# Chapter 3
# Construction of scFv Fragments from Hybridoma or Spleen Cells by PCR Assembly

**Jonas V. Schaefer, Annemarie Honegger, and Andreas Plückthun**

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## Abbreviations

| | |
|---|---|
| BSA | Bovine serum albumin |
| DMSO | Dimethylsulfoxide |
| HRP | Horse radish peroxidase |
| IPTG | Isopropylthiogalactoside |
| PEG | Polyethylene glycol |
| PBS | Phosphate buffered saline |
| scFv | Single-chain Fv fragment |
| cfu | Colony forming units |
| *tet* | Tetracycline |

## 3.1 Introduction

Today, antibodies can be obtained from naive repertoires (Winter et al. 1994; Vaughan et al. 1996) or libraries of fully synthetic genes (Knappik et al. 2000), and in the last decade, numerous libraries have been described (reviewed in Mondon et al. 2008). Nonetheless, hybridomas have remained the predominant source of antibodies, and a wealth of well characterized and even unique clones exist and are continuing to be generated. There is, thus, great interest in immortalizing these clones, in the extreme case, as a computer file of the sequences, as well as in accessing the antibody in a variety of new formats. To obtain enough material

J.V. Schaefer, A. Honegger, and A. Plückthun (✉)
Biochemisches Institut, Universität Zürich, Winterthurerstr. 190, 8057 Zürich, Switzerland
e-mail: plueckthun@bioc.uzh.ch

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for detailed biochemical and biophysical analyses of the deduced antibodies after immunization, their cloning into formats compatible with recombinant expression is beneficial, if not essential. For this purpose, the antibody genes must be cloned, and the binding properties of the recombinant protein have to be verified. In addition to existing hybridomas, the immune response of an animal upon exposure to various antigens may often be of particular scientific interest in itself and also lead to the discovery of new and potent binders. Therefore, there is merit in immortalizing the results from new immunizations as well. In this case, it is not necessary to take the detour of first making hybridomas, but instead, mRNA isolated from spleen can be directly used for the creation of an immune library, from which binders can be subsequently isolated by phage display and their sequences determined.

Once the antibody genes have been successfully cloned and after the presumed binding properties of the recombinant antibodies have been experimentally verified, their sequences can be used for modeling (www.bioc.uzh.ch/antibody/), and their structure subsequently be determined by crystallography (Honegger et al. 2005) or NMR (Freund et al. 1994; Tugarinov et al. 2000). The recombinant single-chain Fv format (Huston et al. 1988; Glockshuber et al. 1990) is an ideal starting point for all engineering efforts, from sensors (Backmann et al. 2005; Morfill et al. 2007) to therapeutic fusion proteins (Di Paolo et al. 2003), or imaging reagents (Adams et al. 1993) to multivalent and multispecific reagents (Plückthun and Pack 1997), just to name a few illustrative examples. Recombinant expression of these proteins also allows one to evolve the affinity further than the immune system normally does, e.g., to low picomolar $K_D$ for scFv fragments (Zahnd et al. 2004; Luginbühl et al. 2006). Finally, some natural antibodies may not be of sufficient stability, which can also be corrected by engineering (Wörn and Plückthun 2001; Ewert et al. 2004). In addition, the murine antibody can be humanized for its use in therapy – a procedure rapidly achievable at the scFv stage.

The key prerequisite for the use of recombinant antibody technologies, starting from immune repertoires or defined hybridomas, is the reliable cloning of functional immunoglobulin genes. Even though hybridomas are considered to express "monoclonal" antibodies, hybridoma clones may encode more than one functional or even nonfunctional heavy or light chains (Kütemeier et al. 1992). As has been reported previously, several kappa chain-secreting hybridomas, possessing X63Ag8.653 myeloma cells as fusion partner, also occasionally transcribe a functional lambda chain, competing with the $V_\kappa$ gene for in-frame scFv antibody assembly (Krebber et al. 1997). As these false or heterogeneous genes might also be amplified and subsequently assembled into the scFv fragments, it is highly recommended to include an enrichment procedure in the cloning protocol. This step can be circumvented and replaced by screening of clones at the scFv level, but the phage enrichment is generally much faster if incorrect sequences abound. Obviously, selection by phage display or by another selection technology such as ribosome display (Hanes and Plückthun 1997; Hanes et al. 1998; for detailed protocols see Schaffitzel et al. 2005; Amstutz et al. 2006; Zahnd et al. 2007) is mandatory when starting from spleens of immunized mice.

