 µl PBST, and finally add 100 µl of HRP substrate. After 10 min, stop the reaction by adding 100 µl of 1M H_3PO_4. Read the plate at 450 nm with a microtiter plate reader.

7. Store the ELISA-positive phage at −20°C for future reference. Identify positive clones in the reference titer plate (Basic Protocol 3, step 18) and prepare a glycerol stock of individual clones for future use.

   Alternatively one may remove phages from the ELISA plate and prepare a glycerol stock of pelleted bacterial cells in the plate for future reference.

SUBCLONING OF VHH FRAGMENTS IN BACTERIAL EXPRESSION VECTOR

BASIC PROTOCOL 5

Expression of single-domain antibodies in bacterial systems

In this protocol we describe in detail the current methods that are used to express sdAbs in bacteria. Bacteria are among the most common expression systems for expressing recombinant proteins. Different approaches for carrying out periplasmic and cytoplasmic expression, as well as small-scale and large-scale expression, are presented. Data related to expression vectors, expression conditions, methods of protein extraction and purification, and yield and purity analysis of sdAbs are presented, and important points that need to be considered before sdAbs that have been expressed in bacteria are used either in vitro or in vivo are highlighted.

NOTE: Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25°C) and analytical-grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials.

Materials

10× PCR buffer (Hoffmann-La Roche)
Expand High-Fidelity *Taq* DNA polymerase (Hoffmann–La Roche)
dNTPs: 10 mM each of dTTP, dATP, dCTP and dGTP (New England Biolabs)
Primers: VHBbs1, VHBbs2, VHBam, M13RP, M13FP (see Table 2.17.2; purchase from Integrated DNA Technology)
Titer reference plates with positive colonies (Basic Protocol 3, step 18)
QIAquick PCR Purification kit (Qiagen)
Restriction enzymes *Bbs*I, *BamH*I (New England Biolabs)
QIAquick Gel Extraction kit (Qiagen)
pSJF2 expression vector (Baral and Arbabi Ghahroudi, 2012; cut with *Bbs*I and *Bam*HI before ligation)
LigaFast Rapid DNA Ligation System (Promega)
TG1 electroporation-competent cells (Support Protocol 4)
SOC medium (see recipe)
LB-Amp plates: Prepare LB agar (see recipe) and add filter-sterilized ampicillin at a final concentration of 100 µg/ml when agar medium has cooled to ~50°C, but before pouring plates
5 U/µl regular *Tag* DNA polymerase (not "Expand")

Thermal Cycler (GeneAmp PCR System 9700, Applied Biosystems) or similar instrument
Sterile toothpicks or 10-µl (P-10) pipet tip
Disposable electroporation cuvettes (BioRad)
MicroPulser electroporator (BioRad Laboratories) or similar instrument
37°C shaking water bath or incubator

32°C incubator
ND-1000 spectrophotometer (Thermo Scientific) or similar instrument (Gallagher and Desjardins, 2008)

Additional reagents and equipment for agarose gel electrophoresis (see Support Protocol 3 and *UNIT 10.4*), DNA sequencing (*UNIT 10.25*), and microvolume determination of DNA concentration (Gallagher and Desjardins, 2008)

PCR amplification of antigen-specific VHH and cloning

All the cloning steps are performed essentially as described elsewhere (Sambrook et al., 1989; Ausubel et al., 2013). The VHH genes used here were identified as binders for an antigen of interest. They were isolated from a phagemid library by panning as described elsewhere (Arbabi Ghahroudi et al., 2009a).

1. Amplify the VHH genes from the phagemid vector (pMED1) produced from the positive clones screened by phage ELISA (Basic Protocols 3 and 4) in a total volume of 50 µl by performing colony PCR using VHHBbs1 or VHHBbs2 and VHHBam primers (*Bbs*I and *BamH*I sites are introduced at the ends of the amplified fragments by using these primers). Prepare each PCR reaction mix with the following ingredients:

   5.0 µl 10× PCR buffer
   1 µl 10 mM dNTPs
   0.5 µl 10 pmol/µl VHBbs1 or VHBbs2
   0.5 µl 10 pmol/µl VHBam primer
   0.5 µl 5 U/µl Expand *Taq* DNA polymerase
   41.5 µl H_2O.

   Touch single colonies from the titer reference plates with sterile toothpicks or a 10-µl (P-10) pipet tip and then swirl around in the PCR tubes to dislodge the bacteria into the PCR reaction solution. Place the reaction tubes into a thermal cycler and perform PCR using the following program:

1 cycle:	5 min	94°C	(initial denaturation)
30 cycles:	30 sec	94°C	(denaturation)
	30 sec	55°C	(annealing)
	30 sec	72°C	(extension)
1 cycle:	7 min	72°C	(final extension).

2. Purify the VHH genes in a final volume of 50 µl water using a QIAquick PCR Purification kit.

3. Digest the purified DNA with *Bbs*I restriction endonuclease for 3 hr at 37°C, and then purify it in a final volume of 50 µl water using a QIAquick PCR Purification kit. Re-digest with *BamH*I restriction endonuclease for an additional 2 hr at 37°C, and re-purify with a QIAquick PCR purification kit in 50 µl of water.

   The same digestion reaction was set up for the pSJF2 vector. However, the vector DNA needs to be gel-purified after the second enzymatic digestion to reduce the chance of self-ligation.

4. Ligate the cut fragment into *Bbs*I/*BamH*I-digested pSJF2 expression vector (Fig. 2.17.3B) using LigaFast Rapid Ligation System.

   This results in addition of C-terminal c-Myc and His_6 tags to the protein.

5. Transform 50 µl of electrocompetent TG1 cells with 3 µl of the ligated material using a MicroPulser electroporator or an equivalent instrument. Transfer the electroporated

cells into a tube that contains 1 ml of SOC medium and incubate it for 1 hr at 37°C with shaking at 180 rpm.

Alternatively, cells can be transformed by subjecting chemically prepared competent cells to a 90-sec heat shock at 42°C (Sambrook et al., 1989).

6. Spread 100 µl of cells onto LB-Amp plates and leave the plates with their lids half open for about 5 min in a laminar-flow hood. Cover, invert, and incubate the plates overnight at 32°C.

7. The following day, perform colony PCR in a volume of 15 µl on the colonies that grew on the overnight plates using the M13RP and M13FP primers:

   a. Prepare a master mix for 24 PCR reactions which consists of the following:

      36 µl 10× PCR buffer
      7.7 µl 10 mM dNTPs
      7.2 µl 10 pmol/µl M13RP
      7.2 µl 10 pmol/µl M13RP
      3.6 µl 5 U/µl *Taq* DNA polymerase
      277.2 µl H_2O.

   b. Aliquot 15 µl from the master mix into each of 24 PCR tubes. Touch single colonies from the titer plates with sterile toothpicks or a 10-µl (P-10) pipet tip and then swirl around in the PCR tubes to dislodge the bacteria into the PCR reaction solution. Place the reaction tubes into a thermal cycler and perform PCR using the following program:

1 cycle:	5 min	94°C	(initial denaturation)
30 cycles:	30 sec	94°C	(denaturation)
	30 sec	55°C	(annealing)
	30 sec	72°C	(extension)
1 cycle:	7 min	72°C	(final extension).

   c. Apply 5 µl of the PCR mix on 1% agarose gels (*UNIT 10.4*) to identify the clones that contain a full insert of approximately 650 bp in size. Purify the remaining PCR mix for the clones which contain the full insert by using a QIAquick PCR Purification kit, and then determine the DNA concentrations with an ND-1000 spectrophotometer (Gallagher and Desjardins, 2008).

8. Confirm the positive clones by further sequencing their VHH genes (*UNIT 10.25*) using M13RP and/or M13FP as primers.

EXPRESSION OF SINGLE-DOMAIN ANTIBODIES IN BACTERIAL SYSTEMS

BASIC PROTOCOL 6

VHH genes are cloned in fusion with the OmpA leader sequence in vector pSJF2H. This sequence directs the expressed protein to the periplasmic space of *E. coli*. The following protein extraction protocol, which is based on an osmotic-shock method (Neu and Heppel, 1965), is designed to increase the permeability of the outer membrane. It therefore enables the sdAbs to be released from the periplasm without the cells being lysed. SdAbs are partially purified by carrying out the periplasmic extraction, since the periplasm contains far less protein than the cytoplasm. It is recommended that the fractions from various steps of the extraction be kept at 4°C until it has been verified by western blotting (Support Protocol 5; also see *UNIT 8.10*) which fractions contain the sdAb.

Materials

Plate with colonies of positive clones (Basic Protocol 5)
B2YT-Amp medium: B2YT medium (see recipe) containing 100 µg/ml filter-sterilized ampicillin

1 M isopropyl-β-D-thio-galactopyranoside (IPTG; Rose Scientific, *http://www.rosesci.com/*) stock solution in H_2O

LB-Amp medium: LB medium (see recipe) containing 100 µg/ml filter-sterilized ampicillin

M9 medium (see recipe) containing 100 µg/ml filter-sterilized ampicillin

10× induction medium (see recipe)

Wash solution: 10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*) containing 150 mM NaCl

Sucrose solution: 10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*) containing 1 mM EDTA and 25% (w/v) sucrose

Shock solution: 10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*) containing 0.5 mM $MgCl_2$, ice cold

Mouse anti-His_6 antibody (GE Healthcare, cat. no. 27-4710-01)

Starting buffer (see recipe)

Lysis buffer (see recipe), ice cold

100 mM phenylmethylsulfonyl fluoride (PMSF; Thermo Scientific)

1 M dithiothreitol (DTT; Sigma-Aldrich)

3 mg/ml lysozyme (USB Corporation), freshly prepared

15 U/µl DNase I (Sigma-Aldrich) in 1 M $MgCl_2$

Incubator with rotary shaking

Beckman Coulter J2-21M/E high-speed centrifuge or equivalent

Sorvall high-speed, swinging-bucket bench-top (RT6000B Refrigerated) centrifuge or equivalent

250-ml Erlenmeyer flask

Cell density meter (Biochrome Ltd., *http://www.biochrom.co.uk/*) or equivalent

Dialysis tubing with MWCO 8000 (Biodesign Inc., *http://biodesignofny.com/*)

0.2 µm GP Express Plus membrane filtration system (Millipore)

Additional reagents and equipment for SDS-PAGE (Support Protocol 5; also see *UNIT 8.4*) and western blotting (immunoblotting; Support Protocol 5; also see *UNIT 8.10*)

For small-scale expression of single-domain antibodies

1a. Use a single colony of a positive clone from Basic Protocol 5 to inoculate 2 ml of B2YT-Amp medium (100 µg/ml ampicillin) in a 15-ml conical centrifuge tube. Incubate the cell suspension in a rotary shaker at a shaking speed of 200 rpm at 37°C overnight.

2a. Centrifuge the cells the following day for 10 min at 2000 × *g*, room temperature. Resuspend the pellet in 2 ml of fresh B2YT-Amp medium.

3a. Add 0.5 ml of the pre-culture to 50 ml of B2YT-Amp medium in a 250-ml Erlenmeyer flask. Incubate the pre-culture at 37°C in a rotary shaker at a shaking speed of 200 rpm overnight until the OD_{600} reaches approximately 0.3 to 0.5 as measured using a cell density meter.

4a. Add IPTG to achieve a final concentration of 0.1 mM. Then, incubate the culture at 37°C with shaking at 200 rpm overnight.

5a. Centrifuge the cells the following day for 20 min at 3400 × *g*, 4°C.

6a. Extract the protein from the periplasm as described in steps 5a to 7a, below.

For large-scale expression of single-domain antibody

1b. Use a single colony of a positive clone from Basic Protocol 5 to inoculate 25 ml of LB-Amp medium. Incubate the cell suspension at 37°C in a rotary shaker with shaking at 240 rpm, overnight.

2b. Transfer the entire overnight culture into 1 liter of M9 medium that has been supplemented with 100 µg/ml ampicillin. Incubate the culture at room temperature with shaking at 180 rpm for 30 hr. Supplement the culture with 100 ml of 10× induction medium and 100 µl of 1 M IPTG and incubate for another 60 hr.

3b. Retain a small aliquot for SDS-PAGE (Support Protocol 5; also see *UNIT 8.4*) and western blotting (immunoblotting; Support Protocol 5; also see *UNIT 8.10*). Centrifuge the remaining culture 20 min at 5000 × *g*, 4°C in a high-speed centrifuge. Keep the supernatant fraction at 4°C.

4b. Resuspend the pellet in 150 ml of wash solution. Centrifuge 10 min at 14,000 × *g*, 4°C. Keep the supernatant fraction at 4°C.

The pellet can then be subjected to periplasmic (steps 5a to 7a) or cytosolic (steps 5b to 13b) protein extraction.

For periplasmic extraction of single-domain antibodies

5a. Resuspend the pellet in 50 ml of sucrose solution and incubate it at room temperature for 10 min. Then, centrifuge 45 min at 14,000 × *g*, 4°C. Store the supernatant fraction at 4°C.

6a. Resuspend the pellet in 50 ml of ice-cold shock solution. Next, incubate it in an ice bath for 10 min. Then, centrifuge 25 min at 14,000 × *g*, 4°C. Keep the supernatant fraction at 4°C.

7a. Verify whether or not the antibody has been expressed by detecting the sdAbs in the fractions that were collected during the steps that are described above. Use an anti-His_6 tag antibody for western blotting (Support Protocol 5; also see *UNIT 8.10*). Pool the fractions that contain sdAb and dialyze them against 6 liters of starting buffer overnight at 4°C. Use an MWCO 8000 dialysis membrane.

For cytosolic extraction of single-domain antibodies

5b. Resuspend the pellet in 100 ml of ice-cold lysis buffer. Keep everything on ice during this process and store at −20°C until further use.

