

Chapter 11

Construction of Human Antibody Gene Libraries and Selection of Antibodies by Phage Display

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Abstract

Recombinant antibodies as therapeutics offer new opportunities for the treatment of many tumor diseases. To date, 18 antibody-based drugs are approved for cancer treatment and hundreds of anti-tumor antibodies are under development. The first clinically approved antibodies were of murine origin or human-mouse chimeric. However, since murine antibody domains are immunogenic in human patients and could result in human anti-mouse antibody (HAMA) responses, currently mainly humanized and fully human antibodies are developed for therapeutic applications.

Here, in vitro antibody selection technologies directly allow the selection of human antibodies and the corresponding genes from human antibody gene libraries. Antibody phage display is the most common way to generate human antibodies and has already yielded thousands of recombinant antibodies for research, diagnostics and therapy. Here, we describe methods for the construction of human scFv gene libraries and the antibody selection.

Key words: scFv, therapeutic antibody, phage display, panning, antibody fragment, antibody gene library.

1. Introduction

In 2006, 18 recombinant antibodies are approved by the FDA (US Food and Drug Administration) for oncology and hematology (1). Rituximab (tradename Rituxan[®]/MabThera) against non-hodgkin-lymphoma (2) Bevacizumab (tradename Avastin[®]) primarily against colon cancer (3) and Trastuzumab (tradename Herceptin[®]) against breast cancer (4) are the top-selling recombinant antibodies in cancer therapy.

In 1986, the first antibody muronomab-CD3 (tradename Orthoclone OKT3[®]) was approved for therapy and was still of murine origin (5). Mouse-derived monoclonal antibodies are problematic as repeated administration of mouse-derived antibodies cause human anti-mouse antibody (HAMA) responses (6). The immunogenicity was step-wise reduced by using chimeric and later humanized antibodies by replacing murine constant and framework regions with the human counterparts, respectively (7). Accordingly, the next therapeutic antibodies were chimeric, e.g. Infliximab (tradename Remicade) (8) or Cetuximab (tradename Erbitux[®]) (9), and later humanized, e.g. Trastuzumab (4). The use of fully human antibodies is thought to further reduce the immunogenicity. In 2002, the first fully human antibody arrived on the market. Adalimumab (tradename Humira[®]) was isolated engineered using antibody phage display (10) by guided selection (11).

Currently, two major strategies are used for the generation of human antibodies: transgenic mice and in vitro selection technologies. Transgenic mice contain the human immunoglobulin repertoire instead of the murine, allowing the generation of human antibodies by the hybridoma technology (12–14). An advantage of transgenic mice is the in vivo affinity maturation. Already, transgenic mice yielded a significant number of antibodies that reached clinical studies. The disadvantages are limitations with respect to toxic and conserved antigens (15).

The alternative is the generation of human antibodies by antibody phage display, which is completely independent from any immune system by an in vitro selection process, called “panning.” The first antibody gene repertoires in phage were generated and screened by using the lytic phage Lambda (16), however, with limited success. The display method most commonly used today is based on the groundbreaking work of Georg P. Smith (17) on filamentous phage display. Here, the genotype and phenotype of oligopeptides were linked by fusing the corresponding gene fragments to the minor coat protein III gene of the filamentous bacteriophage M13. The resulting peptide::pIII fusion protein is expressed on the surface of phage allowing the affinity purification of the peptide and its corresponding gene. In the same way, antibody fragments fused to pIII can be presented on the surface of M13 phage (18–23). Due to limitations of the *Escherichia coli* folding machinery, complete IgG molecules can only be produced or displayed on the surface of phage in rare cases (24, 25). Mainly, smaller antibody fragments are used for antibody phage display: the Fab fragment or the single chain Fv fragment (scFv). Fab fragments consist of two chains, the variable (V_H) and first constant region of the heavy chain (CH1) and the light chain (LC) of the antibody, both linked by a disulphide bond. In contrast, scFv fragments consist of only one polypeptide chain,

composed of the variable region of the heavy chain (VH) and the variable region of the light chain (VL) fused by a short peptide linker. Two different genetic systems have been developed for the expression of the antibody::pIII fusion proteins for phage display. First, the antibody genes can be directly inserted into the phage genome fused to the wildtype pIII gene (18). However, most of the successful systems uncouple antibody expression from phage propagation by providing the genes encoding the antibody::pIII fusion proteins on a separate plasmid (called “phagemid”), containing a phage morphogenetic signal for packaging the vector into the assembled phage particles (19–23). The affinity of antibodies selected by phage display can be increased by additional in vitro affinity maturation steps (26). Despite other in vitro methods like ribosomal display (27, 28), puromycin display (29) or yeast surface display (30), antibody phage display has become the most widely used selection method for human antibodies. An overview about phage display derived human therapeutic antibodies in the clinical development is given by Thie et al. (31).

Depending on the scientific or medical applications, different types of antibody gene libraries can be constructed and used. Immune libraries are constructed from antibody V-genes isolated from IgG secreting plasma cells of immunized donors (20, 32). Immune libraries are typically generated and used in medical research to get an antibody against one particular target antigen, e.g. of an infectious pathogen or an tumor marker. Naive, semi-synthetic and synthetic libraries have been subsumed as “single-pot” libraries, as they are designed to isolate antibody fragments binding to every possible antigen, at least in theory. Naive libraries are constructed from rearranged V genes from B cells (IgM) of non-immunized donors. An example for this library type is the naive human Fab library constructed by de Haard et al. (33). Semi-synthetic libraries are derived from unrearranged V genes from pre B cells (germline cells) or from one antibody framework with genetically randomized complementary determining region (CDR) 3 regions, as described by Pini et al. (34). A combination of naive and synthetic repertoire was used by Hoet et al. (35). They combined light chains from autoimmune patients with a fd fragment containing synthetic CDR1 and CDR2 in the human VH3-23 framework and naive, which originated from autoimmune patients, CDR3 regions. The fully synthetic libraries have a human framework with randomly integrated CDR cassettes (36, 37). All library types – immune, naive, synthetic and their intermediates – are valuable sources for the selection of antibodies for diagnostic and therapeutic purposes. To date, “single-pot” antibody libraries with a theoretical diversity of up to 10^{11} independent clones have been generated (38) to serve as a molecular

repertoire for phage display selection procedures. An overview of antibody gene libraries is given by Hust and Dübel (39) and Hust et al. (40).

2. Materials

2.1. Construction of Antibody Gene Libraries

2.1.1. Isolation of Lymphocytes

1. Phosphate buffered saline (PBS), pH 7.4 (8.0 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄·2H₂O, 0.24 g KH₂PO₄ in 1 L)
2. Lymphoprep (Progen, Heidelberg)
3. mRNA isolation Kit (QuickPrep micro mRNA Purification Kit, GE Healthcare, Munich) or Trizol (Invitrogen, Karlsruhe) for total RNA

2.1.2. cDNA Synthesis

1. Superscript II (Invitrogen) + 5 x RT buffer + 0.1 m DTT
2. Random hexamer oligonucleotide primer (dN₆)
3. dNTP mix (2.5 mM each)

2.1.3. /2.1.4 First and Second Antibody Gene PCR

1. Red Taq (Sigma, Hamburg) + 10 x buffer
2. dNTP mix (10 mM each)
3. Oligonucleotide primer (*see Table 11.1*)
4. Agarose (Serva, Heidelberg)
5. TAE-buffer 50 x (2 M Tris-HCl, 1 M acetic acid, 0.05 M EDTA, pH 8)
6. Nucleospin Extract 2 Kit (Macherey-Nagel, Düren)

2.1.5. First Cloning Step – VL

1. MluI (NEB, Frankfurt)
2. NotI (NEB)
3. Buffer 3 (NEB)
4. BSA (NEB)
5. Calf intestine phosphatase (CIP) (MBI Fermentas, St. Leon-Rot)
6. T4 ligase (Promegq a, Mannheim)
7. 3 M sodium acetate, pH 5.2
8. *E. coli* XL1-Blue MRF⁺ (Stratagene, Amsterdam), genotype: $\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173 \text{ endA1 supE44 thi-1 recA1 gyrA96 relA1 lac}$ [F' *proAB lacI*^q ZΔM15 Tn10 (Tet^r)]
9. Electroporator MicroPulser (BIO-RAD, München)

Table 11.1

Primers used for first and second PCR of antibody genes for antibody gene library construction using phagemids like pHAL14. Restriction sites are underlined

<i>Primer</i>	<i>5' to 3' sequence</i>
First antibody gene PCR VH	
MHVH1_f	cag gtb cag ctg gtg cag tct gg
MHVH1/7_f	car rts cag ctg gtr car tct gg
MHVH2_f	cag rtc acc ttg aag gag tct gg
MHVH3_f1	sar gtg cag ctg gtg gag tct gg
MHVH3_f2	gag gtg cag ctg ktg gag wcy sg
MHVH4_f1	cag gtg car ctg cag gag tcg gg
MHVH4_f2	cag stg cag ctr cag sag tss gg
MHVH5_f	gar gtg cag ctg gtg cag tct gg
MHVH6_f	cag gta cag ctg cag cag tca gg
MHIgMCH1_r	aag ggt tgg ggc gga tgc act
MHIgGCH1_r	gac cga tgg gcc ctt ggt gga
First antibody gene PCR kappa	
MHVK1_f1	gac atc cag atg acc cag tct cc
MHVK1_f2	gmc atc crg wtg acc cag tct cc
MHVK2_f	gat rtt gtg atg acy cag wct cc
MHVK3_f	gaa atw gtg wtg acr cag tct cc
MHVK4_f	gac atc gtg atg acc cag tct cc
MHVK5_f	gaa acg aca ctc acg cag tct cc
MHVK6_f	gaw rtt gtg mtg acw cag tct cc
MHkappaCL_r	aca ctc tcc cct gtt gaa gct ctt
First antibody gene PCR lambda	
MHVL1_f1	cag tct gtg ctg act cag cca cc
MHVL1_f2	cag tct gtg ytg acg cag ccg cc
MHVL2_f	cag tct gcc ctg act cag cct
MHVL3_f1	tcc tat gwg ctg acw cag cca cc
MHVL3_f2	tct tct gag ctg act cag gac cc
MHVL4_f1	ctg cct gtg ctg act cag ccc
MHVL4_f2	cag cyt gtg ctg act caa tcr yc
MHVL5_f	cag sct gtg ctg act cag cc
MHVL6_f	aat ttt atg ctg act cag ccc ca
MHVL7/8_f	cag rct gtg gtg acy cag gag cc
MHVL9/10_f	cag scw gkg ctg act cag cca cc
MHlambdaCL_r	tga aca ttc tgt agg ggc cac tg
MHlambdaCL_r2	tga aca ttc cgt agg ggc aac tg