This chapter largely follows our earlier protocols (Plückthun et al. 1996; Krebber et al. 1997; Burmester and Plückthun 2001). A number of variable antibody domains of hybridomas were accessible with those procedures and reagents whose genes could not be cloned in other experimental setups. The present protocol is based on a standard phage display system, which was optimized for robustness, vector stability, and directional cloning using a single rare cutting restriction enzyme as well as tight control of the expression of the scFv-gene III fusion (Krebber et al. 1997). As the procedures for the construction of scFv fragment libraries from immunized mice and that of cloning one specific antibody from hybridomas are essentially the same, we combined them in just one protocol. However, there are slight differences in the initial preparation of the cells, and high ligation and transformation yields for library cloning are, of course, essential, as explained under "notes."

The current version of this protocol contains improvements in the methods but, most importantly, newly designed primer sequences for the amplification of $V_H$ and $V_L$ genes. They are based on our analysis of a reference set of murine germline sequences found in the most recent version of the IMGT database (http://imgt.cines.fr/textes/vquest/refseqh.html), which thus incorporates most of the knowledge of the mouse genome (for a description of the original database, see Lefranc and Lefranc 2001). Our key criterion was a faithful amplification of the variable region genes preserving as much sequence identity as possible, avoiding the generation of nonnatural residue combinations, which could result in sequences problematic for folding and stability (Honegger and Plückthun 2001; Jung et al. 2001). We also tried to ensure similar annealing temperatures with the different genes, as well as keeping the degeneracy on the DNA level as small as possible. Furthermore, we avoided pronounced secondary structures within the oligonucleotides such as hairpin loops or primer-dimers (which were checked against themselves using the appropriate analysis tools in the Vector NTI software (Invitrogen)). The primers shown below are the result of this iterative process and have also been tested with a slightly different overhang.

The cloning strategy outlined in this protocol (Fig. 3.1) allows the simple conversion of the expression format from the initial scFv fragments to other formats and fusion proteins. Insertion of the assembled scFv gene into the described standard vectors pAK100 and pJB12 leads to the expression of a scFv-gene III fusion applicable for phage display, due to read-through of the amber codons whenever expressed in strains with amber suppressor tRNA such as *Escherichia coli* XL1-Blue. In bacterial strains lacking such suppressor tRNA, the amber stop codons result in translation termination and production of unfused scFv fragments. For purposes of IMAC purification or whenever other fusions will be constructed, it is, however, advantageous to reclone the fragments directly into appropriate vectors (Figs. 3.4 and 3.5) (Plückthun et al. 1996), carrying stronger translation initiation sites. Conversely, it is not advantageous to make expression too strong for phage display, as discussed below. Although not explicitly mentioned, a very similar strategy of cloning (Fig. 3.1) only requiring altered reverse primers can be used for the design of Fab versions of the desired antibodies.