6b. Add 1 ml of 100 mM PMSF to the −20°C frozen suspension to achieve a final concentration of 1 mM and 200 µl of 1 M DTT to achieve a final concentration of 2 mM. Thaw the frozen suspension at room temperature while at the same time occasionally shaking it.

Alternatively, complete EDTA-free protease inhibitor cocktail (Roche) can be used.

7b. Lyse the bacteria by adding 5 ml of freshly prepared 3 mg/ml lysozyme solution to achieve a final concentration of 100 µg/ml.

8b. Incubate the mixture at room temperature for 30 to 50 min while occasionally shaking it until the suspension becomes viscous.

9b. Add 200 to 300 µl of DNase I (15 U/µl stock in 1 M $MgCl_2$).

10b. Incubate the lysate at room temperature for an additional 20 to 30 min until the suspension becomes watery. Save 30 µl of the mixture for western blotting (Support Protocol 5; also see *UNIT 8.10*).

11b. Separate the soluble and insoluble fractions of the lysates by centrifuging the mixture for 20 min at 14,000 × *g*, 4°C. Continue centrifuging until the supernatant becomes clear. Save 30 µl for western blotting (Support Protocol 5; also see *UNIT 8.10*).

12b. Resuspend the pellet in 80 ml of ice-cold lysis buffer and keep it at −20°C. Save 30 µl of the re-suspended pellet for western blotting. Repeat steps 6b to 11b if the pellet contains a lot of protein. This can be checked by western blotting (Support Protocol 5; also see *UNIT 8.10*).

13b. Dialyze the supernatant obtained from step 11b overnight against starting buffer using an MWCO 8000 membrane. Then, filter the dialysate through a 0.22-µm pore size membrane.

14. Whether you have performed the a or b steps, proceed with protein purification (Basic Protocol 7).

BASIC PROTOCOL 7

PURIFICATION OF SINGLE-DOMAIN ANTIBODIES

Affinity purification based on the tag sequences attached to either N-terminal or C-terminal of recombinant proteins including antibody fragments is an efficient strategy which is widely practiced both in research field and for industrial applications. The 6x histidine tag (6xHis tag) is the most common tag used for this purpose; commercially available and easy-to-use magnetic beads and column chromatography have been developed for using this strategy.

Materials

Pure Proteome Nickel Magnetic Beads (Millipore)
Lysis buffer (see recipe)
Cell lysate, dialyzed and filtered (Basic Protocol 6)
Wash solution: 10 mM Tris·Cl, H 8.0 (*APPENDIX 2A*) containing 150 mM NaCl
Elution buffer (see recipe)
5 mg/ml $NiCl_2$
Starting buffer (see recipe)
Phosphate-buffered saline (PBS; see recipe)
Sodium azide (Thermo Scientific Pierce)

Pure Proteome Magnetic Stand (Millipore)
10-ml glass collection tubes (Fisher Scientific, cat. no. 14-961-26)
5 ml HiTrap Chelating HP column (GE Healthcare)
ÄKTA FPLC purification system (GE Healthcare)
Dialysis tubing with MWCO 8000 (Biodesign Inc., *http://biodesignofny.com*
0.2 µm GP Express Plus Membrane filtration system (Millipore)
ND-1000 spectrophotometer (Thermo Scientific; also see Gallagher and Desjardins, 2008) or a similar instrument

Additional reagents and equipment for SDS-PAGE (Support Protocol 5; also see *UNIT 8.4*), dialysis (*APPENDIX 3H*), and microvolume spectrophotometry (Gallagher and Desjardins, 2008)

For small-scale purification by using PureProteome nickel magnetic beads

Pure Proteome Nickel Magnetic Beads represent an efficient and time-saving method for small-scale purification of single-domain antibodies. This protocol is for purifying 1 ml of prepared lysate. However, it can be adjusted for the volume of lysate accordingly.

1a. Vortex the magnetic beads to re-suspend them.

2a. Aliquot 200 µl of the magnetic bead suspension into a 1.5-ml microcentrifuge tube.

Note that 200 µl of PureProteome Nickel Magnetic Bead suspension can bind at least 200 to 600 µg of His-tagged protein.

3a. Place the tube into the PureProteome Magnetic Stand to collect the beads. Carefully remove the storage buffer from the tube with a pipet.

4a. Resuspend the magnetic beads in 500 µl of lysis buffer. Gently mix them for 1 min at room temperature.

5a. Place the tube back into the magnetic stand and remove the buffer.

6a. Add 1 ml of the cell lysate to the magnetic beads and gently mix for 30 min at room temperature.

7a. Place the tube back into the magnetic stand and allow the beads to migrate to the magnet. Invert the tube to remove residual beads from the tube cap while the tube is still seated in the magnetic stand (alternatively, residual liquid can be removed from the cap by briefly centrifuging the tube). Capture the beads and remove the lysate.

8a. Wash the magnetic beads by incubating them in 500 µl of wash buffer while gently mixing them for 1 min at room temperature.

9a. Place the tube back into the magnetic stand and allow the beads to migrate to the magnet. Remove the wash buffer.

10a. Repeat steps 8a and 9a two more times.

11a. Elute the bound protein by adding 100 µl of elution buffer. Incubate the solution while gently mixing it for 2 min at room temperature.

12a. Place the tube back into the magnetic stand. Allow the beads to migrate to the magnet. Then, transfer the eluted fraction into a clean 1.5-ml microcentrifuge tube.

13a. Repeat elution steps 11a and 12a once more.

The first eluent will contain the majority of the recombinant protein. If desired, both eluents can be combined. However, this will result in a lower protein concentration overall.

For large-scale purification on a HiTrap column

Immobilized metal affinity chromatography (IMAC) is a single-step and highly efficient method for the purification of His-tagged proteins. Single-domain antibodies with purity of ≥90% are easily obtained using this methodology. Commercial 1- to 5-ml HiTrap Chelating HP columns from GE Healthcare are routinely used in our laboratory.

1b. Charge the column with Ni^{2+} by applying 30 ml of 5 mg/ml $NiCl_2$ solution. Then, wash the column with 15 ml of deionized water.

2b. Calibrate the column by using 15 ml of starting buffer.

3b. Purify the protein by using an ÄKTA FPLC instrument according to the manufacturer's instructions. Load the column with the dialyzed and filtered protein extract at a rate of 1 ml/min. Wash the column with starting buffer that has been supplemented with 10 mM imidazole, which will remove proteins that are nonspecifically bound to the column. Once a stable baseline is obtained, elute the bound protein by running a program that uses a 10 to 500 mM continuous imidazole gradient in 10 mM HEPES buffer containing 500 mM NaCl (continuous gradient from starting to elution buffer). Collect fractions of eluted proteins.

4b. Analyze the fractions that correspond to the elution peaks on the chromatogram to determine the presence and purity of the VHHs using SDS-PAGE (Laemmli, 1970; Support Protocol 5; also see *UNIT 8.4*). Pool the sdAb fractions and dialyze them (*APPENDIX 3H*) extensively against PBS using an 8000 MWCO dialysis

membrane. Measure the OD_{280} by using a Nanodrop instrument to determine the protein concentration by using molar extinction coefficients (Pace et al., 1995). Then, add sodium azide to give a final concentration of 0.02% and store the sdAbs at 4°C.

SUPPORT PROTOCOL 5

SDS-PAGE AND WESTERN BLOTTING

Materials

70% ethanol for cleaning glass plates of gel-casting apparatus
30% polyacrylamide (BioRad)
1.5 M (pH 8.8) and 0.5 M (pH 6.8) Tris·Cl (*APPENDIX 2A*)
10% (w/v) sodium dodecyl sulfate (SDS) in H_2O
10% (w/v) ammonium persulfate (APS) in H_2O
TEMED (Life Technologies)
Isopropanol (ACP Chemicals, Inc., *http://www.acpchem.com/*)
10× electrophoresis running buffer: 30.35 g Tris, 141.75 g glycine, and 10 g of SDS per liter of water
4× SDS loading buffer (see recipe)
Coomassie blue dye: combine 50 ml isopropyl alcohol, 20 ml acetic acid, 50 mg of Coomassie blue dye, and 130 ml of water
Destaining solution: 10% (v/v) acetic acid, 30% (v/v) methanol, and 60% (v/v) water
1× transfer buffer: 3.03 g Tris hydrochloride, 14.41 g glycine, 40 ml methanol, H_2O up to 1 liter
Phosphate-buffered saline (PBS; see recipe)
PBST: PBS (see recipe) containing 0.1% (v/v) Tween 20
MPBS blocking buffer: 2% (w/v) skim milk in PBS
Mouse anti-His_6 primary antibody (GE Healthcare)
Alkaline phosphatase–conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch)
AP-conjugated substrate kit (BioRad)
Electrophoresis apparatus: e.g., Protean II 16-cm cell (BioRad) or SE 600/400 16-cm unit (Hoefer) with clamps, glass plates, casting stand, and buffer chambers
Spacers
Teflon comb with 1, 3, 5, 10, 15, or 20 teeth
100°C water bath or heat block
Immobilon PVDF membrane (Millipore)
Whatman 3MM filter paper
Trans-Blot SD (BioRad) transfer apparatus for semi-dry western blotting (also see *UNIT 8.4*)
Additional reagents and equipment for electrophoresis (*UNIT 8.4*) and immunoblotting (western blotting; *UNIT 8.10*)

Perform electrophoresis

UNIT 8.4 includes detailed protocols on gel electrophoresis.

1. Use alcohol and Kimwipes to clean the glass. Then, set up the rest of the apparatus according to the manufacturer's instructions (also see *UNIT 8.4*).
2. 2. Prepare two 12.5% resolving gels by combining 3.1 ml of 30% acrylamide, 3 ml of 1.5 M Tris·Cl, pH 8.8, 1.3 ml of distilled, deionized water, 50 µl of 10% SDS, 36 µl of 10% APS, and 5 µl of TEMED.
3. Invert the tube gently after adding all of the components.

4. Pour the mixture into the gel apparatus up to the correct level.
5. Add isopropanol on top of the acrylamide mixture and wait until the gel has polymerized.
6. Remove the isopropanol, wipe the top of the gel apparatus using filter paper, and insert a comb into to the top of the apparatus.
7. Make stacking gel by combining 1 ml of 30% acrylamide, 630 µl of 0.5 M Tris·Cl pH 6.8, 3.6 ml distilled deionized water, 25 µl of 10% SDS, 25 µl of 10% APS, and 5 µl of TEMED. Gently shake the solution. Then, add the stacking gel on top of each resolving gel and let it polymerize.
8. Set up the gel in the tank and make sure that the buffer is not leaking.
9. Pour in 1× running buffer to prevent the gel from drying out.
10. For each sample, use 4× loading dye for 1/3 of the total volume. For example, for a 40 µl sample, add 10 µl of 4× loading dye.
11. Heat each sample in a 100°C water bath or heat block for 5 min. Microcentrifuge briefly before loading on to the gel.
12. Run the gel by setting the current to 20 to 30 mA. Check that the molecular markers separate when the current is flowing.
13. Take the gel and soak it in Coomassie blue dye for 1 hr at room temperature.
14. Destain the gel until the protein bands can be seen clearly by using destaining solution.

Perform western blotting (immunoblotting)

UNIT 8.10 includes detailed protocols on immunoblotting.

15. For western blotting, wet the PVDF membrane in methanol and rinse with distilled water. Immerse the gel, filter paper, and nitrocellulose in transfer buffer. Stack a piece of Whatman 3MM filter paper, transfer membrane, the gel, and another piece of filter paper. Place in the transfer apparatus.

    The proteins in SDS are negatively charged

16. Set the power supply to run at 15 V for 20 min.
17. Take the membrane and wash it four times with PBS after the transfer has been completed.
18. Incubate the membrane in MPBS blocking buffer for 1 hr at room temperature.
19. Wash the membrane three times with PBS and add 10 ml of total primary antibody (mouse anti-His_6 antibody) diluted 1: 3000 in MPBS blocking buffer. Incubate for 1 hr at room temperature.
20. Wash the membrane three times, each time for 10 min with PBST.
21. Apply 1:5000 diluted secondary (AP conjugated goat-anti mouse) for 30 min.
22. Wash the membrane five times with PBST, each time for 5 to 10 min.
23. Add 100 µl each of solution A and solution B in 10 ml of AP substrate buffer (all items from BioRad AP-conjugated substrate kit) to the membrane to develop it. Keep the membrane in the dark for ~20 min but check it occasionally.
24. Stop the reaction by rinsing the membrane with water.

BASIC PROTOCOL 8

GEL-FILTRATION/SIZE-EXCLUSION CHROMATOGRAPHY

Size-exclusion chromatography (SEC) with a Superdex 75 column is used to analyze sdAbs to determine whether they are monomeric or if there are any aggregates present. This step can also be considered as being an extra purification step and allows a buffer exchange to be carried out, which is needed for SPR analysis or CD measurement. Non-aggregating sdAbs give a single, symmetrical peak and is observed for the majority of sdAbs of VHH origin.

Materials

Distilled, deionized H_2O, filtered and degassed
Phosphate-buffered saline (PBS; see recipe), filtered and degassed
IMAC-purified VHHs (Basic Protocol 7)

Superdex 75 size- exclusion column (GE Healthcare)
ÄKTA FPLC purification system (GE Healthcare)

1. Wash a Superdex 75 size exclusion column with 50 ml of filtered and degassed distilled, deionized water, and equilibrate it with 50 ml of filtered and degassed PBS at a pump speed of 0.5 ml/min.
2. Inject and elute 200 µl of purified sdAb VHH and observe the chromatogram.

Some variations may be observed in the elution volumes of monomeric VHH fractions.

It is a rare event to observe aggregating VHHs, which may display asymmetric peaks due nonspecific VHH interaction with the column material.

The graphing software GraphPad Prism (version 4.02 for Windows; GraphPad Software, http://www.graphpad.com) is recommended for chromatographic data analysis and presentation.