(continued)

Table 11.1 (continued)

<i>Primer</i>	<i>5' to 3' sequence</i>
Second antibody gene PCR VH	
MHVH1-NcoI_f	gtcctcgca <u>cc atg gcc</u> cag gtb cag ctg gtg cag tct gg
MHVH1/7-NcoI_f	gtcctcgca <u>cc atg gcc</u> car rts cag ctg gtr car tct gg
MHVH2-NcoI_f	gtcctcgca <u>cc atg gcc</u> cag rtc acc ttg aag gag tct gg
MHVH3-NcoI_f1	gtcctcgca <u>cc atg gcc</u> sar gtg cag ctg gtg gag tct gg
MHVH3-NcoI_f2	gtcctcgca <u>cc atg gcc</u> gag gtg cag ctg ktg gag wcy sg
MHVH4-NcoI_f1	gtcctcgca <u>cc atg gcc</u> cag gtg car ctg cag gag tct gg
MHVH4-NcoI_f2	gtcctcgca <u>cc atg gcc</u> cag stg cag ctr cag sag tss gg
MHVH5-NcoI_f	gtcctcgca <u>cc atg gcc</u> gar gtg cag ctg gtg cag tct gg
MHVH6-NcoI_f	gtcctcgca <u>cc atg gcc</u> cag gta cag ctg cag cag tca gg
MHIgMCH1scFv-HindIII_r	gtcctcgca <u>aag ctt</u> tgg ggc gga tgc act
MHIgGCH1scFv-HindIII_r	gtcctcgca <u>aag ctt</u> gac cga tgg gcc ctt ggt gga
Second antibody gene PCR kappa	
MHVK1-MluI_f1	accgectcc <u>a cgc gta</u> gac atc cag atg acc cag tct cc
MHVK1-MluI_f2	accgectcc <u>a cgc gta</u> gmc atc crg wtg acc cag tct cc
MHVK2-MluI_f	accgectcc <u>a cgc gta</u> gat rtt gtg atg acy cag wct cc
MHVK3-MluI_f	accgectcc <u>a cgc gta</u> gaa atw gtg wtg acr cag tct cc
MHVK4-MluI_f	accgectcc <u>a cgc gta</u> gac atc gtg atg acc cag tct cc
MHVK5-MluI_f	accgectcc <u>a cgc gta</u> gaa acg aca ctc acg cag tct cc
MHVK6-MluI_f	accgectcc <u>a cgc gta</u> gaw rtt gtg mtg acw cag tct cc
MHkappaCLscFv-NotI_r	accgectcc <u>gc ggc cgc</u> gaa gac aga tgg tgc agc cac agt
Second antibody gene PCR lambda	
MHVL1-MluI_f1	accgectcc <u>a cgc gta</u> cag tct gtg ctg act cag cca cc
MHVL1-MluI_f2	accgectcc <u>a cgc gta</u> cag tct gtg ytg acg cag ccg cc
MHVL2-MluI_f	accgectcc <u>a cgc gta</u> cag tct gcc ctg act cag cct
MHVL3-MluI_f1	accgectcc <u>a cgc gta</u> tcc tat gwg ctg acw cag cca cc
MHVL3-MluI_f2	accgectcc <u>a cgc gta</u> tct tct gag ctg act cag gac cc
MHVL4-MluI_f1	accgectcc <u>a cgc gta</u> ctg cct gtg ctg act cag ccc
MHVL4-MluI_f2	accgectcc <u>a cgc gta</u> cag cyt gtg ctg act caa tcr yc
MHVL5-MluI_f	accgectcc <u>a cgc gta</u> cag sct gtg ctg act cag cc
MHVL6-MluI_f	accgectcc <u>a cgc gta</u> aat ttt atg ctg act cag ccc ca
MHVL7/8-MluI_f	accgectcc <u>a cgc gta</u> cag rct gtg gtg acy cag gag cc
MHVL9/10-MluI_f	accgectcc <u>a cgc gta</u> cag scw gkg ctg act cag cca cc
MHLambdaCLscFv-NotI_r	accgectcc <u>gc ggc cgc</u> aga gga sgg ygg gaa cag agt gac
Primer for colony PCR and sequencing	
MHLacZ-Pro_f	ggctcgatgtgtgtgtgg
MHgIII_r	c taa agt ttt gtc gtc ttt cc
MKpelB_f	g cct acg gca gcc gct gg
MKmyc_r	g atc ctc ttc tga gat gag

10. 2 M Glucose (sterile filtered)
11. 2 M Magnesium solution (1 M MgCl, 1 M MgSO₄) (autoclaved)
12. SOC medium, pH 7.0 (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 20 mM Mg solution, 20 mM glucose) (sterilize magnesium and glucose separately; add solutions after autoclavation)
13. 2xYT-medium, pH 7.0 (1.6% (w/v) Tryptone, 1% (w/v) Hefe Extrakt, 0.5% (w/v) NaCl)
14. 2xYT-GAT (2xYT + 100 mM Glucose + 100 µg/ml ampicillin + 20 µg/ml tetracycline)
15. Ampicillin (100 mg/ml stock)
16. Tetracycline (10 mg/ml stock)
17. 9-cm petri dishes
18. 25-cm square petri dishes ("pizza plates")
19. 2xYT-GAT agar plates (2xYT-GAT, 1.5% (w/v) agar-agar)
20. Nucleobond Plasmid Midi Kit (Macherey-Nagel)

2.1.6. Second Cloning Step – VH

1. NcoI (NEB)
2. HindIII (NEB)
3. Buffer 2 (NEB)
4. Glycerol of 99.5% (Roth, Karlsruhe)

2.1.7. Colony PCR

1. Oligonucleotide primer (*see* **Table 11.1**)

2.1.8. Library Packaging and scFv Phage Production

1. 2xTY media, pH 7.0 (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl)
2. 2xTY-GA (2xTY, 100 mM glucose, 100 µg/ml ampicillin)
3. M13K07 Helperphage for monovalent display (Stratagene)
4. Hyperphage for oligovalent display (Progen, Heidelberg)
5. 2xTY-AK (2xTY, containing 100 µg/ml ampicillin, 50 µg/ml kanamycin)
6. Sorval Centrifuge RC5B Plus, rotor GS3 and SS34 (Thermo Scientific, Waltham)
7. Polyethylene glycol (PEG) solution (20% (w/v) PEG 6000, 2.5 M NaCl)
8. Phage dilution buffer (10 mM Tris-HCl, pH 7.5, 20 mM NaCl, 2 mM EDTA)
9. Mouse α-pIII monoclonal antibody PSKAN3 (Mobitec, Göttingen)
10. Goat α-mouse IgG alkaline phosphatase (AP) conjugate (Sigma, Hamburg)

2.2. Antibody Selection (Panning)

2.2.1. Coating of Microtitre Wells

1. Maxisorb microtitre plates oder stripes (Nunc, Langenselbold)
2. PBS (pH 7.4) (8.0 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄·2H₂O, 0.24 g KH₂PO₄ in 1 L)
3. Dimethyl sulfoxide (DMSO)
4. PBST (PBS + 0.1% (v/v) Tween-20)

2.2.2. Panning

1. MPBST (2% skim milk in PBST, prepare fresh)
2. Panning block solution (1% (w/v) skim milk + 1% (w/v) BSA in PBST, prepare freshly)
3. 10 µg/ml Trypsin in PBS
4. *E. coli* XL1-Blue MRF⁺ (Stratagene)
5. M13K07 Helperphage (Stratagene)
6. 2xTY media, pH 7.0 (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl)
7. 2xTY-T (2xTY, containing 50 µg/ml tetracycline)
8. SOB media, pH 7.0 (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, after autoclavation add sterile 1% (v/v) of the 2 M Mg solution)
9. 2 M Mg solution (1 M MgCl + 1 M MgSO₄)
10. SOB-GA (SOB, 100 µg/ml ampicillin, 100 mM glucose)
11. SOB-GA agar plates (SOB-GA + 1,5% (w/v) agar-agar)
12. 15-cm petri dishes
13. 2xTY-GA (2xTY, 100 mM glucose, 100 µg/ml ampicillin)
14. Glycerol (99.5%)

2.2.3. Packaging of Phagemids (scFv Phage Production)

1. 2xTY-AK (2xTY, containing 100 µg/ml ampicillin, 50 µg/ml kanamycin)
2. Polyethylene glycol (PEG) solution (20% (w/v) PEG 6000, 2,5 M NaCl)
3. Phage dilution buffer (10 mM Tris-HCl, pH 7.5, 20 mM NaCl, 2 mM EDTA)

2.2.4. Phage Titration

1. 2xTY-GA agar plates (2xTY-GA + 1.5% (w/v) agar-agar)

2.2.5. ELISA of a Polyclonal Antibody Phage Suspension

1. Bovine serum albumin (BSA) (prepare a 10 mg/ml stock solution in PBS)
2. Anti-M13, horse radish peroxidase (HRP) conjugated monoclonal antibody (GE Healthcare)

2.2.6. Production of Soluble Monoclonal Antibody Fragments in Microtitre Plates

3. TMB substrate solution A, pH 4.1 (10 g citric acid solved in 100 ml water, add 9.73 g potassium citrate add to 1 L water)
4. TMB substrate solution B (240 mg tetramethylbenzidine, 10 ml acetone, 90 ml ethanol, 907 μ l 30% H_2O_2)
5. 1 N H_2SO_4

1. 96-well U-bottom polypropylene (PP) microtitre plates (Greiner Bio-One, Frickenhausen)
2. AeraSeal breathable sealing film (Excel Scientific, Victorville)
3. Thermoshaker PST60-HL4 (Lab4You, Berlin)
4. Potassium phosphate buffer (2.31% (w/v) (0,17 M) KH_2PO_4 + 12.54% (w/v) (0,72 M) K_2HPO_4)
5. Buffered 2xTY, pH 7.0 (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, 10% (v/v) potassium phosphate buffer)
6. Buffered 2xTY-SAI (buffered 2xTY containing 50 mM saccharose + 100 μ g/ml ampicillin + 50 μ M isopropyl-beta-D-thiogalactopyranoside (IPTG))