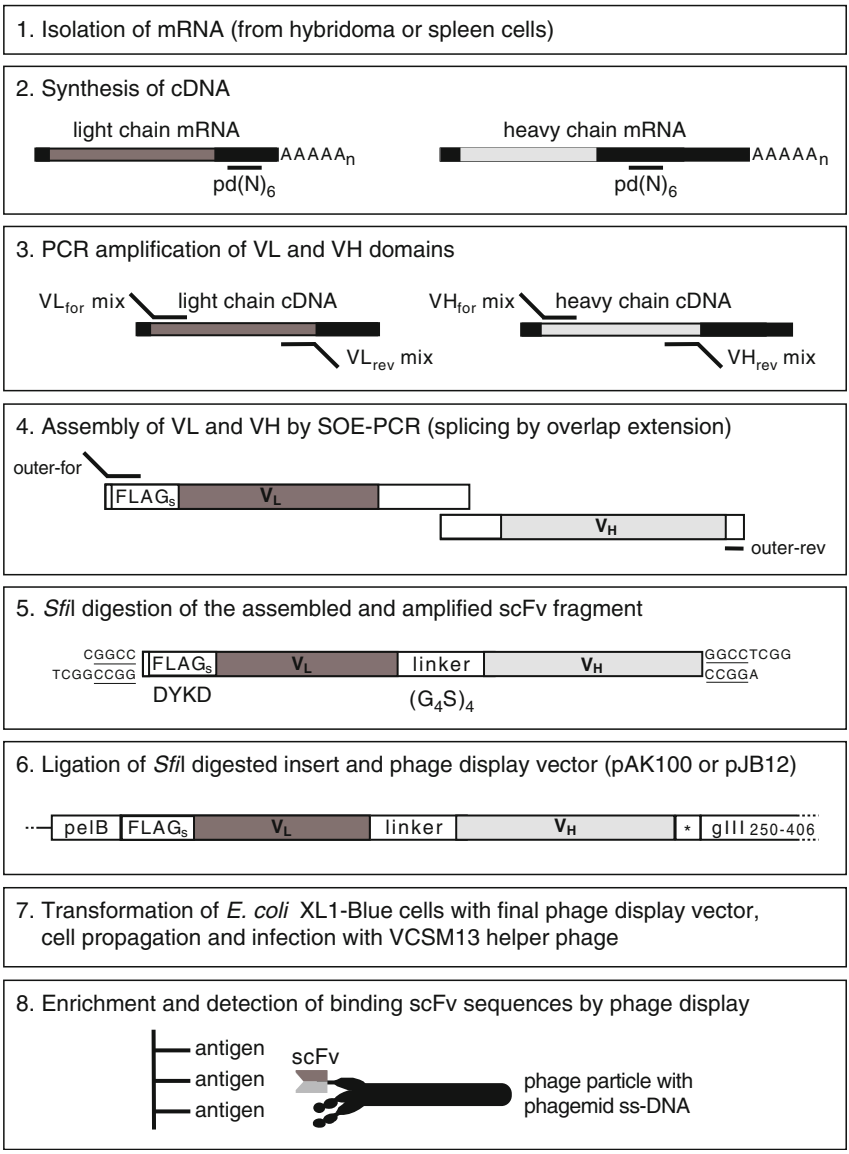



**Fig. 3.1** *Schematic overview of the amplification and cloning procedure*. After its isolation from hybridoma or spleen cells, the mRNA provides the basis for cDNA synthesis, utilizing random hexamer primers. The cDNA is used afterward as template for PCR amplification of $V_L$ and $V_H$ domains (symbolized by the gray boxes, not drawn to scale), which are subsequently assembled by SOE-PCR into the scFv format by the outer primer pair outer-for and outer-rev. For antibody cloning into the phagemid, only the rare cutting enzyme *Sfi*I is used, guaranteeing directional cloning due to the resulting different overhangs at the cleavage site as indicated. In addition, self-ligation of insert or vector molecules is excluded by the asymmetry generated in the cut vector. $FLAG_s$ indicates the shortened *N*-terminal 4-amino acid FLAG tag (Knappik and Plückthun 1994)