BASIC PROTOCOL 9

INCREASING FUNCTIONAL AFFINITY AND POTENCY OF VHHs

VHHs provide excellent opportunities for generating small bivalent/bispecific molecules. This can be accomplished by direct genetic fusion of two VHH domains using a synthetic flexible linker such as Gly/Ser (Huston et al., 1988). It has been demonstrated that bifunctional or trimeric versions of VHH molecules show increased functional affinity (avidity) and strong potency in terms of neutralizing viruses (Hultberg et al., 2011). Bispecific VHHs may also have important applications in cancer therapy, where one antibody arm binds to cancer a cell and the other arm, for example, recruits a T cell to the tumor environment (Holliger and Hudson, 2005). In this protocol, two VHHs of the same or different specificities are linked by a Gly/Ser linker that is introduced through a PCR overlap extension method. The assembled fragments that encode the two VHH domains are cloned into an expression vector. Upon induction in *E. coli*, the dimeric protein is directed to the periplasmic space with the OmpA leader signal, where folding and disulfide bridge formation are facilitated with the aid of bacterial folding machinery.

Materials

5 ng/µl pSJF2-VHH1 plasmid
5 ng/µl pSJF2-VHH2 plasmid
10 pmol/µl Primers: M13RP, M13 FP, DIMBACK1, and DIMFOR1 (see Table 2.17.2)—primers were purchased from Integrated DNA Technology (IDT)
Restriction enzymes *Eco*RI and *Hin*dIII (New England Biolabs)
B2YT medium (see recipe)

Additional reagents and equipment for amplification of VHH genes (Basic Protocol 5), expression of single-domain antibodies in bacterial systems (Basic Protocol 6), and DNA sequencing (*UNIT 10.25*)

Construction of dimeric VHHs by overlap extension PCR

All of the cloning steps are performed essentially as described elsewhere (Sambrook et al., 1989; Ausubel et al., 2013). The VHH genes that are used here were identified as being binders for an antigen of interest. They were isolated from a phagemid library by panning, as described elsewhere (Arbabi Ghahroudi et al., 2009a). Before carrying out this experiment, make sure that there are no internal *Eco*RI or *Hin*dIII restriction enzyme sites in both VHH genes that are used for the assembly reaction. Otherwise, alternative restriction enzyme sites must be introduced in the 5′-end and 3′-end primers for cloning. In bispecific/bivalent VHH formats, the order of the VHHs may have a significant effect on their expression and functional binding. Therefore, it is recommended to perform PCR overlap extension for both VHH1-VHH2 and VHH2-VHH1.

1. Amplify the VHH genes from the pSJF2-VHH1 and pSJF2-VHH2 plasmid vectors (see Basic Protocol 5) in two separate PCR reactions in a total volume of 50 µl by using M13R or DIMFOR1 and DIMBACK1 and M13F primers, respectively. Prepare each PCR reaction mix with the following ingredients:

   5.0 µl 10× PCR buffer
   1.0 µl 10 mM dNTPs
   0.5 µl 10 pmol/µl M13R/M13F
   0.5 µl 10 pmol/µl DIMFOR1/DIMBACK1
   0.5 µl 5 ng/µl pSJF2-VHH1/pSJF2VHH2
   0.5 µl 5 U/µl Expand *Taq* DNA polymerase
   39 µl H_2O.

   For the bivalent construct, only one VHH (pSJF2-VHH1 or pSJF2-VHH2) template will be used in the above and subsequent PCR reaction.

   Place the reaction tubes into a thermal cycler and perform PCR by using the following program:

1 cycle:	5 min	94°C	(initial denaturation)
30 cycles:	30 sec	94°C	(denaturation)
	30 sec	55°C	(annealing)
	30 sec	72°C	(extension)
1 cycle:	7 min	72°C	(final extension).

   The Gly/Ser linker will be introduced at the 3′-end of the first VHH (VHH1) and 5′-end of the second VHH (VHH2) by the two DIMFOR1 and DIMBACK1 primers, respectively. Moreover, the amplified flanking regions at both the 5′-end and 3′-end of the two PCR fragments contain EcoRI and HindIII restriction enzyme sites that are pre-existing in the pSJF2 vectors.

2. Purify the VHH-linker genes in a final volume of 50 µl of water by using a QIAquick PCR Purification kit.

3. Set up the second overlap extension PCR with the following ingredients:

   5.0 µl 10× PCR buffer
   1.0 µl 10 mM dNTPs
   1.5 µl 50 ng/µl VHH1-linker (from step 2)
   1.5 µl 50 ng/µl VHH2-linker (from step 2)
   0.5 µl 5 U/µl Expand *Taq* DNA polymerase
   37.5 µl H_2O.

1 cycle:	5 min	94°C	(initial denaturation)
10 cycles:	30 sec	94°C	(denaturation)
	30 sec	55°C	(annealing)
	30 sec	72°C	(extension).
1 cycle:	7 min	72°C	(final extension).

Then, add 1 µl of an equimolar mixture (5 pmol each) of M13R and M13F to each PCR reaction tube and continue the PCR:

25 cycles:	30 sec	94°C	(denaturation)
	30 sec	55°C	(annealing)
	30 sec	72°C	(extension).
1 cycle:	7 min	72°C	(final extension).

4. Purify the VHH1-linker-VHH2 fragments (~1100 bp in length) in a final volume of 50 µl of water by using a QIAquick PCR Purification kit.

5. Perform a double-digestion of the purified DNA with *Eco*RI/*Hin*dIII restriction endonucleases for 2 hr at 37°C. Then, purify the PCR fragments in a final volume of 50 µl of water using a QIAquick PCR Purification kit.

6. Ligate the cut fragment into *Eco*RI/*Hin*dIII-digested pSJF2 expression vector by using the LigaFast Rapid Ligation System.

   This results in the addition of C-terminal c-Myc and His_6 tags to the protein.

7. Transform 50 µl of electrocompetent TG1 cells with 3 µl of the ligated material using a MicroPulser electroporator or equivalent instrument. Transfer the electroporated cells into a tube that contains 1 ml of SOC medium and incubate it for 1 hr at 37°C with shaking at 180 rpm.

   Alternatively, this procedure can be performed by using chemically prepared competent cells with a 90-sec heat shock at 42°C (Sambrook et al., 1989).

8. Spread 100 µl of the cell suspension on to LB-Amp plates and leave the plates with their lids half open for about 5 min in a laminar-flow hood. Cover, invert, and incubate the plates overnight at 32°C.

9. The following day, perform colony PCR as described in Basic Protocol 5 using the M13RP and M13FP primers. Determine the size of the amplified product by gel electrophoresis using a 1% agarose gel.

   The positive clones that contain the dimeric VHH genes should have a size of around 1100 base pairs.

10. Confirm the positive clones by further sequencing their VHH genes (*UNIT 10.25*) using M13RP and/or M13FP as primers.

11. Express the dimeric clones and extract the periplasmic space proteins by using the osmotic-shock procedure as described in Basic Protocol 6.

BASIC PROTOCOL 10

PREPARATION OF VHH Fc FUSION

Single-domain antibodies, being small molecules, have several desirable properties. However, for some functional aspects of the antibody, the lack of the Fc region could make them less attractive as therapeutic reagents than IgGs. Furthermore, the serum half-life of a sdAb is very short (10 to 15 min; Gainkam et al., 2008; Bell et al., 2010). By making an Fc-fused sdAb, the molecule still remains smaller (approximately 80 kDa) than a whole IgG (~150 kDa), but gains the functions of the desired Fc domain—e.g., human, murine, etc. In addition, the bivalent nature of an Fc fused sdAb can lead to increased functional affinity (Zhang et al., 2009). The following protocol describes the

construction, expression in the HEK-293 cell line, and purification by protein A column of an antigen-specific VHH fused to a mouse Fc fragment, which is called chimeric mouse heavy chain single domain antibody (cmHCAb).

Materials

Construct containing sdab gene (Basic Protocol 5, step 8)
Primers: FC-FW, FC-RE, pTT5-mFc7, and pTT5-mFc8 (see Table 2.17.2)—the primers were purchased from Integrated DNA Technology (IDT).
Restriction enzymes: *Bsr*GI and *Apa*I (New England Biolabs)
pTT5-mFc vector (Zhang et al., 2009)
QIAquick PCR purification kit (Qiagen)
2xYT-Amp plates: 2×YT plates (see recipe) containing 100 µg/ml ampicillin
Maxiprep plasmid extraction kit (Qiagen)
HEK-293 cells (Dr. Yves Durocher, National Research Council Canada)
L FreeStyle F17 medium (Life Technologies): add 10 ml of 10% (100×) Pluronic F-68 stock, 10 ml of 200 mM (100×) L-glutamine stock, and 10 ml of 100× antibiotic/antimycotic stock per liter of Freestyle F17 medium just prior to use
Phosphate-buffered saline (PBS; see recipe)
Polyethyleneimine (PEI; Polyplus, *http://www.polyplus.com/*)
Trypton N1 (TN1, Organotechnie S.A., cat. no. 19553; *http://www.organotechnie.com/*): make 20% stock solution in prewarmed Freestyle F17 culture medium just prior to use
Goat anti-mouse HRP conjugate (Cedarlane)

MicroPulser electroporator (BioRad) or equivalent electroporation device
Disposable Erlenmeyer 125-ml plastic shaker flasks (Corning, cat no. 43143)
5-ml HiTrap protein A HP column (GE Healthcare)
0.22-µm sterilization filter (e.g., Millipore)
0.45 um Steritop filter unit with 500 ml filtrate capacity (Millipore)

Additional reagents for PCR (as described in Basic Protocol 5, step 1) and colony PCR (as described in Basic Protocol 5, step 7), counting cells and determining cell viability (*APPENDIX 3A & 3B*), purification of sdAb on a protein A column (Support Protocol 2), SDS-PAGE (Support Protocol 5; also see *UNIT 8.4*), ELISA (Support Protocol 1), and SPR (Support Protocol 7)

1. Amplify the sdAb gene by PCR by using the primers FC-FW and FC-RE and the PCR conditions described in Basic Protocol 5. Then, purify the PCR product using a QIAquick PCR Purification kit and digest it with the restriction enzymes *Bsr*GI and *Apa*I.
2. Digest pTT5-mFc with restriction enzymes *Bsr*GI and *Apa*I, and purify the vector by using a QIAquick PCR purification kit.
3. Ligate the digested PCR products to the digested pTT5-mFc vector using the LigaFast Rapid DNA Ligation System and transform the ligated material into electrocompetent *E. coli* TG1 cells using a MicroPulser electroporator or an equivalent instrument. Then, plate the cell suspension on a 2YT-Amp plate and incubate overnight.
4. Perform colony PCR (using the conditions described in Basic Protocol 5, step 7) to check for the insert using pTT5-mFc7 and pTT5mFc8 primers (Table 2.17.2). Then, confirm the sequences by sequencing the clones that contain the insert using the same primers.

5. Purify enough plasmid by using a Maxiprep kit, and filter sterilize using a 0.22-µm filter before use.

   We use at least 500 µg of plasmid DNA.

6. Thaw one vial of HEK-293 cells rapidly in a 37°C water bath plate in 20 ml of supplemented FreeStyle F17 medium in a disposable 125-ml plastic flask and incubate at 37°C in a 5% CO_2 incubator.

7. Check the cell viability (*APPENDIX 3A & 3B*) the next day. Dilute the cells to a concentration of 0.8 to 1.2 million/ml in supplemented Freestyle F17 medium.

8. For transfection, grow the cells to ~0.8 to 1.2 million/ml on the day of transfection and check their viability (*APPENDIX 3A & 3B*; viability should be > 95%).

9. Use 1 µg of plasmid DNA/ml of culture (e.g., for 500 ml culture use 500 µg of DNA).

10. Dilute the DNA in PBS totaling 5% of the culture volume (e.g., for 500 ml culture dilute 500 µg DNA in 50 ml of PBS).

11. Add 0.75 µl of PEI (1 mg/ml solution) per 1 µg of DNA to be transfected directly via the PBS solution. Vortex the mixture three times, each time for 10 sec.

12. Leave for 15 min at room temperature. Then, add the mixture to the cells, swirl, and put the cells back into the incubator at 37°C.

13. The next day, add 20% Trypton N1 to make its final concentration 0.5%.

14. Let the cells grow for 5 days before harvesting them.

15. Harvest the culture supernatant by centrifugation for 15 min at 800× *g*, room temperature.

    The supernatant contains the secreted Fc fused sdAb.

16. Purify the Fc fused sdAb on a protein A column essentially as described in Support Protocol 2, step 6 except that the cleared medium is degassed and filtered (using 0.45-µm filter) before applying on protein A column. Load the medium on a 5-ml HiTrap HP protein A column at a rate of 5 ml/min. After washing the column with 25 ml of PBS at 5 ml/min, elute the cmHCAb protein with 50 ml glycine buffer, pH 3.6, collect fractions of 3 ml each, and neutralize with 1 ml Tris·Cl, pH 8.8.

    Recipes for the glycine and Tris buffers are included in the Support Protocol 2 materials list.

17. Analyze the protein A–eluted fractions using SDS-PAGE as described in Support Protocol 5.

18. Analyze the functional binding of the Fc-fused VHH by ELISA (Support Protocol 1; use goat anti-mouse HRP conjugate as the detection antibody) and SPR (Support Protocol 7).

    The ratio of DNA/PEI can be different for different proteins; it can be optimized if needed.

    The yield of Fc-VHH fusion varies and depends on the VHH sequence. We generally obtain 5 to 20 mg/l in a shake flask.

PENTAMERIZATION OF VHHs

SUPPORT PROTOCOL 6

Fusion of sdAbs to the pentamerization domain of verotoxin (VT), also known as Shiga-like toxin, has been shown to be a highly effective means of enhancing binding to

immobilized antigen; these pentavalent sdAbs have been termed pentabodies (Zhang et al., 2004a).