2.2.7. ELISA of Soluble Monoclonal Antibody Fragments

1. Mouse α -His-tag monoclonal antibody (α -Penta His, Qiagen, Hilden)
2. Mouse α -myc-tag monoclonal antibody (9E10, Sigma)
3. Mouse α -pIII monoclonal antibody PSKAN3 (Mobitec)
4. Goat α -Mouse IgG serum, (Fab specific) HRP conjugated (Sigma)
5. Oligonucleotide primers (*see* Table 11.1)

3. Methods

3.1. Construction of Single-Chain Antibody Gene Libraries

Various methods have been employed to clone the genetic diversity of antibody repertoires. After isolation of mRNA from B lymphocytes and preparation of cDNA, the construction of immune libraries is usually done by a two-step cloning or assembly PCR (*see* below). Very large “single pot” naive antibody gene libraries are generally constructed by two or three separate cloning steps. In the two-step cloning strategy, the amplified repertoire of light chain genes is cloned into the phage display vector first. In the second step the heavy chain gene repertoire – as the heavy chain contributes more to diversity, due to its highly variable CDRH3 – is cloned into the phagemids containing the light chain gene repertoire (32, 41–44). In the three-step cloning strategy,

separate heavy and light chain libraries are engineered. The V_H gene repertoire has then to be excised and cloned into the phage display vector containing the repertoire of VL genes (33). Another common method used for the cloning of naive (45, 46), immune (20) or hybridoma (47) scFv phage display libraries is the assembly PCR. The V_H and VL genes including the additional linker sequence are amplified separately and fused by assembly PCR, before the scFv encoding gene fragments are cloned into the vector. Since the CDRH3 is a major source of sequence variety in antibodies (48), the assembly PCR can be combined with a randomization of the CDR3 regions, leading to semi-synthetic libraries. Here, oligonucleotide primers encoding various CDR3 and J gene segments were used for the amplification of the V gene segments of human germlines (49). Hoogenboom and Winter (50) as well as Nissim et al. (51) used degenerated CDRH3 oligonucleotide primers to generate a semi-synthetic heavy chain repertoire derived from human V gene germline segments. Afterwards, this V_H repertoire was combined with an anti-BSA light chain. In some cases a framework of a well known/robust antibody was used as scaffold for the integration of randomly created CDRH3 and CDRL3 (52, 53). Jirholt et al. (54) and Söderlind et al. (55) amplified all CDR regions derived from B cells before shuffling them into this antibody framework by assembly PCR. An entirely synthetic library was described by Knappik et al. (37) who utilized seven different V_H and VL germline master frameworks combined with six synthetically created CDR cassettes. The construction of large naive and semi-synthetic libraries (35, 43, 46, 56–58) requires significant effort to tunnel the genetic diversity through the bottleneck of *E. coli* transformation, e.g. 600 transformations were necessary for the generation of a 3.5×10^{10} phage library (35).

To overcome the bottleneck of *E. coli* transformation, the Cre-lox or lambda phage recombination system has also been employed for library generation (38, 59–61). However, since libraries with more than 10^{10} independent clones have also been accomplished by conventional transformation, most of these complicated methods are rendered unnecessary in particular as they may result in decreased genetic stability.

In summary, antibodies can be selected from either type of library, naive or synthetic (33, 35, 37). If the assembly by cloning or PCR and preservation of molecular complexity is carefully controlled at every step of its construction, libraries of more than 10^{10} independent clones can be generated (35).

The following protocols describe the generation of human naive or immune scFv antibody gene libraries by a two-step cloning strategy already approved for naive (56) and immune libraries (32, 44).

3.1.1. Isolation of Lymphocytes (Peripheral Blood Mononuclear Cells (PBMC))

1. Mix 20 ml fresh blood or EDTA/citric acid-treated blood ($\sim 2 \times 10^7$ cells) of each donor with 20 ml PBS (*see Note 1*).
2. Fill 10 ml Lymphoprep in a 50-ml polypropylen tube. Carefully cover Lymphoprep with 40 ml of the diluted blood using a plastic pipette.
3. Centrifuge the blood with $800 \times g$ for 20 min at RT (without brake!).
4. The lymphocytes form a distinct layer between the Lymphoprep and the medium, whereas the erythrocytes and granulocytes will be pelleted. Carefully aspirate the lymphocytes using a plastic pipette and transfer to a new 50-ml polypropylen tube.
5. Fill up with 50 ml PBS and pellet the lymphocytes with $250 \times g$ for 10 min at RT. Discard the supernatant (be careful, the lymphocyte pellet is not solid).
6. Repeat this washing step to remove most of the thrombocytes.
7. Resuspend the lymphocytes pellet in the supplied extraction buffer of the mRNA isolation kit according to the manufacturers instructions or use 0.5 ml Trizol for total RNA isolation (*see Note 2*). After resuspension using the mRNA extraction buffer or Trizol, the RNA pellet can be stored at -80°C .

3.1.2. cDNA Synthesis

1. Set up mixture for the first strand cDNA synthesis:

Solution or component	volume	Final concentration
mRNA or total RNA	9 μl	50–250 ng (mRNA) or 2–20 μg (total RNA)
Random hexamer oligonucleotide primer (dN ₆) (10 μM)	2.5 μl	1.5 μM
dNTP-Mix (2.5 mM each)	5 μl	500 μM

2. Denature the RNA for 5 min at 70°C . Afterwards directly chill down on ice for 5 min.
3. Add following components:

Solution or component	Volume	Final concentration
RT buffer (5 $\times$)	5 μl	1 $\times$
0.1 M DTT	2,5 μl	10 mM
Superscript II reverse transcriptase (200 U/ μl)	1 μl	200 U

4. Incubate the 25 μ l mixture for 10 min at 25°C for primer annealing. Afterwards incubate 50 min at 42°C for first strand synthesis.
5. Denature the RNA/DNA hybrids and the enzyme for 15 min at 70°C. Store at –20°C (*see* **Note 3**).

3.1.3. First Antibody Gene PCR

1. The cDNA derived from 50–250 ng mRNA or 2–20 μ g total RNA will be used as template to amplify VH and the light chain. Set up the PCR reactions as follows (30 $\times$ mastermix for 27 PCR reactions):

Solution or component	Volume	Final concentration
dH ₂ O	1230 μ l	–
Buffer (10 $\times$)	150 μ l	1 $\times$
dNTPs (10 mM each)	30 μ l	200 μ M each
cDNA	25 μ l	Complete first strand synthesis reaction
RedTaq 1 U/ μ l	60 μ l	2 U

3. Divide the master mix in 450 μ l for VH, 350 μ l for kappa and 550 μ l for lambda.
4. Add to each of the three reactions the corresponding reverse primers (*see* also **Table 11.1**) as follows (use the IgM primer for naive antibody gene libraries or the IgG primer for a immune antibody gene libraries):

Antibody gene	Primer	Volume	Final concentration
VH	MHIgMCH1_r or MHIgGCH1_r (10 μ M)	18 μ l	0.4 μ M
kappa	MHkappaCL_r (10 μ M)	14 μ l	0.4 μ M
lambda	MHlambdaCL_r1/_r2 mix (9:1) (10 μ M)	22 μ l	0.4 μ M

5. Divide the mixture to 9 (VH), 7 (Kappa) and 11 (Lambda) PCR reactions each with 48 μ l and add 2 μ l (10 μ M, 0.4 μ M final concentration) of the subfamily-specific forward primer (*see* also **Table 11.1**):

VH:	(1) MHVH1_f, (2) MHVH1/7_f, (3) MHVH2_f, (4) MHVH3_f1, (5)MHVH3_f2, (6) MHVH4_f1, (7) MHVH4_f2, (8) MHVH5_f, (9)MHVH6_f
Vkappa:	(10) MHVK1_f1, (11) MHVK1_f2, (12) MHVK2_f, (13) MHVK3_f, (14)MHVK4_f, (15) MHVK5_f, (16) MHVK6_f
Vlambda:	(17) MHVL1_f1, (18) MHVL1_f2, (19) MHVL2_f, (20) MHVL3_f1, (21)MHVL3_f2, (22) MHVL4_f1, (23) MHVL4_f2, (24) MHVL5_f, (25)MHVL6_f, (26) MHVL7/8_f, (27) MHVL9/10_f

6. Carry out the PCR using the following program:

94°C	1 min	 30x
94°C	1 min	
55°C	1 min	
72°C	2 min	
72°C	10 min	

7. Separate PCR products by 1.5% TAE agarose gel electrophoresis, cut out the amplified antibody genes (VH: ~380 bp, kappa/lambda: ~650 bp) (*see Note 4*) and purify the PCR products using a gel extraction kit according to the manufacturers instructions. Pool all VH, kappa and lambda subfamilies separately. Determine the DNA concentration. Store the three purified first PCR pools at −20°C.

3.1.4. Second Antibody Gene PCR

1. In the second PCR the restriction sites for library cloning will be added. Set up the PCR reactions as follows (30× mastermix for 27 PCR reactions) (*see Note 5*):

<i>Solution or component</i>	<i>Volume</i>	<i>Final concentration</i>
dH ₂ O	2500 µl	–
RedTaq buffer (10×)	300 µl	1 ×
dNTPs (10 mM each)	60 µl	200 µM each
RedTaq 1 U/µl	120 µl	2 U

2. Divide the master mix in 900 μl for VH, 700 μl for kappa and 1100 μl for lambda.
3. Add to each of the three reactions the corresponding reverse primers (*see* also **Table 11.1**) as follows:

Antibody gene	Primer	Volume	Final concentration
VH	MHIgMCH1scFv-HindIII_r or MHIgGCH1scFv-HindIII_r (10 μM)	18 μl	0.2 μM
kappa	MHKappaCLscFv-NotI_r (10 μM)	14 μl	0.2 μM
lambda	MHLambdaCLscFv-NotI_r (10 μM)	22 μl	0.2 μM

4. Add the corresponding PCR products of the first PCR as follows:

VH	900 ng
Kappa	700 ng
Lambda	1100 ng

5. Divide the solutions to 9 (VH), 7 (Kappa) and 11 (Lambda) PCR reactions, each with 98 μl and add 2 μl (10 μM , 0.2 μM final concentration) of the subfamily-specific forward primer (*see* also **Table 11.1**):

VH:	(1) MHVH1-NcoI_f, (2) MHVH2-NcoI_f, (3) MHVH1/7-NcoI_f, (4) MHVH3-NcoI_f1, (5) MHVH3-NcoI_f2, (6) MHVH4-NcoI_f1, (7) MHVH4-NcoI_f2, (8) MHVH5-NcoI_f, (9) MHVH6-NcoI_f
Vkappa:	(10) MHVK1-MluI_f1, (11) MHVK1-MluI_f2, (12) MHVK2-MluI_f, (13) MHVK3-MluI_f, (14) MHVK4-MluI_f, (15) MHVK5-MluI_f, (16) MHVK6-MluI_f
Vlambda:	(17) MHVL1-MluI_f1, (18) MHVL1-MluI_f2, (19) MHVL2-MluI_f, (20) MHVL3-MluI_f1, (21) MHVL3-MluI_f2, (22) MHVL4-MluI_f1, (23) MHVL4-MluI_f2, (24) MHVL5-MluI_f, (25) MHVL6-MluI_f, (26) MHVL7/8-MluI_f, (27) MHVL9/10-MluI_f

6. Carry out the PCR using the following program:

94°C	1 min
94°C	1 min
57°C	1 min
72°C	2 min
72°C	10 min

 20x

7. Separate the PCR products by 1.5% TAE agarose gel electrophoresis, cut out the amplified antibody genes (VH: ~400 bp, kappa/lambda: ~400 bp) and purify the PCR products using a gel extraction kit according to the manufacturers instructions. Pool all VH, kappa and lambda subfamilies separately. Determine the DNA concentration. Store the three purified second PCR pools at -20°C .