## 3.2 Materials

- $5 \times 10^6$ cells from a growing or frozen hybridoma culture or spleen cells, respectively
- PCR primers (Figs. 3.2 and 3.3) and corresponding plasmids (Figs. 3.4 and 3.5)
- Helper phage (e.g., Stratagene VCSM13 # 200251)
- $F^+$, *sup*E, *rec*A strain (e.g., *E. coli* XL1-Blue) (available in electrocompetent/chemocompetent form from Stratagene)
- Anti-M13 antibody HRP-conjugate (GE Healthcare; # 27-9421-01)
- PEG 6000 (Fluka)
- Sterile, RNase-free equipment: pipet tips, tubes, RNase-free ultra high purity (UHP) water, baked nondisposable glassware, and sterile, disposable plasticware
- Standard molecular biology equipment and reagents for:
  - Determining the isotype of mAbs (Roche IsoStrip Mouse Monoclonal Antibody Isotyping Kit)
  - Purifying RNA (Invitrogen TRIzol reagent and Qiagen RNeasy Mini Kit)
  - Performing a cDNA synthesis reaction (Qiagen QuantiTect Reverse Transcription Kit)
  - Performing PCR reactions
  - Purifying PCR products (Macherey Nagel PCR clean-up Gel Extraction Kit)
  - Cutting and gel-purifying DNA (Sigma-Aldrich GenElute Gel Extraction Kit)
  - Concentrating DNA (Amicon Microcon 30 for volumes less than 500 μl)
  - Ligating and transforming DNA
  - Growing bacteria and phages
  - Conducting an Enzyme Linked Immunosorbent Assay (ELISA)
  - Performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent immunoblotting.

**Fig. 3.1** (continued) and the asterisk symbolizes either the myc tag or the trypsin cleavage site, present in pAK100 and pJB12, respectively. After infection with VCSM13 helper phages, the transformed XL1-Blue cells produce phages, displaying the scFv antibody on their surface. The subsequent enrichment of these phages by panning against the antigen allows the selection of functional antibody sequences from the library generated from the spleen cells. In addition, this approach also supports the isolation of specific scFv fragments if the hybridoma cell line initially contained only a small fraction of mRNA coding for this particular antibody. Phage ELISA then identifies the antigen-binding clones. Subsequently, the binding properties of the unfused scFv in the absence of phage need to be verified after recloning into a more powerful expression vector (Figs. 3.4 and 3.5) and purification from *E. coli* (not shown in the diagram)