Materials

dNTPs: 10 mM dATP, dCTP, dGTP, dTTP (New England Biolabs)
10× PCR buffer (Hoffmann-La Roche)
Primers: VT-*Bbs*I-f and VT-*Apa*I-r (see Table 2.17.2)——purchased from Integrated DNA Technology (IDT).
Plasmid template containing sdAb gene (Basic Protocol 5, step 8)
Expand High-Fidelity *Taq* DNA polymerase (Hoffmann–La Roche)
500 ng/µl pVT2 vector (Zhang et al., 2004b)
Restriction enzymes *Bbs*I and *Apa*I (New England Biolabs)
NEBuffer 4 (New England Biolabs)
QIAquick Gel Extraction Kit (Qiagen)
LigaFast Rapid DNA Ligation System (Promega)
Electrocompetent TG1 *E. coli* cells (see Support Protocol 4)
LB medium (see recipe)
LB-Amp plates: Prepare LB agar (see recipe) and add filter-sterilized ampicillin at a final concentration of 100 µg/ml when agar medium has cooled to ~50°C, but before pouring plates
Mouse anti-VT antibody (National Research Council Canada)
Goat anti mouse–alkaline phosphatase (Jackson Immunoresearch)
Alkaline phosphatase substrate (BioRad Laboratories)

Thermal cycler
ND-1000 spectrophotometer (Thermo Scientific; see Gallagher and Desjardins, 2008)
16°C water bath
MicroPulser electroporator (BioRad) or equivalent electroporation device
Water bath or incubator with rotary shaker

Additional reagents and equipment for PCR (*UNIT 10.20*), microvolume quantitation of DNA (Gallagher and Desjardins, 2008), agarose gel electrophoresis (*UNIT 10.4*), DNA sequencing (*UNIT 10.25*), and SDS-PAGE and western blotting (Support Protocol 5)

1. Amplify the sdAb gene by using primers with *Bbs*I and *Apa*I restriction enzyme sites so that the amplicon will have these restriction sites on the 5′- and 3′-ends of the gene, respectively. Prepare the following reaction mix:

   4 µl 2.5 mM dNTPs
   5 µl 10× PCR buffer
   0.5 µl each 10 pM primer
   1 µl 1 ng/µl plasmid template
   0.5 µl 3.5 U/µl Expand *Taq* DNA polymerase
   39 µl distilled deionized H_2O.

   Perform PCR using the following thermal cycling program:

1 cycle:	5 min	94°C	(initial denaturation)
30 cycles:	30 sec	94°C	(denaturation)
	30 sec	55°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	7 min	72°C	(final extension).

   Detailed protocols for PCR are included in UNIT 10.20.

2. Digest pVT2 with *Bbs*I and *Apa*I:

   6.0 µl 500 ng/µl pVT2 (500 ng/µl)
   2.0 µl NEBuffer 4
   1.0 µl 50 U/µl *Apa*I
   11 µl distilled deionized H_2O.

   Allow *Apa*I to digest the DNA for 2 hr at 25°C. Then, add 1 µl of *Bbs*I (20 U/µl) and let the *Bbs*I digest the DNA overnight at 37°C.

3. Digest the PCR product with *Bbs*I and *Apa*I:

   6.0 µl 500 ng/µl PCR product
   2.0 µl NEBuffer 4
   1.0 µl 50 U/µl *Apa*I (50 U/µl)
   11.0 µl distilled deionized H_2O.

   Perform the digestion as for the vector (see step 2).

4. Gel purify the digested vector and the PCR product using a QIAquick Gel Extraction kit. Measure the DNA concentration using a ND-1000 spectrophotometer (Gallagher and Desjardins, 2008).

5. Prepare the following reaction mix to ligate the PCR product into pVT2:

   4.0 µl ~100 ng/µl cut pVT2 (from step 2)
   4.0 µl (~50 ng/µl) cut PCR product (from step 3)
   1.0 µl 10× T4 DNA ligase buffer (from LigaFast kit)
   1.0 µl 1 U/µl T4 DNA ligase (from LigaFast kit).

   Allow the ligation reaction to proceed for 1 hr at room temperature or for 4 hr at 16°C.

6. Transform 50 µl of *E. coli* TG1 electrocompetent cells with 0.5 to 1 µl of the ligation mixture using a MicroPulser or equivalent instrument. Immediately afterward, add 1 ml LB medium and incubate at 37°C with shaking at 180 rpm for 1 hr. Plate 2, 10, and 100 µl of the culture on an LB-Amp. Incubate the plates at 32°C overnight.

7. The next day, perform colony PCR as described in Basic Protocol 5 (step 7) and check for the insert size by agarose gel electrophoresis (*UNIT 10.4*).

   Use the same primers for colony PCR as were used in step 1 of this protocol.

8. Isolate plasmids from clones with PCR products of ~400 bp and confirm the DNA sequences (*UNIT 10.25*).

9. Use the clones with correct sequences for protein expression and purification as described in Basic Protocol 6.

10. Analyze the IMAC fractions by using SDS-PAGE and western blotting as described in Support Protocol 5. Use mouse anti-VT for detection by western blotting. Detect the primary antibody using goat anti mouse–alkaline phosphatase and an alkaline phosphatase substrate.

BASIC PROTOCOL 11

CHARACTERIZATION OF ANTIGEN-SPECIFIC VHHs

Characterization of the purified recombinant VHHs in terms of specific binding to their target antigens is mainly performed by ELISA, whereby pure antigens are passively adsorbed onto the plastic wells. After sufficient blocking of the plastic surfaces with a proper blocking reagent such as BSA or nonfat dry milk, purified VHH fractions are

added and incubated for 1 to 2 hr. Specific binding of the VHHs is detected using a mouse anti-6His tag monoclonal antibody after proper washing away of nonspecific binders and other possible contaminants.

Materials

Purified antigen (100 µg/ml stock)
Phosphate-buffered saline (PBS; see recipe)
Purified VHH solution (Basic Protocol 7, step 4b)
PBST: PBS (see recipe) containing 0.05% Tween 20
Anti-His_6 mouse monoclonal antibody (GE Healthcare, cat. no. 27-4710-01)
HRP substrate solutions for ELISA (GE Healthcare)
ELISA plates: 96-well MaxiSorp microtiter plates (VWR)

Additional reagents and equipment for blocking ELISA plate (see Basic Protocols 3 and 4) and ELISA procedure (Support Protocol 1)

1. Coat ELISA plate with antigen (typically 2 to 5 µg/ml in PBS).
2. The next day, block each well with buffer B as described in Basic Protocols 3 and 4.
3. Remove the blocking agent, wash each well five times, each time with 300 µl PBST, and pat dry.
4. Add 100 µl of VHH protein solution. Incubate the wells for 1.5 hr at room temperature.
5. Add 100 µl/well of HRP-conjugated anti-His_6 mouse monoclonal antibody (1:5000 dilution).
6. Discard the unbound antibody and proceed with detection of the antibody (see Support Protocol 1).

Background binding is frequently encountered in ELISA and can be due to a number of reasons including insufficient blocking or the nature of blocking reagent, or non-optimal amounts of primary and/or secondary antibodies.

SURFACE PLASMON RESONANCE (SPR) AND EPITOPE MAPPING

SUPPORT PROTOCOL 7

Standard ELISA methods can be employed to determine the binding specificities and approximate affinities of sdAb-antigen interactions. However, accurate binding affinities and information on the kinetics of binding can be determined by surface plasmon resonance (SPR) analyses (also see *UNIT 18.1*). In a typical SPR experiment, one of the two binding molecules is immobilized on a surface. The other binding partner is then injected at different known concentrations. The binding of the partner increases the mass of the bound material on the surface, and this mass difference can be detected and used to calculate the binding affinity. The same method, with slight modifications, can used to analyze whether two antibodies bind to the same epitope or to a different epitope. In this assay, first, one antibody is injected onto the surface with the immobilized antigen on the surface. Once saturation is achieved, the second antibody is injected. If both of the antibodies bind to the same epitope, injection of the second antibody does not give an increase in signal. On the other hand, if they bind to different epitopes, a signal increase will be seen with the injection of the second antibody. The affinities of the two antibodies can influence the outcome of the experiment. Therefore, it is critical to analyze which antibody should be injected first.

Materials

HBS-E buffer: 10 mM HEPES (pH 7.4) containing 150 mM NaCl, and 3 mM EDTA (this buffer can be purchased from GE Healthcare; if it is not purchased from GE Healthcare, it should be thoroughly degassed before use)
Surfactant P20 (GE Healthcare)

IMAC-purified VHH protein (Basic Protocol 7, step 4b)
Protein assay kit
Amine coupling kit that contains *N*-hydroxysuccinimide (NHS), *N*-ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), and ethanolamine (GE Healthcare)
Antigen (same antigen used for llama immunization in Basic Protocol 1)
Immobilization buffer: 10 mM sodium acetate buffer, pH 4.0 (GE Healthcare, cat. no. BR 100349)
sdAb-Fc fusion (Basic Protocol 10)
sdAb pentabodies (Support Protocol 6)
10 mM phosphate buffer, pH 7.3: dissolve 1.4 g Na_2HPO_4 and 0.24 g KH_2PO_2 (pH should be 7.3) in 1 liter of distilled H_2O, filter-sterilize, and store at room temperature.
Superdex 75 10/300 GL and Superdex 200 10/300 GL gel filtration columns (GE Healthcare; also see Basic Protocol 8)
BIACORE 3000 (GE Healthcare) or another surface plasmon resonance instrument with similar capabilities
CM5 sensor chips (GE Healthcare)
BIAevaluation software 4.1 (GE Healthcare)
10-mm quartz cuvette
A J-850 CD spectrometer (JASCO)
GraphPad Prism software (*http://www.graphpad.com/*)
Additional reagents and equipment for size-exclusion chromatography (Basic Protocol 8) and determination of circular dichroism (CD) spectrum (Pain, 2005)

1. Isolate non-aggregated monomeric sdAbs prior to SPR analysis by Superdex 75 size exclusion column chromatography (Basic Protocol 8). Equilibrate the columns with 2 to 5 column volumes of HBS-E buffer that contains 0.005% surfactant P20 at a pump speed of 0.5 ml/min. Then, inject 200 µl of IMAC-purified protein and collect the monomer peak fractions. Following this, determine the protein concentrations.
2. Carry out SPR experiments at 25°C by using a BIACORE 3000 instrument with HBS-E that contains 0.005% surfactant P20 as the running buffer.
3. Activate the CM-dextran surface on a CM5 sensor chip with a 7-min injection of a mixture of 50 mM NHS and 200 mM EDC at a flow rate of 5 µl/min. Inject 50 µg/ml antigen that has been diluted in immobilization buffer (10 mM sodium acetate buffer, pH 4.0) for 3 min and block the surface with a 7-min injection of 1 M ethanolamine (pH 8.5).

   The surface density should be high enough to achieve maximum binding valency if the objective is to determine the highest functional affinity of bivalent or multivalent antibody.

4. Analyze the sdAb interaction with antigen by using an appropriate reference surface. Inject suitable amounts of six or more different concentrations of the sdAb, sdAb-Fc fusion, or pentabody over both the active and reference surfaces at a flow rate of 50 µl/min to collect a data set for K_D determination.
5. Analyze the data by using BIAevaluation software 4.1. Calculate the K_Ds for sdAb binding to antigen by steady-state affinity fitting and/or from rate constants.

Epitope mapping

6. Isolate non-aggregated monomeric sdAbs prior to SPR analysis by Superdex 75 size exclusion chromatography (Support Protocol 8). Equilibrate the column with 2 to 5 column volumes of HBS-E buffer that contains 0.005% surfactant P20 at a pump speed of 0.5 ml/min. Then, inject 200 µl of IMAC-purified protein and collect the monomer peak fractions. Finally, determine the protein concentrations.

7. Carry out SPR experiments at 25°C by using a BIACORE 3000 instrument with HBS-E that contains 0.005% surfactant P20 as the running buffer.
8. Activate the CM-dextran surface on a CM5 sensor chip with a 7-min injection of a mixture of 50 mM NHS and 200 mM EDC at a flow rate of 5 µl/min. Inject 50 µg/ml antigen that has been diluted in 10 mM acetate buffer (pH 4.0) for 3 min and block the surface with a 7-min injection of 1 M ethanolamine (pH 8.5).
9. Inject 80 µl of the first VHH that has been diluted in HBS-E buffer that contains 0.005% surfactant P20 to a concentration of 20× its K_D at 40 µl/min.
10. Inject the second VHH (80 µl total volume, 20× K_D) at 40 µl/min over the antigen surface that has already been saturated with the first VHH.
11. Repeat the experiment for all possible paired combinations of the VHHs in both orientations.
12. Analyze the data for competitive or noncompetitive binding

T_m measurement

As a routine practice in our lab, we measure the T_m of each sdAb, which is an indicator of its thermostability. The T_m measurement is performed by analyzing the circular dichroism (CD) spectrum (Pain, 2005) of the sdAb.