3.1.5. First Cloning Step – VL

1. Prepare a plasmid preparation of pHAL14 vector for library cloning.
2. Digest the vector and the VL PCR products (always perform single enzyme digest of the vector in parallel to check whether the digestion is complete, *see* **Notes 5 and 6**):

<i>Solution or component</i>	<i>Volume</i>	<i>Final concentration</i>
dH ₂ O	83-x μl	–
pHAL14 or VL	x μl	5 or 2 μg
NEB buffer 3 (10 $\times$)	10 μl	1 $\times$
BSA (100 $\times$)	1 μl	1 $\times$
NEB MluI (10 U/ μl)	3 μl	30 U
NEB NotI (10 U/ μl)	3 μl	30 U

3. Incubate at 37°C for 2 h. Control the digest of the vector by using a 5 μl aliquot on 1% TAE agarose gel electrophoresis. If the vector is not fully digested, extend the incubation time.
4. Inactivate the enzymes at 65°C for 10 min.

5. Add 0.5 μl CIP (1 U/ μl) and incubate at 37°C for 30 min. Repeat this step once.
6. Purify the vector and the PCR product using a PCR purification Kit according the manufacturers instructions and elute with 50 μl elution buffer or water. The short stuffer fragment containing multiple stop codons between MluI and NotI in pHAL14 will be removed. Determine the DNA concentration.
7. Ligate the vector pHAL14 (4255 bp) and VL (~380 bp) as follows (*see* **Note 5**):

Solution or component	Volume	Final concentration
dH ₂ O	89-x-y μl	–
pHAL14	x μl	1000 ng
VL (kappa or lambda)	y μl	270 ng
T4 ligase buffer (10 $\times$)	10 μl	1 $\times$
T4 ligase (3 U/ μl)	1 μl	3 U

8. Incubate at 16°C overnight.
9. Inactivate the ligation at 65°C for 10 min.
10. Precipitate the ligation with 10 μl 3 M sodium acetate, pH 5.2, and 250 μl ethanol, incubate for 2 min at RT and centrifuge for 5 min at 16,000 $\times g$ and 4°C.
11. Wash the pellet with 500 μl 70% (v/v) ethanol and pellet the DNA for 2 min at 16,000 $\times g$ and 4°C. Repeat this step once and resolve the DNA pellet in 35 μl dH₂O.
12. Thaw 25 μl electrocompetent XL1-Blue MRF' on ice and mix with the ligation reaction.
13. Transfer the 60 μl mix to a prechilled 0.1 cm cuvette. Dry the electrode of the cuvette with a tissue paper.
14. Perform a 1.7 kV pulse using an electroporator (*see* **Note 7**). Immediately, add 1 ml 37°C prewarmed SOC medium, transfer the suspension to a 2 ml cap and shake for 1 h at 600 rpm and 37°C.
15. To determine the amount of transformants, use 10 μl (=10⁻² dilution) of the transformation and perform a dilution series down to 10⁻⁶ dilution. Plate out a 10⁻⁶ dilution on 2xYT-GAT agar plates and incubate overnight at 37°C.
16. Plate out the remaining 990 μl on 2xYT-GAT agar “pizza plate” and incubate overnight at 37°C.
17. Calculate the amount of transformants, which should be 1 $\times$ 10⁶–5 $\times$ 10⁸ cfu. Control colonies for full-size insert by colony PCR (*see* **Section 3.1.7**).

18. Float off the colonies on the “pizza plate” with 40 ml 2xYT medium using a drigalsky spatula. Use 5 ml bacteria solution for midi plasmid preparation according to the manufacturer’s instructions. Determine the DNA concentration.

3.1.6. Second Cloning
Step – VH

1. Digest the pHAL14-VL repertoire and the VH PCR products (always perform single enzyme digest of the vector in parallel, *see* **Notes 5** and **6**):

Solution or component	Volume	Final concentration
dH ₂ O	81-x µl	–
pHAL14-VL or VH	x µl	5 or 2 µg
NEB buffer 2 (10×)	10 µl	1×
BSA (100×)	1 µl	1×
NEB NcoI (10 U/µl)	3 µl	30 U
NEB HindIII (20 U/µl)	5 µl	100 U

2. Incubate at 37°C for 2 h (*see* **Note 8**). Control the digest of the vector by using a 5 µl aliquot on 1% agarose gel electrophoresis.
3. Inactivate the digestion at 65°C for 10 min.
4. Add 0.5 µl CIP (1 U/µl) and incubate at 37°C for 30 min. Repeat this step once.
5. Purify the vector and the PCR product using a PCR purification Kit according the manufacturers instructions and elute with 50 µl elution buffer or water. The short stuffer fragment between NcoI and HindII in pHAL14 will be removed. Determine the DNA concentration.
6. Ligate the vector pHAL14-VL (~4610 bp) and VH (~380 bp) as follows (*see* **Note 5**):

Solution or component	Volume	Final concentration
dH ₂ O	89-x-y µl	–
pHAL14	x µl	1000 ng
VH	y µl	250 ng
T4 ligase buffer (10×)	10 µl	1×
T4 ligase (3 U/µl)	1 µl	3 U

7. Incubate at 16°C overnight.
8. Inactivate the ligation at 65°C for 10 min.
9. Precipitate the ligation with 10 µl 3 M, pH 5.2, sodium acetate and 250 µl ethanol, incubate for 2 min at RT and centrifuge for 5 min at 16,000×g and 4°C.

10. Wash the pellet with 500 μl 70% (v/v) ethanol and pellet the DNA for 2 min at $16,000\times g$ and 4°C . Repeat this step once and resolve the pellet in 35 μl dH₂O.
11. Thaw 25 μl electrocompetent XL1-Blue MRF' on ice and mix with the ligation reaction.
12. Transfer the 60 μl mix to a prechilled 0.1 cm cuvette. Dry the electrode of the cuvette with a tissue paper.
13. Perform a 1.7 kV pulse using an electroporator (*see Note 7*). Immediately, add 1 ml 37°C prewarmed SOC medium, transfer to a 2-ml cap and incubate for 1 h at 600 rpm.
14. To determine the amount of transformants, use 10 μl ($=10^{-2}$ dilution) of the transformation and perform a dilution series down to 10^{-6} dilution. Plate out a 10^{-6} dilution on 2xYT-GAT agar plates and incubate overnight at 37°C .
15. Plate out the remaining 990 μl on 2xYT-GAT agar “pizza plate” and incubate overnight at 37°C .
16. Calculate the amount of transformants (1×10^6 – 5×10^8 should be reached to be included into the final library). Control colonies for full size insert by colony PCR (*see Section 3.1.7*).
17. Float off the colonies on the “pizza plate” with 40 ml 2xYT medium using a drigalsky spatula. Use 800 μl bacteria solution ($\sim 1 \times 10^{11}$ bacteria) and 200 μl glycerol for glycerol stocks. Make 5–25 glycerol stocks per sublibrary and store at -80°C .
18. When all transformations are done, thaw one aliquot of each sublibrary on ice, mix all sublibraries (*see also Note 5*) and make new aliquots for storage at -80°C (*see also Note 9*).

3.1.7. Colony PCR

1. Choose 10–20 single colonies per transformation. Set up the PCR reaction per colonies as follows (*see Table 11.1* for primer sequences):

<i>Solution or component</i>	<i>Volume</i>	<i>Final concentration</i>
dH ₂ O	16,7 μl -x μl	
RedTaq buffer (10 $\times$)	2 μl	1 $\times$
dNTPs (10 mM each)	0,4 μl	je 200 μM
MHLacZPro_f 10 μM	0,2 μl	0,1 μM
MHgIII_r10 μM	0,2 μl	0,1 μM
RedTag (1 U/ μl)	0,5 μl	1 U
template	picked colonie from dilution plate	

2. Control the PCR by 1.5% TAE agarose gel electrophoresis.
3. The PCR products should be ~ 1100 bp when including VH and VL, ~ 750 bp when including only VL or VH and 375 bp if the vector contains no insert. Each used sublibrary should have more than 80% full-size inserts to be included into the final library.