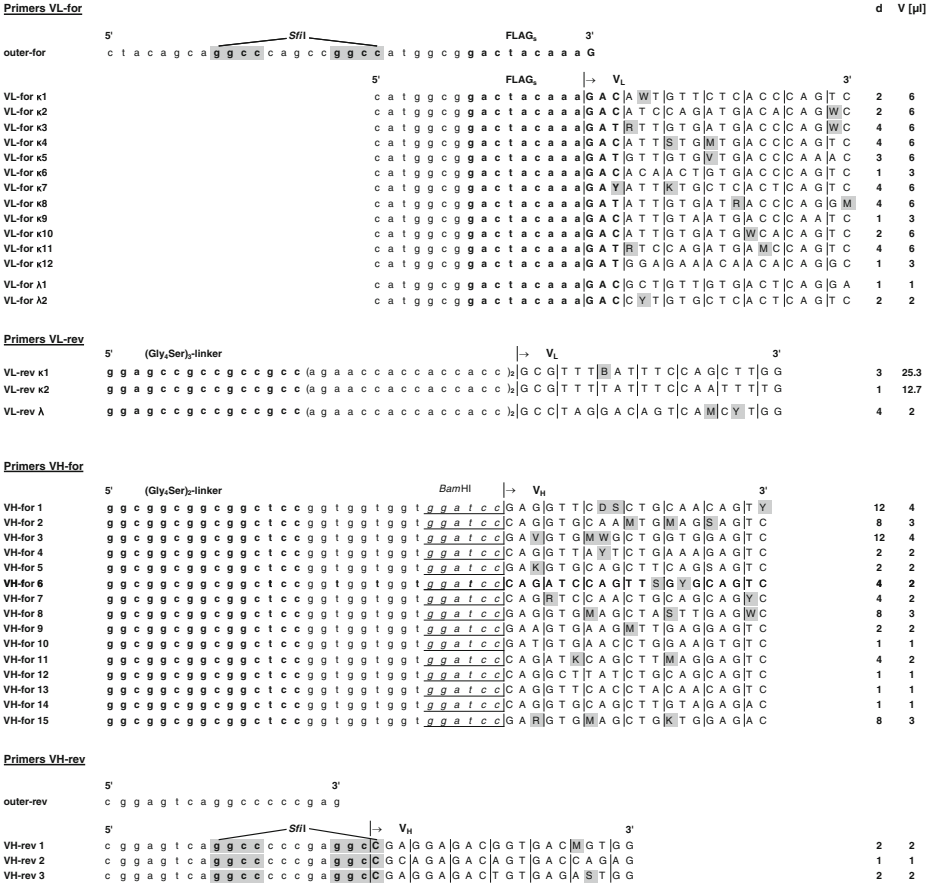



**Fig. 3.2** *List of primers used for assembling mouse scFv fragments*. The depicted oligonucleotides direct the assembly of scFv fragments in the orientation $V_L$-$(G_4S)_4$-$V_H$, being compatible with the vectors presented in Fig. 3.4. They have been newly derived from an analysis of the complete set of mouse sequences, and are thus different from the previously reported sets (Burmester and Plückthun 2001; Krebber et al. 1997). The sequences are given using the IUPAC nomenclature of mixed bases (shown as capital letters with gray background, R = A or G; Y = C or T; M = A or C; K = G or T; S = C or G; W = A or T; H = A or C or T; B = C or G or T; V = A or C or G; D = A or G or T). A column lists the d-fold degeneration encoded in each primer, d being the number of unique sequences. The "VL-for" primers VL-for κ1 to VL-for κ12 encode a stretch of 20 bases, hybridizing to the mature mouse antibody κ sequences (in capital letters). The preceding sequence that encodes the shortened FLAG sequence (Knappik and Plückthun 1994) is shown in bold. Since the FLAG tag uses the fixed *N*-terminal aspartate of the mature antibody (encoded by GAY), only three additional amino acids are necessary. The FLAG codons are then preceded by the codons specifying the end of the *pelB* signal sequence. The "VL-for" primers VL-for λ1 and VL-for λ2 for mouse lambda chains are constructed in an analogous manner (the *N*-terminal glutamate of the mature mouse λ sequence is replaced by aspartate (encoded by GAC) to generate a FLAG tag). The "VL-rev" primer sequences are complementary to the J-elements of kappa or lambda chains (capital letters) and encode three repeats of the $Gly_4Ser$ sequence, with the terminal one (bold) possessing a different codon usage to minimize incorrect overlaps during the PCR

## 3.3 Method

### *3.3.1 Isolation of mRNA and cDNA Synthesis*

1. Take $5 \times 10^6$ cells from a frozen or growing hybridoma culture (for isotype determination, use the Roche IsoStrip Mouse Monoclonal Antibody Isotyping Kit) or spleen cells, respectively (see note). Perform a total RNA preparation, combining homogenization of cells in the presence of TRIzol Reagent (Invitrogen) with RNA purification using the Qiagen RNeasy Mini Kit as described by the manufacturers. According to the supplier, the latter kit can be used for up to $1 \times 10^7$ cells, but in order to get highly pure mRNA, take only $5 \times 10^6$ cells.

   *Note*: For RNA preparation from mouse spleen (typically yielding $5 \times 10^7$ B-cells each), first separate it from connective tissue with sharp forceps or scissors (if frozen, also cut the frozen tissue into smaller pieces and pulverize using a mortar) and homogenize it using the Tissue Lyser (Qiagen) or similar homogenizers in the presence of 1 ml TRIzol Reagent per 50 mg of tissue. Make sure not to use too many cells as spleens are typically rich in nucleases, and, therefore, enough RNAse-deactivating components from the TRIzol Reagent should be present in the solution. TRIzol Reagent is a commercial monophasic preparation of guanidinium isothiocyanate and phenol and only the addition of chloroform separates the solution in two phases. If desired, poly$A^+$ mRNA can subsequently be isolated from the total RNA using the Oligotex Direct mRNA Mini Kit (Qiagen) – however, in most cases, this should not be necessary for the subsequent production of cDNA. Therefore, we do not recommend including this additional purification step, as it might lead to loss of mRNAs present only in smaller quantities. Since specific amplification primers are used, we consider it rather advantageous to work with a higher total amount of RNA.
2. Separate the RNA from DNA and proteins by phenol-chloroform extraction with subsequent silica membrane purification as described by the manufacturer (Invitrogen). Transfer the upper aqueous phase to a new, RNase-free tube. Add an equal volume of 100% ethanol dropwise, as its presence is required for the RNeasy columns to bind the RNA during the initial application. Transfer up to 700 µl of the mixture, including any precipitate that may have formed,