13. Perform a buffer exchange (if needed) to have the sdAb in 10 mM phosphate buffer (pH 7.0) by performing Superdex 75 size exclusion chromatography.
14. Collect the sdAb fraction and use the pure protein for CD analysis.
15. Collect the CD spectrum from 250 to 200 nm at protein concentrations between 2.5 to 5 µM using a 10-mm quartz cuvette and a J-850 CD spectrometer.
16. Collect data with a bandwidth of 1.0 nm and a scanning speed of 50 nm-min^{-1} with two scans to determine the CD profile.
17. For T_m determination, use the same conditions but do a single scan.
18. Measure the CD spectra at 2°C intervals from 25° to 91°C to determine the thermal denaturation of the protein at a temperature shift speed of 1°C-min^{-1}.
19. Plot the ellipticity of the proteins at 217 nm against the temperature and calculate the melting temperature (T_m) by using the Boltzmann Sigmoidal equation and GraphPad Prism software.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***APPENDIX 5****.*

B2YT medium

16 g Bacto tryptone,
10 g Bacto yeast extract,
5 g NaCl,
4 ml glycerol
12.32 g of K_2HPO_4
2.22 g KH_2PO_4
Add H_2O to 1 liter
Sterilize by autoclaving
Store up to 1 month at room temperature

Elution buffer

10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) buffer, pH 7.0
500 mM imidazole
500 mM NaCl
Store up to 1 month at room temperature

Induction medium, 10×

12 g Bacto tryptone,
24 g Bacto-yeast extract
4 ml glycerol
Add H_2O to 100 ml
Sterilize by autoclaving
Store up to 1 month at room temperature

LB agar

10 g Bacto tryptone
5 g Bacto yeast extract
10 g NaCl
15 g of Bacto-agar
1 liter H_2O

Sterilize by autoclaving. Before pouring the medium into the plates, allow it to cool to 50°C. To make LB-Amp plates, add filter-sterilized ampicillin at a final concentration of 100 µg/ml after the plates have cooled to 50°C but before pouring the plates.

Luria-Bertani (LB) medium

10 g Bacto tryptone,
5 g Bacto yeast extract,
10 g NaCl
Add H_2O to 1 liter
Sterilize by autoclaving
Store up to 1 month at room temperature

Lysis buffer

50 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
25 mM NaCl
2 mM EDTA
Store up to 1 month at 4°C

M9 minimal medium and plates

100 ml 10× M9 salts (see recipe)
900 ml H_2O
Sterilize by autoclaving and store up to 2 months at 4°C
Add the following ingredients before use:
1 ml of filter-sterilized 1 M $MgCl_2$
0.1 ml of 1 M $CaCl_2$
5 ml of 1 mg/ml thiamine-HCl (vitamin B1)
10 ml of 20% (w/v) glucose
20 ml of 20% (w/v) casamino acids

To prepare M9 plates, add 15 g/liter Bacto-agar just before autoclaving; add supplements after cooling (below 50°C) and pour plates immediately.

M9 salts, 10×

60 g Na_2HPO_4,
30 g K_2HPO_4,
10 g NH_4Cl
5 g NaCl
Add H_2O to 1 liter
Sterilize by autoclaving
Store up to 1 month at room temperature

PEG/NaCl

20% (v/v) PEG 8000
146.1 g NaCl
Add H_2O to 1 liter
Autoclave to sterilize
Store at 4°C for up to 3 months

Phosphate-buffered saline (PBS)

10 mM Na_2HPO_4
2 mM KH_2PO_4
137 mM NaCl
2.7 mM KCl
pH should be 7.4
Autoclave to sterilize if required
Store at room temperature for up to 6 months

SDS loading buffer, 4×

4 ml glycerol
0.4 g SDS
5 ml 1.5 M Tris·Cl, pH 6.8 (*APPENDIX 2A*)

Add water to 10 ml and add bromphenol blue to a concentration of 0.2% (w/v), such that a 1× solution is dark enough to permit easy monitoring of the gel when it is being run. Just before use, add 50 µl of 2-mercaptoethanol per ml of buffer.

SOC medium

First, prepare SOB medium:
20 g Bacto tryptone (BD Difco)
5 g Bacto yeast extract (BD Difco)
0.5 g NaCl
Adjust pH to 7.0
Add H_2O to 1 liter
Sterilize by autoclaving
Store up to 1 month at room temperature or 3 months at 4°C
SOC medium: SOC medium is made immediately prior to use by adding 1 ml filter-sterilized 1M $MgCl_2$, 1 ml filter-sterilized 1M $MgSO_4$, and 1 ml of filter-sterilized 2 M glucose per 100 ml SOB medium.

Starting buffer

10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) buffer, pH 7.0
500 mM NaCl
Store up to 3 months at room temperature

Tris-Acetate-EDTA (TAE) buffer, 50× stock solution

242 g Tris base
57.1 ml glacial acetic acid

Add distilled water to 600 ml and stir until dissolved. Then, add 100 ml 0.5 M EDTA, pH 8.0 (*APPENDIX 2A*) and distilled water to a final volume of 1 liter. Store up to several months at room temperature.

1× working solution: Dilute 1 part 50× stock with 49 parts water. Store the working solution at room temperature up to several weeks.

2xYT medium and plates

2xYT medium:
16 g tryptone
10 g yeast extract
5 g NaCl
Add H_2O to 1 liter

Autoclave to sterilize. Store in 100 ml-bottles at room temperature for up to 1 month. Add selective antibiotics if required: e.g., 100 µg/ml ampicillin, added from 100 mg/ml stock (filter sterilized and stored in 1-ml aliquots at –20°C) or 50 µg/ml kanamycin (added from 50 mg/ml stock, filter sterilized and stored in 1-ml aliquots at –20°C) after the medium has cooled below 50°C. Also add nutritional supplement (e.g., glucose) after the medium temperature is below 50°C.

2xYT plates: 15 g agar per liter of 2xYT medium (see above). Autoclave, cool to ~55°C, add ampicillin if required to a final concentration of 100 µg/ml, pour plates, and store at 4°C for up to 1 month.

COMMENTARY

Background Information

Antibody engineering techniques and phage-display technology were established in the early 1990s and resulted in the generation and isolation of antibody fragments such as Fabs and scFvs, which were shown to be similar to their parental counterpart, namely, immunoglobulin G (IgG) in terms of antigen-binding properties. However, the construction of antibody-fragment libraries was a major challenge, since it required the genetic assembly of antibody domains by using a synthetic DNA linker in the case of scFvs or the separate cloning of individual heavy and light chains followed by in vivo assembly in *E. coli* in the case of Fabs. The instability of antibody libraries and unpredictable expression patterns of the constructs initiated parallel efforts to look for less complicated, smaller, binding domains. Initially, Sally Ward and her colleagues at the Medical Research Council (MRC) in the U.K. examined the binding properties of a VH domain against lysozyme that had been isolated from a mouse VH library and, found them to have nM-range affinity (Ward et al., 1989). However, the solubility of such domain antibodies was of major concern due to their exposed hydrophobic VH-VL interfaces. The discovery of heavy-chain antibodies in *Camelidae* by Raymond Hamers and his colleagues in 1993 (Hamers-Casterman et al., 1993) revitalized the idea of single-domain antibodies. It was subsequently shown that these naturally occurring antibodies have variable domains that are uniquely positioned to serve as single-domain antibodies (Desmyter et al., 1996; Arbabi Ghahroudi et al., 1997). Their structural features, which are genetically inherited with heavy-chain antibodies, include key amino acid substitutions at the VL-VH interface (V37F/Y, G44E, L45R, and W47G) and the exclusion of CH1 domains from the heavy chain by RNA splicing. These modifications permit the natural existence of the VHH domain as the sole paratope of heavy-chain antibodies. By applying genetic engineering techniques, Arbabi Ghahroudi and colleagues at the Vrije Universiteit Brussel (Free University of Brussels) in Belgium showed that VHH domain libraries could be constructed from the heavy-chain antibody repertoire of an immunized dromedary and displayed on the

surface of filamentous phages. The abbreviation VHH was first coined in 1993-1994 by the research team to distinguish the heavy-chain antibody variable domain from the conventional VH (Arbabi Ghahroudi, 1996). By panning, specific phage antibodies were enriched against two target antigens, and VHH binders of lysozyme (cAbLy2 and cAbLys3) and tetanus toxoid (cAb-TT1 and cAb-TT2) were isolated and characterized for the first time (Arbabi Ghahroudi, 1996; Desmyter et al., 1996; Arbabi Ghahroudi et al., 1997). The abbreviation cAb was initially suggested by the same research team for camelid VHHs to distinguish them from conventional domain antibodies or dAbs. These findings established the concept of domain antibodies as the smallest known antigen-recognition molecules, an idea that was initially suggested by Ward et al. (1989). Bacterial expression, purification, and characterization of antigen-specific VHH-domain antibodies revealed that VHHs maintain a typical immunoglobulin fold (β–pleated sheet folding), behave essentially as monomers with little or no tendency to aggregate even at micromolar concentrations, have affinities in the nanomolar range, and are quite specific for their target antigens as shown by SPR experiments.

One of the most intriguing questions that arose following the discovery of heavy-chain antibodies was the nature of the antigen:VHH complex. In fact, the crystal structure of cAbLys3 at high resolution shed light on yet another important feature of these nano-size domains (a roughly cylindrical structure of about 2.5 nm in diameter and 4 nm in height). Surprisingly, it was shown that a longer than average CDR3 loop of cAbLys3 (24 amino acids in length) penetrated into the active site of lysozyme, a cavity that was previously assumed to be inaccessible to conventional antibodies (De Genst et al., 2006b). Additionally, an extra disulfide bridge between CDR1 and CDR3 stabilized the long CDR3 loop; the average CDR3 loop in mouse and human VHs is between 9 and 12 residues, while in *Camelidae* it was found to be 17 amino acids (Conrath et al., 2003). The longer than average CDR3 loop length, extension of the CDR1 loop, and, consequently, adaptation of novel canonical conformations (different from the known canonical structures observed in human or mouse VHs), as well as additional hotspots for somatic hypermutations in CDR1 and a higher rate of hypermutation during affinity maturation, are thought to compensate for limitations in the paratope repertoire of heavy-chain antibodies that are due to the absence of the VL domain (Muyldermans et al., 2009).

The potential in vivo application of VHHs as toxin neutralizers was also shown in animal models, where mice were challenged with $10LD_{50}$ doses of tetanus toxin alone or pre-incubated with anti-tetanus toxoid VHHs (cAb-TT1 and cAb-TT2). Preliminary data showed that mice that received 40 μg of cAb-TT1 were protected over a period of 5 days (Arbabi Ghahroudi, 1996). These studies, as well as results from a number of other investigations (for a review see Muyldermans, 2001; Ewert et al., 2002; Harmsen et al., 2007; Wesolowski et al., 2009a), concluded that VHH single-domain antibodies have a number of unique and exclusive features including: (a) small size and monomeric nature; (b) ease of engineering and genetic fusion; (c) high levels of expression in bacteria and yeast (in the range of milligrams to grams per liter of cultured bacteria or yeast); (d) exquisite target specificity and high affinity (low nanomolar to picomolar) for protein targets; (e) high solubility, up to micromolar concentrations; (f) outstanding stability under non-physiological conditions such as high temperature, high pressure, extreme pH, and the presence of proteases and denaturing reagents; and, most uniquely, (g) recognition of enzyme cavities and cryptic/hidden epitopes on cell surfaces that are inaccessible to conventional antibodies.

Conventional antibodies

A typical IgG antibody consists of two heavy chains (H) and two light chains (L) (Padlan, 1994). The N-terminal domains of both H- and L-chains are variable and are called the variable region (Williamson, 1976), abbreviated as VH and VL. The constant domains are abbreviated CH and CL. L-chains contain only one CL domain, whereas CH comprises three to four domains numbered as CH1, CH2, and so on. The variable domains are not uniformly variable. They are composed of relatively invariant regions, called framework regions or FR, of about 15 to 30 amino acids, separated by shorter, 9 to 12 amino acid–long regions, which are hypervariable (Kabat et al., 1978). The VH and VL both contain three such hypervariable regions (Wu et al., 1975). These hypervariable regions form the antigen-binding site of the antibody (Kabat et al., 1976), and are also called complementary-determining regions (CDRs) (Padlan, 1996). The $(CH2\text{-}CH3)_2$ make up the Fc domain of antibodies, which recruits cytotoxic effector functions.

Heavy-chain antibodies

Hamers and co-workers have demonstrated that the sera of the *Camilidae* family (camels, llamas, and dromedaries) contain a substantial proportion (ranging between 35% to 70% of the total IgG) of antibodies that do not have light chain (Hamers-Casterman et al., 1993). These antibodies are homodimers of two heavy chains, so they are called heavy-chain only antibodies (HCAb). These antibodies have a molecular weight of ~95 kDa instead of ~150 kDa, the molecular weight of conventional antibodies. In HCAbs, the CH1 domain, which in conventional antibodies interacts with light chain, is absent, explaining the absence of light chain (Padlan, 1994; Arbabi Ghahroudi et al., 1997).

These unique HCAbs interact with the antigen by a single variable domain, referred to as VHH (for variable domain of the heavy-chain of HCAb) instead of the paired VH and VL domains for conventional antibodies. This discovery has made it possible to isolate soluble and functional VHH-sdAbs (Arbabi Ghahroudi et al., 1997). An sdAb is the smallest naturally available intact antigen-binding fragment harboring the full antigen-binding capacity of the original HCAbs, and have evolved to be fully functional in the absence of light chain. Despite the absence of the VH-VL combinatorial diversity, these sdAbs exhibit a broad antigen-binding capability.

In fact, the reduced complexity of the VHH paratope, which has three instead of six antigen binding loops, allows these antibodies to adopt new canonical structures like protruding loops, broadening the repertoire of the antigen-binding site (Transue et al., 1998). When the VHs of conventional antibodies and the VHHs of HCAb are compared, some very significant differences are observed in the amino acid sequence. In VH, there are some amino acids that are involved in CH1-VL interactions, and they are well conserved, whereas in VHH these amino acids are substituted to avoid the possible interaction with VL. Leu11 of VH (in Kabat numbering; Kabat et al., 1991) is substituted with Ser11 in camel VHH. Furthermore, three other amino acids, (Gly44, Leu45, and Trp47), which are crucial for hydrophobic interaction with VL, are substituted by more hydrophilic amino acids (Glu44, Arg45 or Cys45 and Gly47), making interaction with VL unlikely. Moreover, VHHs often possess two extra Cys compared to VHs, one in CDR3 and another in CDR1 (or in framework-2 at position 45), and these extra Cys can form an inter-loop disulfide bridge.