3.1.8. Library Packaging and scFv Phage Production

1. To package the library, inoculate 400 ml 2xTY-GA in a 1 L Erlenmeyer flask with 1 ml antibody gene library stock. Grow at 250 rpm at 37°C up to an O.D._{600 nm} ~ 0.5 .
2. Infect 25 ml bacteria culture ($\sim 1.25 \times 10^{10}$ cells) with 2.5×10^{11} colony forming units (cfu) of the helper phage M13K07 or Hyperphage according to a multiplicity of infection (moi) = 1:20 (*see Note 10*). Incubate 30 min without shaking and the following 30 min with 250 rpm at 37°C.
3. To remove the glucose which represses the lac promoter of pHAL14 and therefore the scFv::pIII fusion protein expression, harvest the cells by centrifugation for 10 min at $3200 \times g$ in 50-ml polypropylene tubes.
4. Resuspend the pellet in 400 ml 2xTY-AK in a 1 L Erlenmeyer flask. Produce scFv-phage overnight at 250 rpm and 30°C.
5. Pellet the bacteria by centrifugation for 10 min at $10,000 \times g$ in two GS3 centrifuge tubes. If the supernatant is not clear, centrifuge again to remove remaining bacteria.
6. Precipitate the phage from the supernatant by adding 1/5 volume PEG solution in two GS3 tubes. Incubate for 1 h at 4°C with gentle shaking, followed by centrifugation for 1 h at $10,000 \times g$.
7. Discard the supernatant, resolve each pellet in 10 ml phage dilution buffer in SS34 centrifuge tubes and add 1/5 volume PEG solution.
8. Incubate on ice for 20 min and pellet the phage by centrifugation for 30 min at $10,000 \times g$.
9. Discard the supernatant and put the open tubes upside down on tissue paper. Let the viscous PEG solution move out completely. Resuspend the phage pellet in 1 ml phage dilution buffer. Titre the phage preparation (*see Section 3.2.4*). Store the packaged antibody phage library at 4°C.
10. The library packaging should be controlled by 10% SDS-PAGE, Western-Blot and anti-pIII immunostain (mouse anti-pIII 1:2000, goat anti-mouse IgG AP conjugate 1:10,000). Wildtype pIII has a calculated molecular mass

of 42.5 kDa, but it runs at an apparent molecular mass of 65 kDa in SDS-PAGE. Accordingly, the scFv::pIII fusion protein runs at about 95 kDa.

3.2. Antibody Selection (Panning)

The in vitro procedure for isolating antibody fragments by their binding activity was called “panning,” referring to the gold washers tool (62). The antigen is immobilized to a solid surface, such as nitrocellulose, e.g. (63), magnetic beads, e.g. (64), column matrixes, e.g. (19) or most widely used or plastic surfaces with high protein binding capacity as polystyrene tubes, respectively, microtitre wells, e.g. (65). The antibody phage are incubated with the surface-bound antigen, followed by stringent washing to remove the vast excess of non-binding antibody phage. Subsequently, the bound antibody phage will be eluted and reamplified by infection of *E. coli*. The selection cycle will be repeated by infection of the phagemid bearing *E. coli* colonies from the former panning round with a helperphage to produce new antibody phage, which can be used for further panning rounds until a significant enrichment of antigen specific phage is achieved. The number of antigen-specific antibody phage clones should increase with every panning round. Usually 2–3 panning rounds, sometimes up to 6, are necessary to select specifically binding antibody fragments.

The first step in the evaluation process of potential binders is often done – but not a requisite – by an ELISA of the polyclonal phage preparations from each panning round against the target antigen and control proteins, e.g. BSA. In the next step, antibody clones are isolated from panning rounds showing a significant enrichment of specific antigen binding in the polyclonal phage ELISA. These antibodies clones are produced as soluble monoclonal antibody fragments in microtitre plates followed by an antigen ELISA. An ELISA with monoclonal phage preparations should be omitted because often antibody clones that bind only as scFv-phage or scFv-pIII fusion but not as soluble scFv fragment are identified. The complete panning and screening procedure is given in Fig. 11.1.

The following protocols describe the selection of recombinant antibody fragments from antibody gene libraries by phage display and the screening of the selected antibody fragments.

3.2.1. Coating of Microtitre Plate Wells

1. (A) Protein antigen: For the first panning round, use 2–10 µg protein/well per panning; for the following rounds use 0.1–1 µg protein/well for more stringent conditions. Dissolve the antigen in 150 µl PBS and incubate it into a microtitre plate well overnight at 4°C (see Note 11).
(B) Oligopeptide antigen: Use 100–500 ng oligopeptide for each panning round. Dissolve the oligopeptide in 150 µl

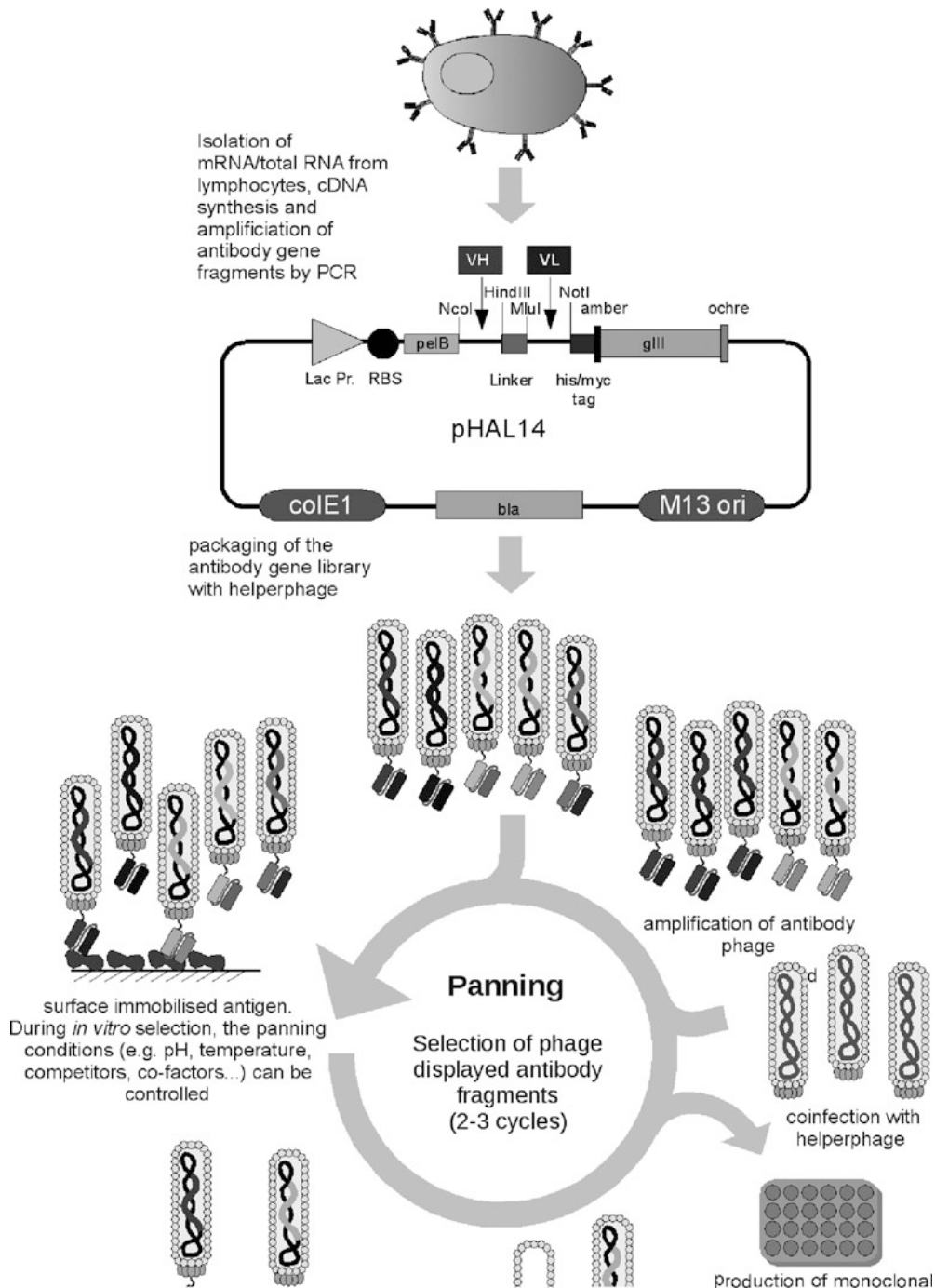


Fig. 11.1. Schematic overview about of the construction of antibody gene libraries and selection of antibodies ("panning") by phage display.

PBS, transfer into the microtitre plate well and incubate overnight at 4°C (*see Note 12*).

2. Wash the coated microtitre plate wells 3× with PBST using an ELISA washer (*see Note 13*).

3.2.2. Panning

1. (A) Block the antigen coated wells with MPBST for 2 h at RT. The wells must be completely filled. Afterwards, wash the blocked antigen coated wells 3× with PBST (*see Note 13*).

(B) *You need to perform this step only in the first panning round but it also can be done in the following rounds!* In parallel, block an additional well (without antigen!) per panning with MPBST for 1 h at RT for preincubation of the antibody gene library. The wells must be completely filled. Wash 3× times with PBST (*see Note 13*). Incubate 10^{11} – 10^{12} antibody phage from the library in 150 µl panning block for 1 h at RT. This step removes unspecific binders, which often occur from the antibody gene libraries due to incorrect folding of individual antibodies.

2. Carry over the preincubated antibody phage library to the blocked wells or fill 10^{11} – 10^{12} amplified phage solved in 150 µl panning block from the former panning round in the blocked wells. Incubate at RT for 2 h for binding of the antibody phage.
3. Remove the unspecifically bound antibody phage by stringent washing. Therefore, wash the wells 10× with an ELISA washer in the first panning round. In the following panning rounds increase the number of washing steps (20× in the second panning round, 30× in the third panning round. . .) (*see Note 13*).
4. Elute bound antibody phage with 200 µl Trypsin solution for 30 min at 37°C (*see Note 14*).
5. Use 10 µl of the eluted phage for titration (*see Section 3.2.4*).
6. Inoculate 50 ml 2xTY-T with an overnight culture of *E. coli* XL1-Blue MRF' in 100-ml Erlenmeyer flasks and grow at 250 rpm and 37°C.
7. Infect exponential ($\text{O.D.}_{600\text{ nm}} \sim 0.5$, after 2–3 h) growing 20 ml XL1-Blue MRF' culture with the remaining 190 µl of the eluted phage. Incubate 30 min at 37°C without shaking and the following 30 min with 250 rpm at 37°C.
8. Harvest the infected bacteria by centrifugation for 10 min at $3200\times g$ in 50 ml polypropylene tubes. Resolve the pellet in 250 µl SOB-GA and plate the bacteria suspension on SOB-GA agar plates (15-cm petri dish). Grow overnight at 37°C (*see Note 15*).

9. Harvest the grown colonies by suspending in 2.5 ml 2xTY-GA with a Drigalsky spatula.
10. Use 100 μ l of the harvested bacteria for the amplification of the eluted phage (*see* **Section 3.2.3**).
11. Make a glycerol stock of the panning round by adding 250 μ l glycerol to 750 ml of the harvested bacteria. Mix and store at -80°C .