**Fig. 3.2** (continued) assembly reaction. Please note that for these primers, the two identical linker repeats are presented by a parenthesis with the subscript 2. The "VH-for" primers encode the other part of the linker (overlap with VL-rev shown in *bold*) as well as a *Bam*HI recognition site (*underlined*). The 20 bases given in capital letters hybridize with the mature mouse VH sequences. The last 20 nucleotides (nt) at the 3′ end of the "VH-rev" primers hybridize with the JH region. The first nt shown in capital letters will cause a silent mutation at the end of $V_H$ in order to code for the first nt of the second *Sfi*I recognition site (*bold and highlighted*). The final assembly of the scFv gene by SOE-PCR is carried out with the outer-for and outer-rev primer set. The outer primer outer-for encodes the first *Sfi*I site (*bold and highlighted*). The last column lists the volume that should be used when mixing the primers (see text)

| $V_L$ | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| VL-for κ1 | D | I,N | V | L | T | Q | S |
| VL-for κ2 | D | I | Q | M | T | Q | S,T |
| VL-for κ3 | D | I,V | V | M | T | Q | S,T |
| VL-for κ4 | D | I | L,V | L,M | T | Q | S |
| VL-for κ5 | D | V | V | L,M,V | T | Q | T |
| VL-for κ6 | D | T | T | V | T | Q | S |
| VL-for κ7 | D | I | L,V | L | T | Q | S |
| VL-for κ8 | D | I | V | I,M | T | Q | A,D |
| VL-for κ9 | D | I | V | M | T | Q | S |
| VL-for κ10 | D | I | V | M | S,T | Q | S |
| VL-for κ11 | D | I,V | Q | M | N,T | Q | S |
| VL-for κ12 | D | G | E | T | T | Q | A |
| VL-for λ1 | D | A | V | V | T | Q | E |
| VL-for λ2 | D | P,V | V | L | T | Q | S |

amino acid position

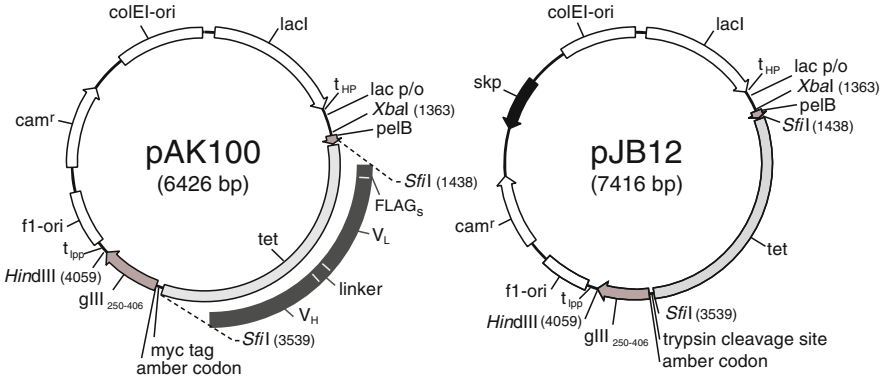
| 102 | 103 | 104 | 105 | 106 | 107 | 108 | $V_L$ |
|---|---|---|---|---|---|---|---|
| T | K | L | E | I,M | K | R | VL-rev κ1 |
| T | K | L | E | I | K | R | VL-rev κ2 |
| A,T | K,R | L,V | T | V | L | G | VL-rev λ |