Advantages of sdAbs

The single-domain nature of sdAbs provides some special characteristics compared to antigen-binding fragments derived from conventional antibodies. The favorable characteristics of sdAbs include (i) easy cloning and selection of high-affinity binders, (ii) small size (~15 kDa), (iii) high expression yield and easy purification, (iv) strict monomeric behavior, (v) high stability at low/high pH and high temperature, (vi) low to no immunogenicity in humans, (vii) ability to target regions that are less antigenic (such as active sites of enzymes and cavities on the surface of viruses, bacteria and parasites) compared to conventional antibodies, and (viii), finally, easy ability to be tailored with toxins against cancer cells or infectious agents (Revets et al., 2005). Moreover, because of their small size, the sdAbs (i) can recognize some cryptic epitopes, which are not accessible to larger antibody molecules (Stijlemans et al., 2004; Baral et al., 2006), (ii) can penetrate tissues and solid tumors or pass through the blood-brain barrier more rapidly than whole antibodies (Cortez-Retamozo et al., 2002; Abulrob et al., 2005), (iii) have a faster clearance rate from the blood circulation (Bell et al., 2010), which leads to differences of selectivity, and (iv) are also promising molecular-recognition units for generating more complex bivalent (Els Conrath et al., 2001), pentavalent (Zhang and Mackenzie, 2012; Zhang et al., 2004b), and bispecific constructs or immunofusions for tumor targeting and drug delivery (Cortez-Retamozo et al., 2004). Together, these features make tsdAbs very attractive for biotechnological, diagnostic, and therapeutic applications (Cortez-Retamozo et al., 2002, 2004; Tanha et al., 2002)

Generation and production of recombinant antigen-specific sdAbs

Being a single-chain fragment, sdAbs are easy to retrieve from a VHH library from an animal immunized with a single antigen or with multiple antigens. There are two ways of acquiring functional sdAbs. One is to obtain naïve or non-immune sdAbs (Tanha et al., 2002), which can be isolated quickly from a naïve or nonimmune VHH library but which often have low to medium affinities. The other way is to acquire immune sdAbs (Li et al., 2009), which usually have high affinities but necessarily require a lengthy immunization and library construction process. A schematic drawing with the workflow, starting with immunization of a llama, through library

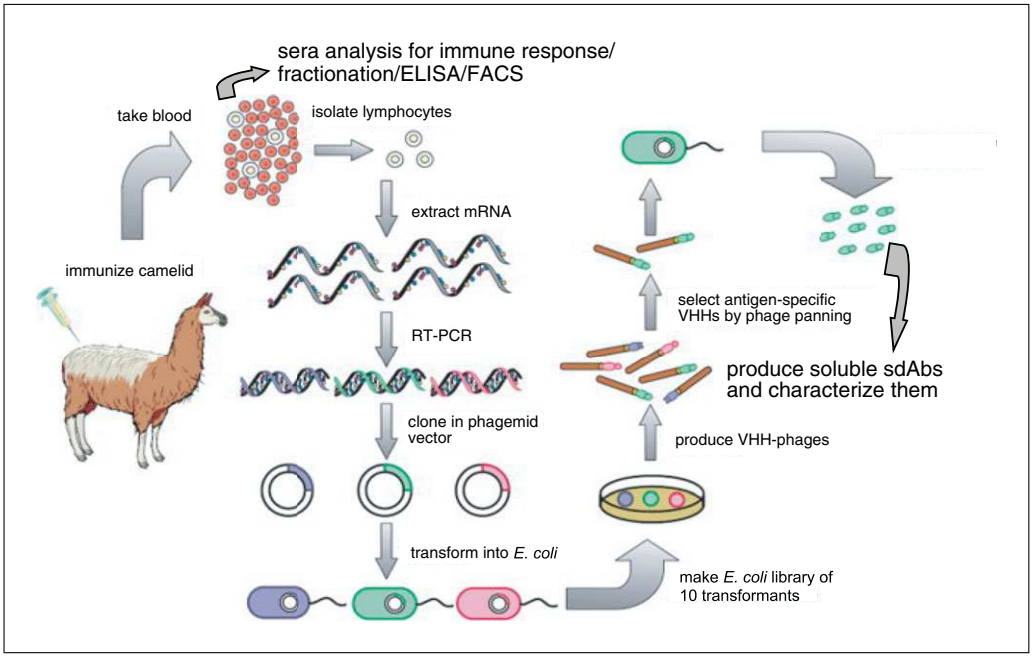

Figure 2.17.4 Schematic drawing of immunization, library construction, and panning.

construction and panning, to isolate a binding sdAb is presented in Figure 2.17.4. Such an immune library is useful only for isolating sdAbs against the few antigens which were used to immunize the llama. Different strategies have been employed for broadening the utility of the library, such as whole-cell immunization (Baral et al., 2011) experimental infection (Saerens et al., 2008), whereby sdAbs have been successfully isolated without the use of pure antigen in the immunization. A general immunization protocol is described in Basic Protocol 1. Routinely, to make a VHH library, a pool of peripheral blood lymphocytes is used, and by reverse transcription cDNA is synthesized using total RNA/mRNA that has been isolated from the peripheral blood lymphocytes. A detailed protocol for library generation is described in Basic Protocol 2. Such a VHH library can be screened for the presence of antigen-specific binders by biopanning (Arbabi Ghahroudi et al., 1997; Kretzschmar and von Ruden, 2002). A methodology for such panning is described in Basic Protocol 3. Once the appropriate binders have been selected, they can be further produced in bacteria or fungal or other eukaryotic expression systems (van der Vaart, 2002; Joosten et al., 2003; Agrawal et al., 2012; Baral and Arbabi Ghahroudi, 2012), including plants (Ismaili et al., 2007). In order to obtain soluble sdAbs, the selected VHHs are re-cloned into an expression vector (Conrath et al., 2001; Baral and Arbabi Ghahroudi, 2012). Typical sdAbs expression yields are between 5 and 20 mg per liter bacterial culture (Arbabi Ghahroudi et al., 1997). Similar types of sdAbs that are derived from human VHs or VLs (Jespers et al., 2004b; To et al., 2005) are particularly promising for their potential use in immunotherapy because of their human origin. However, the solubility of these human sdAbs is one of the main problems with them. Several approaches have been reported to obtain soluble VH or VL sdAbs (To et al., 2005; Arbabi Ghahroudi et al., 2009a; Agrawal et al., 2012). However, the generation and isolation of the VH- or VL-based sdAbs are out of the scope of this unit.

Since the pioneering work on camelid heavy-chain antibodies and the isolation of VHHs in the 1990s, there have been close to 1000 scientific publications (by the end of 2012) that cover a wide range of research on camelid and non-camelid sdAbs. These cover the genetic origin and evolutionary aspects of VHHs, the antigen-binding repertoire, germline database and somatic hypermutation, structural properties and domain characterization, and expression of VHHs in a wide range of prokaryotic and eukaryotic hosts, as well as biotechnological and medical applications (see below). A number of excellent reviews in this field have elaborated on many aspects of the structural features and applications of sdAbs, and are suggested for further reading on this subject (Holliger and Hudson, 2005; Eyer and Hruska, 2012; Finlay and Almagro, 2012; Vincke et al., 2012).

Following the discovery and characterization of camelid heavy-chain antibodies and

VHH domains, several major developments have occurred in this field of research. First, Greenberg and colleagues identified a set of similar heavy chain–only antibodies in cartilaginous fish (Wobbegong and nurse sharks; Greenberg et al., 1995). It was then demonstrated that the new antigen-binding receptors of shark heavy-chain antibodies, termed V-NAR, mimic many features of camelid VHHs and are therefore attractive for bioengineering and therapeutic applications (Nuttall et al., 2001, 2004). Second, by using a camelid VHH as a model framework, mutations were introduced into a human VH (Davies et and Reichman, 1994) and rabbit VH (Aires da Silva et al., 2004) at key VH:VL contact residues (37, 44, 45, and 47 by Kabat numbering). The "camelization" of the selected human VH domains showed moderate improvements in its solubility and thermal stability without affecting antigen-binding capacity (Davies and Riechmann, 1994). Following a similar strategy, camelized synthetic libraries were created and panned against target antigens, and soluble VH binders to target antigens with micromolar binding affinity were identified (Martin et al., 1997; Tanha et al., 2001). In another development, sdAbs with sequence features of VH domains were isolated from *Camelidae* libraries and were shown to be stable and functional (Tanha et al., 2002; Monegal et al., 2009). Moreover, the existence of a new class of VH domains in mice (Zou et al., 2007) and llamas (Deschacht et al., 2010) that can exist as independent VHs or combine with VL domains was reported. This third development sparked a renewal in engineering efforts to isolate soluble and stable human domain antibodies (VHs and VLs). In this regard, a number of approaches have been taken, most notably: (i) the isolation of an independent VH domain from an scFv library from a melanoma patient (Cai and Garen, 1996); (ii) the selection of a VH scaffold with a unique VH/VL interface by searching for least dependency on the VL domain for generation of a semi-synthetic library (Reiter et al., 1999); and (iii) the use of an intercellular antibody capture (IAC) method (Tanaka and Rabbitts, 2003) and a combination of heat denaturation and protein A selection (Jespers et al., 2004a) as an innovative methodology to select non-aggregating human sdAbs from a multivalent phage-display library. In a different approach, surface engineering of the VH:VL interface using conventional biophysical methods and phage-display technology led to the isolation of monomeric and soluble VHs with high thermostability and reversible unfolding characteristics (Barthelemy et al., 2008). Finally, To et al. (2005) developed a phage-selection method to isolate several dozen soluble human VH and VL frameworks. Subsequently, a semi-synthetic library with randomized CDR1 and CDR3 was constructed by using one of the VH scaffolds and the isolation of anti-amylase sdAbs with favorable biophysico-chemical properties was reported (Arbabi Ghahroudi et al., 2009b).

The resulting human domain antibodies from the above-mentioned studies were essentially monomeric and expressed well in bacteria with good solubility and binding properties despite lacking camelid hallmark residues. Nonetheless, it is certain that extensive engineering efforts are needed to isolate such domain antibodies with favorable biophysico-chemical properties that are suitable for medical or other biotechnological applications, which is a time-consuming and costly process and which may deter the biopharmaceutical industry from exploiting these molecules at the present time.

Sir Greg Winter, a pioneer of recombinant antibody technology predicted the "increasing future use of smaller antibodies" empowered by bispecific nature, conjugated with cytotoxic drugs and/or appendages to extend serum half-life (Winter, 2011). It is now a view commonly held by many investigators in this field of research that sdAbs (camelid, shark, and human) are a perfectly suited as part of the "smaller antibodies" group due to their aforementioned unique structural, biophysical, and biochemical properties (Holt et al., 2003; Wesolowski et al., 2009b; Vanlandschoot et al., 2011; Nuttall, 2012; Vincke et al., 2012). Large companies such as Merck Serono, Boehringer Ingelheim, and Novartis have already started to invest hundreds of millions of dollars in the development of empowered camelid single-domain antibodies as anti-cancer and anti-infectious diseases therapeutic reagents (Wesolowski et al., 2009b; also see Fierce Biotech, September 27, 2012; *http://www.fiercebiotech.com*)

Critical Parameters

Llama immunization and serum response monitoring

The *Camelidae* family (dromedaries, camels, llamas, and alpacas) harbor a substantial number of heavy-chain IgGs in their serum ranging from 45% (llamas) to 75% (dromedaries) of the total IgG amounts

(Daley et al., 2005; Maass et al., 2007). Secondly, hematological data on camelid sera showed that lymphocytes constitute a larger fraction of total white blood cells (leukocytes) in dromedaries compared with llamas (De Genst et al., 2006a). VHHs that are derived from dromedaries have on average a longer CDR3 region, frequently stabilized by an additional disulfide bond, than other camelid species. This is advantageous for isolating VHHs with enzyme inhibitor activities or with the ability to neutralize or block infectious reagents such as viruses, bacteria, or parasites (Baral et al., 2006; Wesolowski et al., 2009a). When immunizing for the purpose of isolating useful sdAbs against antigens of interest, factors such as the nature of the immunogens (e.g., antigen conservation), immunogenic potential, antigen purity, antigen stability, antigen quantity, and the route of immunization need to be considered. The health of animals and the number of boosts are other key factors that need to be considered.

VHH phage-display library construction from a heavy-chain antibody repertoire

As for the source of antibody-producing cells, lymph nodes, spleen, or blood are generally used as sources of lymphocytes for somatic fusion to a myeloma partner (hybridoma technology) or the construction of antibody libraries. Although blood is considered to be a less rich source of lymphocytes, it has been used as almost the only source of lymphocytes, because of minimum risk to the health of the animal, for VHH library construction, and is recommended as the source of lymphocytes. Isolated lymphocytes should be immediately subjected to RNA extraction or frozen in liquid nitrogen and stored at −80°C until further use. Upon the extraction of total RNA, cDNA is synthesized by using either random or oligo(dT) primers, and is then used as template in PCR reaction mixtures that contains camelid IgG-specific primers. The first set of 5′-end primers are designed based on pooled sequencing data of the VHH repertoire (Harmsen et al., 2000; Nguyen et al., 2000; Tanha et al., 2002; Arbabi Ghahroudi et al., 2009b), which shows considerable variability in the N-terminal part of the framework 1 region (FR1). On the contrary, the 3′-end primer is rather conserved, since there is little variation in the C-terminal part of the framework 4 region (FR4). Finally, after the first PCR, the heavy-chain gene repertoire is gel purified and used as a template for the amplification of VHH fragments, which are then used for library construction. Alternatively, heavy-chain antibody hinge-specific primers have been used in single-step PCR in some studies for the construction of VHH libraries (Maass et al., 2007). However, the possibility that these primers will not cover the entire heavy-chain antibody repertoire of the llama cannot be ruled out. The vector that is used for the library construction here is phagemid pMED1 (with ampicillin-resistance marker; Hoogenboom et al., 1993; Arbabi Ghahroudi et al., 2009a; Fig. 2.17.3A). It is the 33 or 3 + 3 vector/library system (Smith and Scott, 1993), in which bacteria that produce recombinant VHH-gene III are superinfected by a helper phage (e.g., M13KO7), that provides the wild-type pIII. VHH transcription is under the control of the *lacZ* inducible promoter and the fusion proteins are directed into the periplasmic space of *E. coli* by using the *pel*B leader signal sequence (Hoogenboom et al., 1991; Better et al., 1988). Another feature of the pMED1 phagemid vector is the presence of an amber stop codon between VHH and gene III, which allows expression of the VHH in both fusion form (by using TG1 SupE strain) or in soluble form (by using non-suppressor HB 2151 strain). The fact that the suppression power in TG1 is incomplete (only 20% to 30% efficient; Dai et al., 2003) and that the VHH is expressed at low levels by using the natural leakiness property of the *lacZ* promoter ensure monovalent display of VHH on the phage surface (Garrard et al., 1991; Griffiths and Duncan, 1998). However, more than 90% of the packaged phages particles harbor the phagemid genome, due to a mutational defect in the intergenic region (IG) of the M13 KO7 helper phage (Vieira and Messing, 1987). Alternatively, for the construction of naïve libraries, a type 3 phage vector (Smith and Scott, 1993) such as fd-tetM (Arbabi Ghahroudi et al., 2009a; Zacher et al., 1980) is used in which the recombinant VHH gene is cloned at the N-terminal of the wild-type gene and therefore five copies of VHH per phage are displayed (for construction of a camelid naïve library, see Kumaran et al., 2012).