3.2.3. Packaging of Phagemids (scFv Phage Production)

1. For the next panning round the eluted phage must be packaged and reamplified. Inoculate 30 ml 2xTY-GA in a 100 ml Erlenmeyer flask with 100 μ l harvested bacteria ($\text{O.D.}_{600} < 0.1$). Grow at 250 rpm at 37°C up to an $\text{O.D.}_{600\text{ nm}} \sim 0.5$.
2. Infect 5 ml bacteria culture ($\sim 2.5 \times 10^9$ cells) with 5×10^{10} cfu of the helper phage M13K07 ($\text{moi} = 1:20$). Incubate for 30 min without shaking at 37°C and for the following 30 min at 250 rpm at 37°C .
3. To remove the glucose, harvest the cells by centrifugation for 10 min at $3200 \times g$ in 50-ml polypropylene tubes.
4. Resuspend the pellet in 30 ml 2xTY-AK and transfer into a 100-ml Erlenmeyer flask. Produce the phage for 16 h at 250 rpm and 30°C .
5. Pellet the bacteria by centrifugation for 10 min at $3200 \times g$ in 50-ml polypropylene tubes. If the supernatant is not clear, centrifuge again to remove remaining bacteria.
6. Precipitate the phage in the supernatant by adding 1/5 volume PEG solution in 50-ml polypropylene tubes. Incubate for 1 h at 4°C with gentle shaking.
7. Pellet the phage by centrifugation for 1 h at $3200 \times g$ and 4°C . Discard the supernatant and put the open tubes upside down on tissue paper. Let the viscous PEG solution move out completely. Resuspend the phage pellet in 500 μ l phage dilution buffer. Titre the phage preparation and use it for the next panning round. Store the remaining phage at 4°C .

3.2.4. Phage Titration

1. Inoculate 5 ml 2xTY-T in a 100 ml Erlenmeyer flask with XL1-Blue MRF' and grow overnight at 37°C and 250 rpm.
2. Inoculate 50 ml 2xTY-T with 500 μ l overnight culture and grow at 250 rpm at 37°C up to $\text{O.D.}_{600} \sim 0.5$ (*see* **Note 16**).
3. Make serial dilutions of the phage suspension in PBS. The number of eluted phages depends on several parameters (e.g. antigen, library, panning round and washing stringency). In case of a successful enrichment, the titre of eluted phage usually is $10^3 - 10^5$ phage per well after the first panning round

and increases two to three orders in magnitude per additional panning round (*see* **Note 17**). The phage preparation after reamplification of the eluted phage have a titre of about 10^{12} – 10^{14} phage/ml.

4. Infect 50 μ l bacteria with 10 μ l phage dilution and incubate 30 min at 37°C (*see* **Note 18**).
5. You can perform titrations in two different ways:
 - (A) plate the 60 μ l infected bacteria on 2xTY-GA agar plates (9 cm petri dishes).
 - (B) pipette 10 μ l (in triplicate) on 2xTY-GA agar plates. Here, about 20 titering spots can be placed on one 9-cm petri dish. Dry drops under workbench.
6. Incubate the plates overnight at 37°C.
7. Count the colonies and calculate the cfu or cfu/ml titre according to the dilution.

3.2.5. ELISA of a Polyclonal Antibody Phage Suspension

1. To investigate the enrichment of antigen specific antibody phage after a panning round, coat microtitre plate wells with 100–1000 ng antigen per well for each panning round (for method *see* **Section 3.1**). As a control, coat other wells with 100–1000 ng BSA in 150 μ l PBS overnight at 4°C (*see* **Note 19**).
2. Wash the coated microtitre plate wells 3 $\times$ with PBST using an ELISA washer (for washing procedure *see* **Section 3.2** and **Note 13**).
3. Block the antigen-coated wells with MPBST for 2 h at RT. The wells must be completely filled.
4. Wash the coated microtitre plate wells 3 $\times$ with PBST (for washing procedure *see* **Section 3.2** and **Note 13**).
5. Resuspend 10^9 and 10^{10} cfu/well antibody phage from each panning round in 150 μ l 2%MPST and incubate them for 1.5 h on the antigen and the BSA control, respectively.
6. Wash the microtitre plate wells 3 $\times$ with PBST (for washing procedure *see* **Section 3.2** and **Note 13**).
7. Incubate each well with 100 μ l HRP-conjugated anti-M13 antibody 1:5000 diluted in 2% MPBST for 1.5 h.
8. Wash the microtitre plate wells 3 $\times$ with PBST (for washing procedure *see* **Section 3.2** and **Note 13**).
9. Shortly before use, mix 19 parts TMB substrate solution A and 1 part TMB substrate solution B. Add 100 μ l of this TMB solution to each well and incubate for 1–15 min.
10. Stop the substrate reaction by adding 100 μ l 1 N sulphuric acid. The colour turns from blue to yellow.
11. Measure the extinction at 450 nm using an ELISA reader.

**3.2.6. Production
of Soluble Monoclonal
Antibody Fragments in
Microtitre Plates**

1. Fill each well of a 96-well U-bottom polypropylene microtitre plate with 150 μ l 2xTY-GA.
2. Pick 92 clones with sterile tips from the desired panning round (*see* **Note 20**) and inoculate each well (*see* **Note 21**). Seal the plate with a breathable sealing film.
3. Incubate overnight in a microtitre plate shaker at 37°C and 1000 rpm.
4. (A) Fill a new 96-well polypropylene microtitre plate with 150 μ l 2xTY-GA and add 10 μ l of the overnight cultures. Incubate for 2 h at 37°C and 1000 rpm.
(B) Add 30 μ l glycerol solution to the remaining 140 μ l overnight cultures. Mix by pipetting and store this master-plate at -80°C.
5. Pellet the bacteria in the microtitre plates by centrifugation for 10 min at 3200 $\times g$ and 4°C. Remove 180 μ l glucose containing media by carefully pipetting (do not disturb the pellet).
6. Add 180 μ l buffered 2xTY-SAI (containing saccharose, ampicillin and 50 μ M IPTG) and incubate overnight at 30°C and 1000 rpm (*see* **Note 22**).
7. Pellet the bacteria by centrifugation for 10 min at 3200 $\times g$ in the microtitre plates. Transfer the antibody fragment containing supernatant to a new polypropylene microtitre plate and store at 4°C.

**3.2.7. ELISA of Soluble
Monoclonal Antibody
Fragments**

1. To analyse the antigen specificity of the monoclonal soluble antibody fragments, coat 100–1000 ng antigen per well overnight at 4°C. As control coat 100–1000 ng BSA per well (*see* **Section 3.1, Note 19**).
2. Wash the coated microtitre plate wells 3 $\times$ with PBST (for washing procedure *see* **Section 3.2** and **Note 3**).
3. Block the antigen coated wells with MPBST for 2 h at RT. The wells must be completely filled.
4. Fill 50 μ l MPBST in each well and add 50 μ l of antibody solution (*see* **Section 3.6**). Incubate for 1.5 h at RT (or overnight at 4°C).
5. Wash the microtitre plate wells 3 $\times$ with PBST (for washing procedure *see* **Section 3.2** and **Note 3**).
6. Incubate 100 μ l mouse 9E10 α -myc tag antibody solution for 1.5 h (appropriate dilution in MPBST).
7. Wash the microtitre plate wells 3 $\times$ with PBST (for washing procedure *see* **Section 3.2** and **Note 13**).
8. Incubate 100 μ l goat α -mouse HRP conjugate (1:10,000 in MPBST).

9. Wash the microtitre plate wells $3\times$ with PBST (for washing procedure *see* **Section 3.2** and **Note 3**).
10. Shortly before use, mix 19 parts TMB substrate solution A and 1 part TMB substrate solution B. Add 100 μl of this TMB solution into each well and incubate for 1–15 min.
11. Stop the colour reaction by adding 100 μl 1 N sulphuric acid. The colour turns from blue to yellow.
12. Measure the extinction at 450 nm using an ELISA reader.
13. Identify positive candidates with a signal (on antigen) $10\times$ over noise (on control protein, e.g. BSA) (*see* **Note 23**).
14. DNA sequencing of binders is performed with appropriate oligonucleotide primers (MKpelB_f and MKmyc_r or with MHLacZ-Pro_f). The antibody sequences can be analysed by VBASE2 (www.vbase2.org) (**66**).

4. Notes

1. Be careful with human blood samples since it is potentially infectious (HIV, hepatitis, etc.)!
2. Both methods, mRNA or total RNA isolation, work well.
3. Check the cDNA quality using standard glyceraldehyde 3-phosphate dehydrogenase (GAPDH) oligonucleotide primers. Do not use cDNA preparations if the GAPDH fragment could not be amplified by PCR.
4. The VH amplifications of VH subfamilies sometimes results also in longer PCR products. Cut out only the ~ 380 bp fragment. The amplifications of kappa subfamilies should always give a clear ~ 650 bp fragment. When amplifying lambda subfamilies often other PCR products are generated, especially the amplification of the lambda2 subfamily results often in slushy bands. If some subfamilies are bad amplified and no clear ~ 650 bp fragment is detectable, use only the ~ 650 bp fragments from the well amplified subfamilies. Additional comment: since the first PCR amplifies the full LC, it can be used also to construct a Fab or scFab (**67**) libraries from this material.
5. For a very large naive antibody gene library perform as many PCRs as sufficient to perform 20 light chains ligations/transformations and about 100 VH ligations. For an immune library 4 light chains ligations/transformations and 8 VH ligations are usually sufficient. Prepare and digest

also adequate amounts of pHAL14 and VL for the first cloning step and pHAL14-VL library and VH for the second cloning step. Keep kappa and lambda in all steps (cloning, packaging) separately and mix only after phage production before panning.

6. Always perform single digests using only one enzyme in parallel to control the success of the restriction reaction. Analyse the digestion by TAE agarose gel electrophoresis by comparing with the undigested plasmid. Use only material where both single digests are successful and where no degradation is visible in the double digest.
7. The pulse time should be between 4 and 5 ms for optimal electroporation efficiency.
8. Often the HindIII digestion is incomplete after 2 h. Then, inactivate the enzymes by heating up to 65°C for 10 min, add additional 5 µl of HindIII and incubate overnight. You can use also higher concentrated HindIII. Alternatively, perform the NcoI digest first for 2 h, inactivate the digest and afterwards perform the HindIII digest.
9. To minimize loss of diversity, avoid to many freeze and thaw steps, e.g. when constructing an immune library make eight transformations in parallel and directly package the immune library.
10. The use of Hyperphage as helperphage instead of M13K07 offers oligovalent phage display, facilitates the selection of specific binders in the first and most critical panning round by avidity effect (32, 44, 68, 69). The Hyperphage should be only used for library packaging. For the following panning rounds use M13K07 to enhance the stringency of the panning process.
11. If the protein is not binding properly to the microtitre plate surface, try bicarbonat buffer (50 mM NaHCO₃, pH 9.6).
12. More hydrophobic oligopeptides may need to be dissolved in PBS containing 5% DMSO. If biotinylated oligopeptide is used as antigen for panning, dissolve 100 ng Streptavidin in 150 µl PBS and coat overnight at 4°C. Coat two wells for each panning, one well is for the panning, the second one for the preincubation of the library to remove streptavidin binders! Sometimes it is necessary to use free streptavidin during panning for competition to remove streptavidin binders. Pour out the wells and wash 3× with PBST. Dissolve 100–500 ng biotinylated oligopeptide in PBS and incubate for 1 h at RT. Alternatively, oligopeptides with a terminal cysteine residue can be coupled to BSA and coated overnight at 4°C.