| $V_H$ | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| VH-for 1 | E | V | H,L,Q,R | L | Q | Q | F,L,S |
| VH-for 2 | Q | V | Q | L,M | K,Q | E,Q | S |
| VH-for 3 | D,E | V | K,M,Q | L | V | E | S |
| VH-for 4 | Q | V | I,T | L | K | E | S |
| VH-for 5 | D,E | V | Q | L | Q | E,Q | S |
| VH-for 6 | Q | I | Q | F,L | A,V | Q | S |
| VH-for 7 | Q | I,V | Q | L | Q | Q | P,S |
| VH-for 8 | E | V | K,Q | L | L,V | E | S,T |
| VH-for 9 | E | V | K | I,L | E | E | S |
| VH-for 10 | D | V | N | L | E | V | S |
| VH-for 11 | Q | I,M | Q | L | K,Q | E | S |
| VH-for 12 | Q | A | Y | L | Q | Q | S |
| VH-for 13 | Q | V | H | L | Q | Q | S |
| VH-for 14 | Q | V | Q | L | V | E | T |
| VH-for 15 | E | V | K,Q | L | V,L | E | T |

amino acid position

| 107 | 108 | 109 | 110 | 111 | 112 | 113 | $V_H$ |
|---|---|---|---|---|---|---|---|
| T | T | V | T | V | S | S | VH-rev 1 |
| T | L | V | T | V | S | A | VH-rev 2 |
| T | S,T | L | T | V | S | S | VH-rev 3 |

**Fig. 3.3** *Deduced amino acid sequence of the V domain, hybridizing part of primers from Fig. 3.2*. The residues of $V_L$ and $V_H$ are numbered according to Kabat et al. (1991). All forward primers determine only the first 2 nt of residue 7, whereas the reverse primers determine the last 2 nt of position 102 or 107, respectively. Therefore, the alternative translations at those latter positions given do not indicate that they encode a mixed codon




| pAK / pJB vector series | | phage display | Skp coexpression | enhanced expression | trypsin cleavage site | IMAC purification | C-terminal detection | direct detection | dimerization |
|---|---|---|---|---|---|---|---|---|---|
| pAK100 | XbaI SfiI SfiI EcoRI HindIII — pelB tet myc * gIII 250-406 | ● | | | | | | | |
| pAK300 | XbaI SfiI SfiI HindIII — pelB tet (His)6 | | | | | ● | ● | | |
| pAK400 | XbaI SD T7g10 SfiI SfiI HindIII — lac p/o pelB tet (His)6 | | | ● | | ● | ● | | |
| pAK500 | XbaI SfiI SfiI EcoRI HindIII — pelB tet dHLX (His)5 | | | | | ● | ● | | ● |
| pAK600 | XbaI SfiI SfiI EcoRI HindIII — pelB tet alkaline phosphatase | | | | | | | ● | ● |
| pJB12 | XbaI SfiI SfiI EcoRI HindIII — pelB tet KDIR * gIII 250-406 | ● | ● | | ● | | | | |
| pJB23 | XbaI SfiI SfiI EcoRI HindIII — pelB tet (His)6 | | ● | | | ● | ● | | |
| pJB33 | XbaI SD T7g10 SfiI SfiI EcoRI HindIII — lac p/o pelB tet (His)6 | | ● | ● | | ● | ● | | |

**Fig. 3.4** *Overview of pAK/pJB vector series*. The pAK/pJB vector series (see also Fig. 3.5) can be used either for phage display (pAK100 and pJB12) by the strategy outlined in Fig. 3.1, or for the expression of the antibody in a variety of formats. All vectors contain a chloramphenicol resistance cassette ($cam^r$) and additionally a tetracycline resistance "stuffer" cassette (*tetA* and *tetR*; 2,101 bp), which will be replaced by the antibody gene (the *tet* cassette allows the monitoring of complete *Sfi*I digested vector by plating of transformed DH5α cells on tetracycline plates). Furthermore, these vectors contain the *lacI* repressor gene, a strong upstream terminator ($t_{HP}$) to avoid read-through and premature expression, the *lac* promoter/operator and the *pelB* (pectate lyase gene of *Erwinia carotovora*) leader sequence (modified to contain a *Sfi*I site) as well as a

to one RNeasy spin column and continue according to the manufacturer's instructions.