In contrast to scFvs and Fabs, where combinatorial diversity plays a major role in generating a large and complex library, the size of a VHH library is directly correlated with the number of lymphocytes used to extract RNA, while taking into account that only 30% to 40% of the total IgG molecules are heavy-chain antibodies, which likely reflects the relative proportion of heavy-chain IgG-producing B

cells versus conventional antibody producers. There should be a reasonable correlation between the complexity of the library and the number of lymphocytes used for the extraction of RNA. We typically use around 10^6 to 10^7 leukocytes, and therefore a library size of 10^6 to 10^7 individual different clones is most likely attained. This is in agreement with the average sizes of the VHH libraries that are described in the literature.

It is important to calculate or estimate the complexity of the constructed library, which is determined by three different factors: (a) the size of the library (the number of colonies obtained after transformation by serial dilution); (b) the number of clones with correct VHH lengths; and (c) the number of clones with VHH unique sequences. The complexity of the VHH libraries following the abovementioned protocol is generally above 95% of all the individually tested colonies.

VHH phage-display library screening

Screening of a VHH library is yet another critical step in the isolation of optimal sdAbs. In principle, it mimics the process of in vivo antibody generation: antigen interaction between circulating B lymphocytes and, most frequently, an epitope of a foreign antigen followed by the clonal amplification of B cells with bound antigen in secondary lymphoid tissues. The end result is differentiation into two cell populations: antibody-producing plasma cells and memory cells. The latter cells undergo further affinity maturation upon encountering the same antigen, and therefore produce antibodies with much higher affinities (Winter et al., 1994). Similarly, individual phage particles display unique antibody sequences with the encoding VHH genes in their genomes. The antibody:antigen interaction may occur in a simple solid-phase ELISA well, in an immunotube, on the surface of streptavidin magnetic beads, or in situ, i.e., on the surface of cells cultured in vitro or as they exist in vivo. The critical point in using any platform strategy is to ensure that the antigen resembles its natural form as closely as possible, is in sufficient quantity, and is easily accessible to the pool of phage antibodies. Depending on the antigen nature, coating conditions, including the type of buffer that is used, need to be optimized. Phage antibodies need to be prepared with great care to avoid salt carry-over or phage aggregates. During each round of panning, accurate monitoring of bacterial growth upon infection with eluted phages is highly recommended, as bacterial cultures are prone to contamination from various sources, in particular, lytic phages that can get amplified easily yet are very difficult to eradicate. The panning cycle of the phage antibody:antigen interaction, phage elution, and amplification in *E. coli* TG1 are generally repeated three to four times. Phage enrichment (E), which is a reliable indicator for the success of panning, is calculated by using the following formula:

X1 = Output phages/Input phages × 100 (first round)
X2 = Output phages/Input phages × 100 (second round)
X3 = Output phages/Input phages × 100 (third round)
X4 = Output phages/Input phages × 100 (fourth round)
E1 = X2/X1, and E2 = X3/X2, and
E3 = X4/X3.

Final enrichment: $E_{\text{total}} = \text{E1} \times \text{E2} \times \text{E3}$, which varies between 10^3 to 10^6 and is determined by several factors including the nature of the immune response, the complexity of the library, and the success of panning.

The success of panning will eventually be determined by phage ELISA screening of individual phage antibodies that are produced by colonies isolated after the final round of panning. It is recommended to initially screen between 24 and 48 colonies, then extend the screening to 100 to 200 colonies if the antigen is available in sufficient quantity. Colony PCR is another technique by which one may monitor the panning progress, and also needs to be done on the colonies that are selected for phage ELISA. Finally, by sequencing the PCR products and analyzing the data, unique sequences can be identified and selected for subcloning and protein expression.

VHH subcloning, expression, and purification

The expression of sdAbs, in contrast to other antibody fragments such as Fabs and scFvs, is rather simple and straightforward. The single-domain nature of these antibodies fits in well with the *E. coli* expression system, where folding capability and disulfide-bridge formation capacity for eukaryotic proteins is limited (Arbabi Ghahroudi et al., 2005). VHH antibodies can either be expressed in the cytoplasm or can be directed to the periplasmic space of *E. coli* by using a bacterial leader signal. The latter strategy provides a better environment for domain folding and disulfide-bridge formation (Arbabi Ghahroudi et al.,

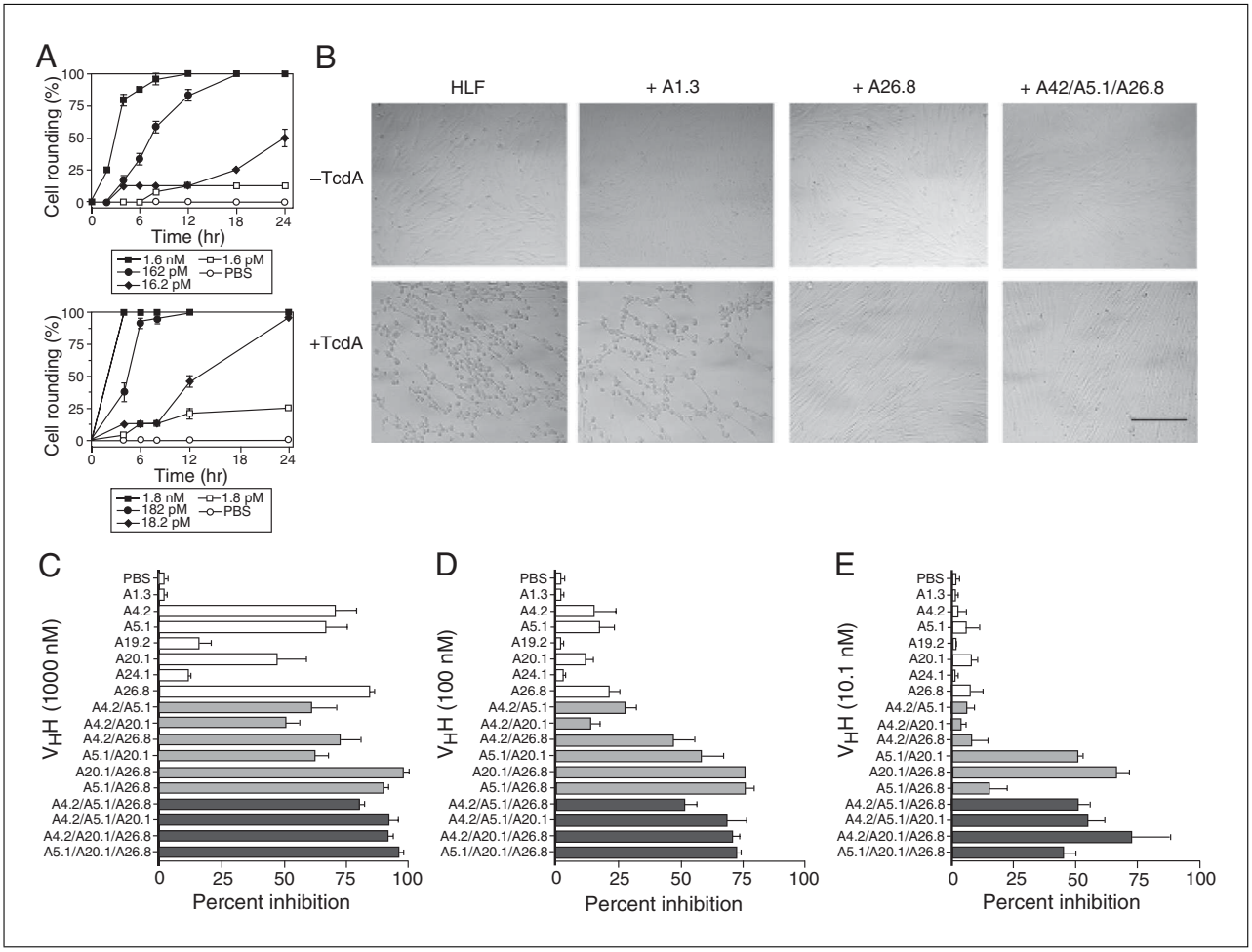

Figure 2.17.5 Single-domain antibodies as toxin neutralizers. (**A-B**) Percentage of HLF cell rounding was assayed in the presence or absence of TcdA and/or four sdAbs (A1.3, A26.8, A42 and A5.1). (**C-E**) Percentage of cell rounding inhibition (relative to cells receiving TcdA only) was measured at three different VHH concentration (1000, 10, 10 nM) and VHHs were used as singles, pairs, or triplet combinations. The efficacy of toxin neutralization is increased when a combination of VHH antibodies are used. Reprinted from Hussack et al. (2011) with permission for the American Society for Biochemistry and Molecular Biology.

2005). Moreover, the accumulated proteins are easily extracted using a simple osmotic-shock method. This has the advantage of minimal bacterial protein contamination, and consequently makes the purification process very easy to perform and quite efficient. Alternatively, VHHs can be produced in sufficient quantities in the bacterial cytoplasm and still be functional. However, for large-scale cytoplasmic expression, we recommend using T7-based expression vectors for cytoplasmic VHH accumulation as inclusion bodies, which can then easily be purified from other bacterial proteins. However, the VHH inclusion bodies need to undergo unfolding and refolding processes, which are complicated and time-consuming. In this chapter, a medium-level expression system for VHH production is presented and discussed. The vector pSJF2 (Baral and Arbabi Ghahroudi, 2012; Fig. 2.17.3B) is a pUC-derivative, ampicillin-resistant vector and has the following features: (a) a *lac* promoter that allows for the control of VHH expression; (b) an OmpA leader signal; and (c) c-Myc and His-tags at the C-terminal of the protein. By using this vector and an optimized expression protocol, VHH expression levels of 10 to 100 mg per liter of bacterial culture are routinely obtained in our laboratory.

Purification of recombinant VHHs is important for evaluating their interaction with antigen. Immobilized metal affinity chromatography (IMAC) is considered to be the most effective purification method and is widely used for purifying histidine-tagged recombinant proteins, including antibody fragments (for a review, see Block et al., 2009). A combination of periplasmic extraction followed by IMAC purification results in consistent yields (10 to 100 mg/liter) and purity (>90% or above) of VHH

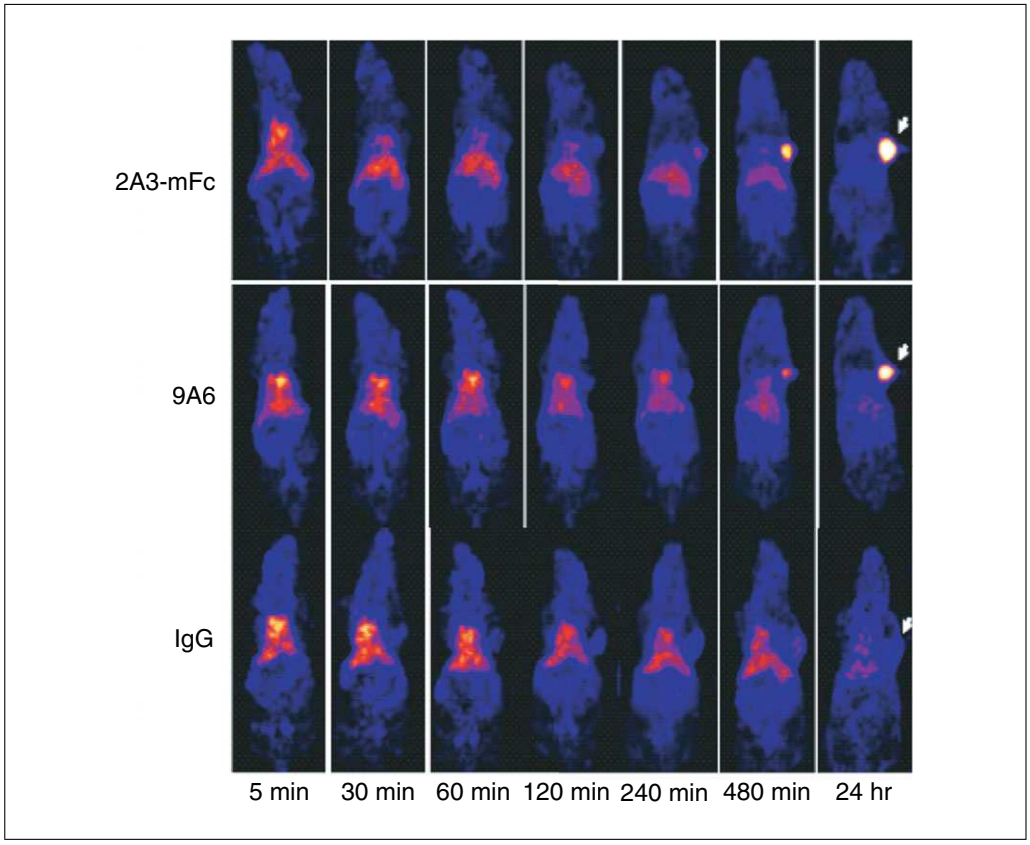

Figure 2.17.6 Single-domain antibodies as cancer diagnostic reagents. PET imaging of BxPC3 tumors with ^{64}Cu-2A3-mFc shows that 2A3-Fc has potential as a tumor-imaging agent. As controls, a CEACAM6-specific murine monoclonal antibody (9A6) with almost identical affinity (positive control) and a murine IgG (as negative control) were used. Reprinted from Niu et al. (2012) with permission from Elsevier.