13. The washing should be performed with an ELISA washer (e.g. TECAN Columbus Plus) to increase the stringency and reproducibility. To remove antigen or blocking solutions wash 3× with PBST (“standard washing protocol” for TECAN washer). If no ELISA washer is available, wash manually 3× with PBST.
After binding of antibody phage, wash 10× with PBST (“stringent bottom washing protocol” in case of TECAN washer). If no ELISA washer is available, wash manually 10× with PBST and 10× with PBS. For stringent off-rate selection increase the number of washing steps or additionally incubate the microtitre plate in 1 L PBS for several days.
14. Phagemids like pSEX81 (41) or pHAL14 (32, 44, 56) have coding sequences for a trypsin specific cleavage site between the antibody fragment gene and the gIII. Trypsin also cleaves within antibody fragments but does not degrade the phage particles including the pIII that mediates the binding of the phage to the F pili of *E. coli* required for the infection. We observed that proteolytic cleavage of the antibody fragments from the antibody::pIII fusion by trypsin not only increases the elution but also enhances the infection rate of eluted phage particles, especially when using Hyperphage as helperphage (68–70).
15. The high concentration of glucose is necessary to efficiently repress the lac promoter controlling the antibody::pIII fusion gene on the phagemid. Low glucose concentrations lead to an inefficient repression of the lac promoter and background expression of the antibody::pIII fusion protein. Background antibody expression is a strong selection pressure frequent causing mutations in the phagemid, especially in the promoter region and the antibody::pIII fusion gene. Bacteria with this kind of mutations in the phagemids proliferate faster than bacteria with non-mutated phagemids. Therefore, the 100 mM glucose must be included in every step of *E. coli* cultivation except during the phage production!
16. If the bacteria have reached O.D.₆₀₀ ~ 0,5 before they are needed, store the culture immediately on ice to maintain the F pili on the *E. coli* cells for several hours. M13K07 helperphage (kan⁺) or other scFv-phage (amp⁺) can be used as positive control to check the infectibility of the *E. coli* cells.
17. If the antibody gene library is packaged using Hyperphage, the titre of the eluted phage after the second panning may

not increase as strongly or even decreases slightly due to the change from oligovalent to monovalent display.

18. Check all solutions for phage contamination. To check the PBS or PEG solutions use 10 μ l of these solution for *E. coli* “infection.” In parallel plate out non-infected XL1-Blue MRF’ to check the bacteria. We recommend to clean the working place each time with virus inactivating solutions (e.g. Barrycidal 36, BIO-HIT, Germany) and to use filter tips for pipetting!
19. Antibody phage which bind unspecifically are usually enriched during panning. These unspecific binding often results from misfolded or incomplete antibodies. They often bind to BSA, Streptavidin and plastic surfaces.
20. Use the polyclonal antibody phage ELISA to select the suitable panning round for picking monoclonals.
21. We recommend picking only 92 clones. Use the wells H3, H6, H9 and H12 for controls. H3 and H6 are negative controls – these wells will not be inoculated and not used for the following ELISA with soluble antibodies. We inoculate the wells H9 and H12 with a clone containing a phagemid encoding a known antibody fragment. In ELISA, the wells H9 and H12 are coated with the antigen corresponding to the control antibody fragment in order to check scFv production and ELISA.
22. The appropriate IPTG concentration for induction of antibody or antibody::pIII expression depends on the vector design. A concentration of 50 μ M was well suited for vectors with a Lac promoter like pSEX81 (41), pIT2 (71), pHENIX (72) and pHAL14 (32, 44, 56). The method for the production of soluble antibodies works with vectors with (e.g. pHAL14) and without (e.g. pSEX81) an amber stop codon between antibody fragment and gIII. If the vector has no amber stop codon the antibody::pIII fusion protein will be produced (73). Buffered culture media and the addition of saccharose enhances the production of many but not all scFvs. We observed that antibody::pIII fusion proteins and antibody phage sometimes show differences in antigen binding in comparison to soluble antibody fragments because some antibodies can bind the corresponding antigen only as pIII fusion. Therefore, we recommend to perform the screening procedure only by using soluble antibody fragment, to avoid false positive binders.
23. The background (noise) signals should be about O.D.₄₅₀ $\sim$ 0.02 after 5–30 min TMB incubation time.

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References

1. Dübel, S. (2007) Recombinant therapeutic antibodies. *Appl Microbiol Biotechnol* **74**, 723–729.
2. Coiffier, B. (2008) Monoclonal antibodies in the treatment of malignant lymphomas. *Adv Exp Med Biol* **610**, 155–176.
3. Cao, Y. (2008) Molecular mechanisms and therapeutic development of angiogenesis inhibitors. *Adv Cancer Res* **100**, 113–131.
4. Jones, S. E. (2008) Metastatic breast cancer: the treatment challenge. *Clin Breast Cancer* **8**, 224–233.
5. Chatenoud, L., and Bluestone, J. A. (2007) CD3-specific antibodies: a portal to the treatment of autoimmunity. *Nat Rev Immunol* **7**, 622–632.
6. Courtenay-Luck, N. S., Epenetos, A. A., Moore, R., Larche, M., Pectasides, D., Dhokia, B., and Ritter, M. A. (1986) Development of primary and secondary immune responses to mouse monoclonal antibodies used in the diagnosis and therapy of malignant neoplasms. *Cancer Res* **46**, 6489–6493.
7. Moroney, S., and Plückthun, A. (2005) Modern antibody technology: the impact on drug development. In: Knäblein, J. (ed.) *Modern Biopharmaceuticals*. Wiley-VCH, Weinheim, pp. 1147–1186.
8. Moss, M. L., Sklair-Tavron, L., and Nudelman, R. (2008) Drug insight: tumor necrosis factor-converting enzyme as a pharmaceutical target for rheumatoid arthritis. *Nat Clin Pract Rheumatol* **4**, 300–309.
9. Dalle, S., Thieblemont, C., Thomas, L., and Dumontet, C. (2008) Monoclonal antibodies in clinical oncology. *Anti-Cancer Agents Med Chem* **8**, 523–532.
10. Alonso-Ruiz, A., Pijoan, J. I., Ansuategui, E., Urkaregi, A., Calabozo, M., and Quintana, A. (2008) Tumor necrosis factor alpha drugs in rheumatoid arthritis: systematic review and metaanalysis of efficacy and safety. *BMC Musculoskeletal Disord* **9**, 52.
11. Osbourn, J., Groves, M., and Vaughan, T. (2005) From rodent reagents to human therapeutics using antibody guided selection. *Methods* **36**, 61–68.
12. Fishwild, D. M. et al. (1996) High-avidity human IgG kappa monoclonal antibodies from a novel strain of minilocus transgenic mice. *Nat Biotechnol* **14**, 845–851.
13. Lonberg, N., and Huszar, D. (1995) Human antibodies from transgenic mice. *Int Rev Immunol* **13**, 65–93.
14. Jakobovits, A. (1995) Production of fully human antibodies by transgenic mice. *Curr Opin Biotechnol* **6**, 561–566.
15. Winter, G., and Milstein, C. (1991) Man-made antibodies. *Nature* **349**, 293–299.
16. Huse, W. D., Sastry, L., Iverson, S. A., Kang, A. S., Alting-Mees, M., Burton, D. R., Benkovic, S. J., and Lerner, R. A. (1989) Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* **246**, 1275–1281.
17. Smith, G. P. (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* **228**, 1315–1317.
18. McCafferty, J., Griffiths, A. D., Winter, G., and Chiswell, D. J. (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **348**, 552–554.
19. Breitling, F., Dübel, S., Seehaus, T., Klewinghaus, I., and Little, M. (1991) A surface expression vector for antibody screening. *Gene* **104**, 147–153.
20. Clackson, T., Hoogenboom, H. R., Griffiths, A. D., and Winter, G. (1991) Making antibody fragments using phage display libraries. *Nature* **352**, 624–628.
21. Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P., and Winter, G. (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acids Res* **19**, 4133–4137.
22. Marks, J. D., Hoogenboom, H. R., Bonnert, T. P., McCafferty, J., Griffiths, A. D., and Winter, G. (1991) By-passing immunization.

- Human antibodies from V-gene libraries displayed on phage. *J Mol Biol* 222, 581–597.
23. Barbas, C. F., Kang, A. S., Lerner, R. A., and Benkovic, S. J. (1991) Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc Natl Acad Sci USA* 88, 7978–7982.
24. Mazor, Y., Van Blarcom, T., Mabry, R., Iverson, B. L., and Georgiou, G. (2007) Isolation of engineered, full-length antibodies from libraries expressed in *Escherichia coli*. *Nat Biotechnol* 25, 563–565.
25. Simmons, L. C. et al. (2002) Expression of full-length immunoglobulins in *Escherichia coli*: rapid and efficient production of aglycosylated antibodies. *J Immunol Methods* 263, 133–147.
26. Thie, H., Voedisch, B., Dübel, S., Hust, M., and Schirrmann, T. (2009) Affinity maturation by phage display. *Methods Mol Biol* 525, 309–322.
27. Hanes, J., and Plückthun, A. (1997) In vitro selection and evolution of functional proteins by using ribosome display. *Proc Natl Acad Sci USA* 94, 4937–4942.
28. He, M., and Taussig, M. J. (1997) Antibody-ribosome-mRNA (ARM) complexes as efficient selection particles for in vitro display and evolution of antibody combining sites. *Nucleic Acids Res* 25, 5132–5134.
29. Roberts, R. W., and Szostak, J. W. (1997) RNA-peptide fusions for the in vitro selection of peptides and proteins. *Proc Natl Acad Sci USA* 94, 12297–12302.
30. Boder, E. T., and Wittrup, K. D. (1997) Yeast surface display for screening combinatorial polypeptide libraries. *Nat Biotechnol* 15, 553–557.
31. Thie, H., Meyer, T., Schirrmann, T., Hust, M., and Dübel, S. (2008) Phage display derived therapeutic antibodies. *Curr Pharm Biotechnol* 9, 439–446.
32. Pelat, T., Hust, M., Laffly, E., Condemine, F., Bottex, C., Vidal, D., Lefranc, M., Dübel, S., and Thullier, P. (2007) High-affinity, human antibody-like antibody fragment (single-chain variable fragment) neutralizing the lethal factor (LF) of *Bacillus anthracis* by inhibiting protective antigen-LF complex formation. *Antimicrob Agents Chemother* 51, 2758–2764.
33. de Haard, H. J., van Neer, N., Reurs, A., Hufton, S. E., Roovers, R. C., Henderikx, P., de Bruïne, A. P., Arends, J. W., and Hoogenboom, H. R. (1999) A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. *J Biol Chem* 274, 18218–18230.
34. Pini, A., Viti, F., Santucci, A., Carnemolla, B., Zardi, L., Neri, P., and Neri, D. (1998) Design and use of a phage display library. Human antibodies with subnanomolar affinity against a marker of angiogenesis eluted from a two-dimensional gel. *J Biol Chem* 273, 21769–21776.
35. Hoet, R. M. et al. (2005) Generation of high-affinity human antibodies by combining donor-derived and synthetic complementarity-determining-region diversity. *Nat Biotechnol* 23, 344–348.
36. Hayashi, N., Welschof, M., Zewe, M., Braunagel, M., Dübel, S., Breitling, F., and Little, M. (1994) Simultaneous mutagenesis of antibody CDR regions by overlap extension and PCR. *Biotechniques* 17, 310–315.
37. Knappik, A., Ge, L., Honegger, A., Pack, P., Fischer, M., Wellenhofer, G., Hoess, A., Wölle, J., Plückthun, A., and Virnekäs, B. (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J Mol Biol* 296, 57–86.
38. Sblattero, D., and Bradbury, A. (2000) Exploiting recombination in single bacteria to make large phage antibody libraries. *Nat Biotechnol* 18, 75–80.
39. Hust, M., and Dübel, S. (2004) Mating antibody phage display with proteomics. *Trends Biotechnol* 22, 8–14.
40. Hust, M., Dübel, S., and Schirrmann, T. (2007) Selection of recombinant antibodies from antibody gene libraries. *Methods Mol Biol* 408, 243–255.
41. Welschof, M., Terness, P., Kipriyanov, S. M., Stanescu, D., Breitling, F., Dörsam, H., Dübel, S., Little, M., and Opelz, G. (1997) The antigen-binding domain of a human IgG-anti-F(ab')₂ autoantibody. *Proc Natl Acad Sci USA* 94, 1902–1907.
42. Johansen, L. K., Albrechtsen, B., Andersen, H. W., and Engberg, J. (1995) pFab60: a new, efficient vector for expression of antibody Fab fragments displayed on phage. *Protein Eng* 8, 1063–1067.
43. Little, M. et al. (1999) Generation of a large complex antibody library from multiple donors. *J Immunol Methods* 231, 3–9.
44. Kirsch, M., Hülseweh, B., Nacke, C., Rülker, T., Schirrmann, T., Marschall, H., Hust, M., and Dübel, S. (2008) Development of human antibody fragments using antibody phage display for the detection and diagnosis of Venezuelan equine encephalitis virus (VEEV). *BMC Biotechnol* 8, 66.
45. McCafferty, J., Fitzgerald, K. J., Earnshaw, J., Chiswell, D. J., Link, J., Smith, R., and

- Kenten, J. (1994) Selection and rapid purification of murine antibody fragments that bind a transition-state analog by phage display. *Appl Biochem Biotechnol* **47**, 157–171.
46. Vaughan, T. J., Williams, A. J., Pritchard, K., Osbourn, J. K., Pope, A. R., Earnshaw, J. C., McCafferty, J., Hodits, R. A., Wilton, J., and Johnson, K. S. (1996) Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nat Biotechnol* **14**, 309–314.
47. Krebber, A., Bornhauser, S., Burmester, J., Honegger, A., Willuda, J., Bosshard, H. R., and Plückthun, A. (1997) Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. *J Immunol Methods* **201**, 35–55.
48. Shirai, H., Kidera, A., and Nakamura, H. (1999) H3-rules: identification of CDR-H3 structures in antibodies. *FEBS Lett* **455**, 188–197.
49. Akamatsu, Y., Cole, M. S., Tso, J. Y., and Tsurushita, N. (1993) Construction of a human Ig combinatorial library from genomic V segments and synthetic CDR3 fragments. *J Immunol* **151**, 4651–4659.
50. Hoogenboom, H. R., and Winter, G. (1992) By-passing immunisation. Human antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro. *J Mol Biol* **227**, 381–388.
51. Nissim, A., Hoogenboom, H. R., Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D., and Winter, G. (1994) Antibody fragments from a 'single pot' phage display library as immunochemical reagents. *EMBO J* **13**, 692–698.
52. Desiderio, A., Franconi, R., Lopez, M., Villani, M. E., Viti, F., Chiaraluce, R., Consalvi, V., Neri, D., and Benvenuto, E. (2001) A semi-synthetic repertoire of intrinsically stable antibody fragments derived from a single-framework scaffold. *J Mol Biol* **310**, 603–615.
53. Barbas, C. F., Bain, J. D., Hockstra, D. M., and Lerner, R. A. (1992) Semisynthetic combinatorial antibody libraries: a chemical solution to the diversity problem. *Proc Natl Acad Sci USA* **89**, 4457–4461.
54. Jirholt, P., Ohlin, M., Borrebaeck, C. A., and Söderlind, E. (1998) Exploiting sequence space: shuffling in vivo formed complementarity determining regions into a master framework. *Gene* **215**, 471–476.
55. Söderlind, E. et al. (2000) Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries. *Nat Biotechnol* **18**, 852–856.
56. Schütte, M., Thullier, P., Pelat, T., Wezler, X., Rosenstock, P., Hinz, D., Kirsch, M.I., Hasenberg, M., Frank, R., Schirrmann, T., Gunzer, M., Hust, M., and Dübel, S. (2009) Identification of a putative Crf splice variant and generation of recombinant antibodies for the specific detection of *Aspergillus fumigatus*. *PLoS ONE* **4**, e6625.
57. Løset, G. A., Løbersli, I., Kavlie, A., Stacy, J. E., Borgen, T., Kausmally, L., Hvattum, E., Simonsen, B., Hovda, M. B., and Brekke, O. H. (2005) Construction, evaluation and refinement of a large human antibody phage library based on the IgD and IgM variable gene repertoire. *J Immunol Methods* **299**, 47–62.
58. Sheets, M. D. et al. (1998) Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. *Proc Natl Acad Sci USA* **95**, 6157–6162.
59. Waterhouse, P., Griffiths, A. D., Johnson, K. S., and Winter, G. (1993) Combinatorial infection and in vivo recombination: a strategy for making large phage antibody repertoires. *Nucleic Acids Res* **21**, 2265–2266.
60. Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M., Waterhouse, P., Crosby, W. L., Kontermann, R. E., Jones, P. T., Low, N. M., and Allison, T. J. (1994) Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J* **13**, 3245–3260.
61. Geoffroy, F., Sodoyer, R., and Aujame, L. (1994) A new phage display system to construct multicombinatorial libraries of very large antibody repertoires. *Gene* **151**, 109–113.
62. Parmley, S. F., and Smith, G. P. (1988) Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. *Gene* **73**, 305–318.
63. Hawlisch, H., Müller, M., Frank, R., Bautsch, W., Klos, A., and Köhl, J. (2001) Site-specific anti-C3a receptor single-chain antibodies selected by differential panning on cellulose sheets. *Anal Biochem* **293**, 142–145.
64. Moghaddam, A., Borgen, T., Stacy, J., Kausmally, L., Simonsen, B., Marvik, O. J., Brekke, O. H., and Braunagel, M. (September 2003) Identification of scFv antibody fragments that specifically recognise the heroin metabolite 6-monoacetylmorphine but not morphine. *J Immunol Methods* **280**, 139–155.
65. Hust, M., Maiss, E., Jacobsen, H., and Reinard, T. (2002) The production of a genus-specific recombinant antibody (scFv)

- using a recombinant potyvirus protease. *J Virol Methods* **106**, 225–233.
66. Retter, I., Althaus, H. H., Münch, R., and Müller, W. (2005) VBASE2, an integrative V gene database. *Nucleic Acids Res* **33**, D671–D674.
67. Hust, M., Jostock, T., Menzel, C., Voedisch, B., Mohr, A., Brenneis, M., Kirsch, M. I., Meier, D., and Dübel, S. (2007) Single chain Fab (scFab) fragment. *BMC Biotechnol* **7**, 14.
68. Rondot, S., Koch, J., Breitling, F., and Dübel, S. (2001) A helper phage to improve single-chain antibody presentation in phage display. *Nat Biotechnol* **19**, 75–78.
69. Soltes, G., Hust, M., Ng, K. K. Y., Bansal, A., Field, J., Stewart, D. I. H., Dübel, S., Cha, S., and Wiersma, E. J. (2007) On the influence of vector design on antibody phage display. *J Biotechnol* **127**, 626–637.
70. Hust, M., Meysing, M., Schirrmann, T., Selke, M., Meens, J., Gerlach, G., and Dübel, S. (2006) Enrichment of open reading frames presented on bacteriophage M13 using hyperphage. *Biotechniques* **41**, 335–342.
71. Goletz, S., Christensen, P. A., Kristensen, P., Blohm, D., Tomlinson, I., Winter, G., and Karsten, U. (2002) Selection of large diversities of antiidiotypic antibody fragments by phage display. *J Mol Biol* **315**, 1087–1097.
72. Finnern, R., Pedrollo, E., Fisch, I., Wieslander, J., Marks, J. D., Lockwood, C. M., and Ouwehand, W. H. (1997) Human autoimmune anti-proteinase 3 scFv from a phage display library. *Clin Exp Immunol* **107**, 269–281.
73. Mersmann, M., Schmidt, A., Tesar, M., Schöneberg, A., Welschhof, M., Kiprianov, S., Terness, P., Little, M., Pfizenmaier, K., and Moosmayer, D. (1998) Monitoring of scFv selected by phage display using detection of scFv-pIII fusion proteins in a microtiter scale assay. *J Immunol Methods* **220**, 51–58.