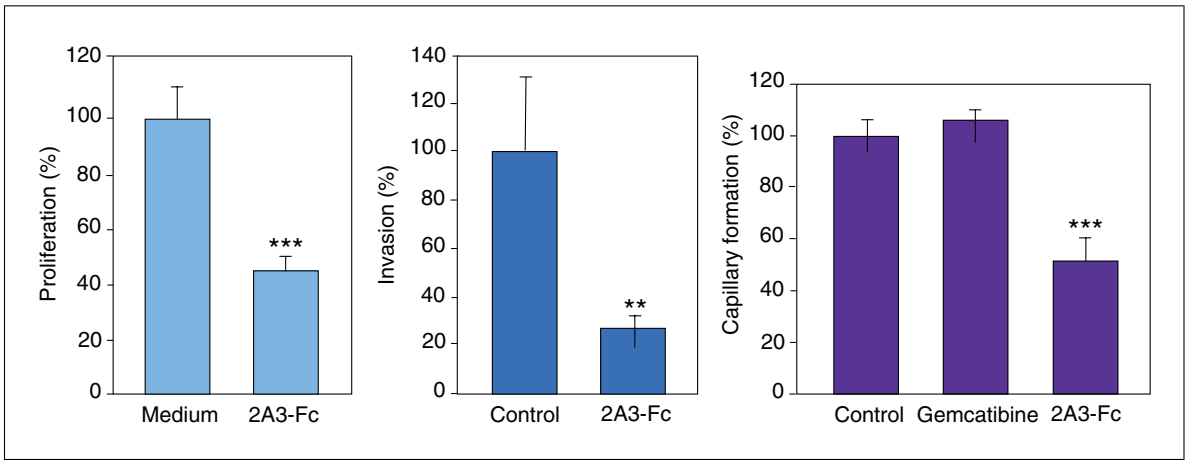

Figure 2.17.7 Single-domain antibodies as cancer therapeutic reagents. 2A3-Fc has an effect on proliferation, invasion, and capillary formation of endothelial cells. Reprinted from Cheng et al. (2012) with permission from Elsevier.

domains, yielding protein that is suitable for characterization.

VHH specificity and affinity determination by soluble ELISA and SPR

Characterization of purified VHH antibodies begins with solid-phase ELISA, and specific binding to the target antigen is often detected by anti-His tag antibodies. The molecular interaction and binding kinetics between VHHs and antigens are quantitatively determined by an optical technique called surface plasmon resonance (SPR), which detects changes in the mass upon binding of a

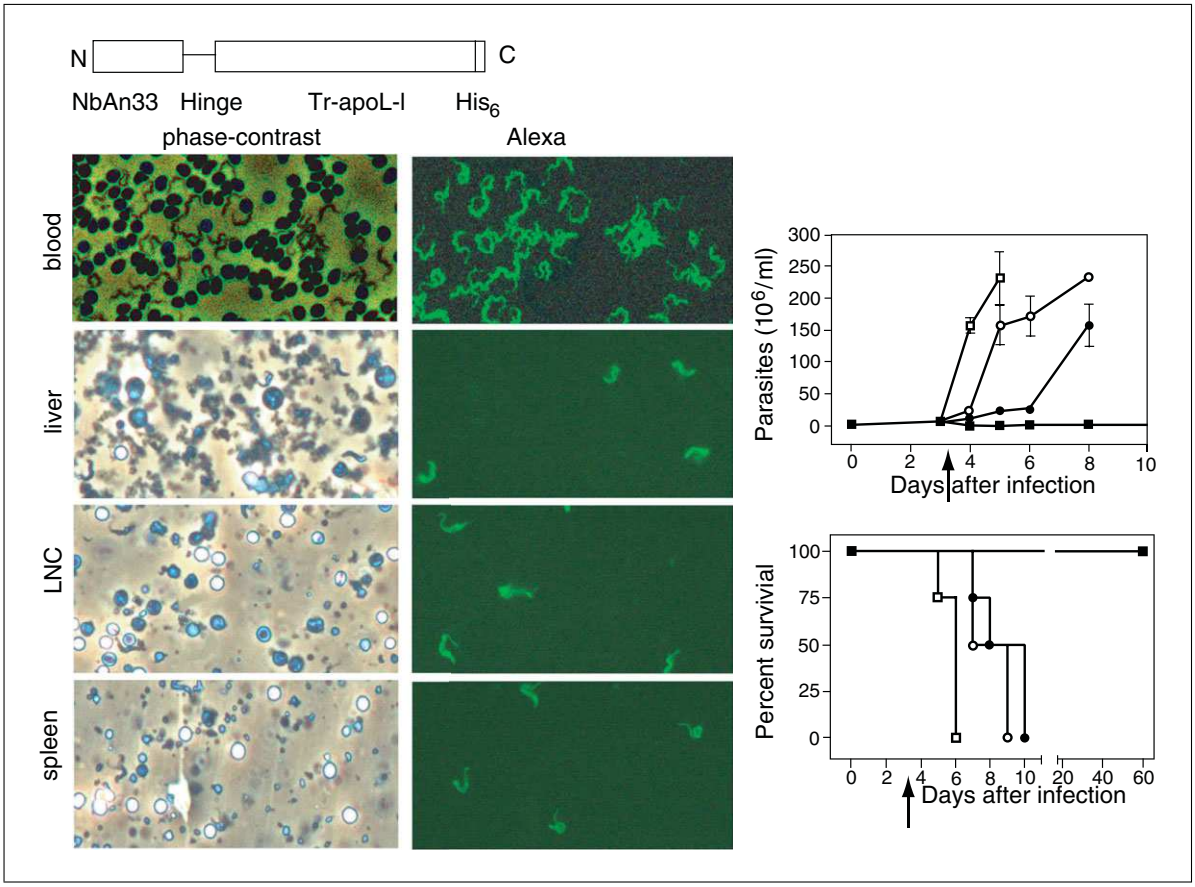

Figure 2.17.8 Single-domain antibodies as therapeutic reagents against infectious disease. sdAB can target a therapeutic molecule to an infectious agent. Left panel: Targeting specificity of an engineered, sdAb-based Alexa 488-labeled immunotoxin (NbAn33) to *Trypanosoma brucei rhodesiense*. Right panel, parasitemia (top graph) and survival of mice (bottom graph) infected with *T. b. rhodesiense* and treated with two different does of NbAn33 (10 μg, filled circles; 20 μg, filled squares). As control, 100 μg of the same construct with a non-related sdAb (open circles) was used. Reprinted from Baral et al. (2006) with permission from Nature Publishing Group.

mobile molecule (analyte) to an immobilized molecule (ligand) on a thin gold film. Small changes in the refractive index at the gold surface are measured in real time with precision, and resonance/response units (RU) are plotted versus time after subtracting any the background response. With SPR, equilibrium affinity can be reproducibly measured. Binding kinetics are analyzed by using BIAevaluation software. The calculated association and dissociation rate constants are used to obtain the affinity constant of the interaction (MacKenzie et al., 1996; Drescher et al., 2009).

Epitope mapping of isolated VHHs by SPR

The binding of VHHs to non-overlapping or overlapping epitopes on the protein (immunogen) surface can be determined by SPR. The method used here is called a 'Biacore co-injection experiment' and relies on the saturation of the antigen surface by the first VHH antibody followed by injection of the second VHH antibody at concentrations of 20 to 50 times their K_D. The experiments are repeated for all possible paired combinations of antibodies, and data are collected and evaluated as described in the Support Protocol 7.

Use of single-domain antibodies

Camelid single-domain antibodies have been successfully used in various fields of research. Applications include: use as immunological reagents and nanoprobes; use as crystallization chaperones; immune affinity chromatography; modulation of immune function; immunosensor reagents; immunoconjugates and multivalent/multispecific anticancer and anti-infectious reagents; enzyme inhibitors and hidden/cryptic epitope binders; blood-brain barrier carriers or transmigrators; protein-based oral therapeutics; immunotoxin and liposome/gold-conjugates; toxin neutralizers; and anti-cancer and anti–infectious disease therapeutic molecules (Muyldermans, 2001, 2009; Ewert et al., 2002; Revets et al.,

2005; Baral et al., 2006; Harmsen et al., 2007; Van Bockstaele et al., 2009; Wesolowski et al., 2009a; De Groeve et al., 2010; Vanlandschoot et al., 2011; Eyer and Hruska, 2012; Schoonooghe et al., 2012). Here we present a few typical examples of VHH applications as bacterial growth inhibitors and toxin neutralizers, cancer diagnostic reagents, cancer therapeutic reagents, and therapeutic reagents against infectious disease.

Single-domain antibodies as toxin neutralizers

Single-domain antibodies have been reported to neutralize different toxins such as scorpion toxin (Hmila et al., 2012), cobra toxin (Chavanayarn et al., 2012), and botulinum toxin (Tremblay et al., 2010). Here, we provide sdAbs that neutralize *Clostridium difficile* toxin (Fig. 2.17.5). *C. difficile* is a leading cause of nosocomial infections and is a major concern for healthcare professionals. In a recent study by Hussack et al (2011), it has been shown that sdAbs are potent neutralizers of the cytopathic effects of toxin A of *C. difficile* in an in vitro assay. The neutralizing potency was further enhanced when sdAbs were used in pairs or as triplets. The authors believe that the sdAbs are attractive systemic therapeutics, but are more so as oral therapeutics in the destabilizing environment of the gastrointestinal tract.

Single-domain antibodies as cancer diagnostic reagents

The utility of sdAbs as cancer-imaging agents has been described in different tumor models (Devoogdt et al., 2012; Pruszynski et al., 2013). Here, we provide two such examples of sdAbs with potential applications in cancer imaging (also see Fig. 2.17.6). An anti-CEACAM6 sdAb named 2A3 has shown tumor imaging capability both in vitro as an sdAb (Baral et al., 2011) or in vivo as an Fc fusion-format (Niu et al., 2012). The authors found that the heavy-chain antibody 2A3-mFc is superior to the sdAb and an anti-CEACAM6 conventional mAb with regard to tumor detection and pharmacokinetics, and shows great potential to be developed for CEACAM6-targeted pancreatic cancer imaging and therapy (Niu et al., 2012). Another example of an sdAb and its Fc fusion as a tumor imaging agent was reported by Bell et al. (2010). In this study, the authors utilized an sdAb against EGFR, and showed that the Fc fused sdAb against EGFR has good potential as an imaging agent.

Single-domain antibodies as cancer therapeutic reagents (Examples, Anti-CECAM6 VHHs)

It has been shown that sdAbs could be targeted specifically to tumor cells (Cortez-Retamozo et al., 2002), which together with the possibility of generation of bispecific VHH constructs (Els Conrath et al., 2001) is of major interest for tumor therapy and imaging (Cortez-Retamozo et al., 2004). It has been demonstrated that sdAbs can be used as molecular recognition units in the preparation of immunotoxins (Stijlemans et al., 2004). One example where an sdAb targets cancer antigens is that of CEACAM6-targeting sdAbs. An sdAb, 2A3, targeting CEACAM6 (Baral et al., 2011), has been shown to have anti-cancer potential (Fig. 2.17.7). It was determined that treating BxPC3 tumor cells with 2A3/2A3-Fc reduces BxPC3 proliferation, invasion, and MMP-9 activity. It also inhibits HUVEC cell angiogenesis (Cheng et al., 2012). Therefore, 2A3 can be a useful addition to Gemcitabine in treating pancreatic cancer when Gemcitabine alone fails to inhibit angiogenesis. Another sdAb, called AFAI, which also targets CEACAM6 (Mai et al., 2006), is being developed as a therapeutic candidate against lung cancer.

Single-domain antibodies as therapeutic reagents against infectious disease

The potential application of sdAbs in therapeutic applications against viral diseases was reviewed by Vanlandschoot (Vanlandschoot et al., 2011). Here, we provide one example of a sdAb as a therapeutic candidate against a blood protozoan parasite, the trypanosome. In this case, an sdAb, named Nb-An33 (Stijlemans et al., 2004), recognizes the oligo-mannose moiety of the variant specific surface glycoprotein (VSG) of the parasite (Fig. 2.17.8). This sdAb has been shown to recognize a buried and conserved epitope of VSG that is not accessible to larger molecules such as conventional IgGs. The NbAn33 was genetically fused to a truncated version of the APOL1 protein, which is a human serum protein that is trypanocidal. The results of the study showed that treatment with this conjugated Tr-APOL1 cured mice that were infected with either NHS-resistant *T. rhodesiense* or NHS-sensitive *T. brucei* (Baral et al., 2006). The treatment also had a beneficial effect when used in the chronic stage of trypanosomiasis, although in this case complete elimination of the parasite was not possible. The authors believe that they have developed a new

trypanocidal modality that is amenable to treating human African trypanosomiasis (HAT), either alone or in combination with less curative doses of other trypanocidal drugs. They also anticipate that this modality is amenable to use against other infectious agents depending on the availability of specific targeting sdAbs and a host-derived natural-defense molecule.

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