*Note*: RNA in harvested tissue is not protected from degradation until the sample is mixed with TRIzol Reagent, flash-frozen or disrupted and homogenized in the presence of RNase-inhibiting or protein-denaturing reagents. Therefore, proceed with this step as fast as possible. Generally, DNase digestion is not required with RNeasy Kits since its silica membrane efficiently removes most of the DNA. However, if desired, residual DNA can be removed by optional on-column DNase digestion using the RNase-Free DNase Set (Qiagen). It is important not to overload the RNeasy spin column, as this will significantly reduce RNA yield and quality.

3. Elute the purified RNA by the addition of 30 µl RNase-free water. The mRNA solution is now ready for cDNA synthesis or can alternatively be stored at −80°C for up to one month.

   *Note*: Diethylpyrocarbonate (DEPC)-treated UHP water can also be used. However, as DEPC is a suspected carcinogen the use of filtrated RNase-free water is recommended.

4. For reverse transcription, take approximately 0.1–0.5 µg RNA and 1 µl random hexamer primers provided in the kit in 20 µl total reaction volume. The precise procedure is described in the QuantiTect Reverse Transcription Kit (Qiagen).

   *Note*: Ribonuclease H activity of Quantiscript Reverse Transcriptase specifically degrades only the RNA in RNA:DNA hybrids, but it has no effect on pure RNA. Hence, an additional RNA degradation step using another RNase H enzyme is not necessary prior to subsequent PCR reaction.

   *Note*: Specific primers hybridizing to the constant regions can be used as well, e.g., if only a particular antibody class should be amplified from spleen cells. In general, however, the random hexamer primers work robustly.

**Fig. 3.4** (continued) downstream terminator ($t_{lpp}$). The rationale for these elements has been described in detail previously (Krebber et al. 1997). The origins for phage replication and plasmid replication are as described in Ge et al. (1995). The antibody gene is fused in frame either to $gIII_{250–406}$ for phage display, to a his tag for IMAC purification (Lindner et al. 1992) and *C*-terminal detection with a recombinant anti-his tag scFv-phosphatase fusion protein (Lindner et al. 1997), to dimerization helices (Pack et al. 1993, Plückthun and Pack 1997, see also chapter 7) or to alkaline phosphatase for both dimerization and direct detection (Lindner et al. 1997). In pAK100, the in-frame fusion contains a myc tag (Munro and Pelham 1986), offering an additional detection possibility next to the short *N*-terminal 4-amino acid FLAG tag (DYKD; Knappik and Plückthun 1994) present in all the vectors being encoded by the primers shown in Fig. 3.2. The plasmid pJB12 contains a trypsin cleavage site (KDIR) and can therefore be conveniently used for selection of high-affinity binders as described by Dziegiel et al. (1995) and Johansen et al. (1995). The asterisk in these two vectors pAK100 and pJB12 represents an amber codon. The scFv expression level in pAK400 and pJB33 is enhanced due to the strong Shine Dalgarno sequence SDT7g10 (from T7 phage gene 10). Because of the compatibility of the vectors, this feature can easily be introduced in all of them. In the pJB vector series the co-expressed periplasmic protein Skp (Bothmann and Plückthun 1998), encoded on this vector, can increase the functional yield of antibody fragments expressed in the periplasm without the need of cotransformation with another plasmid coding for further chaperones. This feature can also be introduced into any of the other vectors. The complete sequences of all vectors are available from the authors upon request

## 3.3.2 PCR Amplification and scFv Assembly

### 3.3.2.1 PCR Amplification of $V_L$ and $V_H$ Domains

1. Use the primers described in Fig. 3.2, which have been dissolved in 100 μM stock solutions in either sterile water or sterile TE buffer to prepare appropriate mixtures (VL-for mix, VL-rev mix, VH-for mix, and VH-rev mix). Mix them according to the degree of degeneration, indicated as "d" in Fig. 3.2 (equaling the number of different unique sequences encoded by mixed bases in the primer) by adding the stated volumes (in μl) towards the final primer mix. The fraction of lambda-specific primers in both the forward and reverse $V_L$ mixture amounts for ~5% of the total volume, accounting for the low percentage of this light chain type in mouse antibodies. The nominal total primer concentration of these mixtures is still 100 μM, ranging from 3 to 40 μM for each of the individual oligonucleotides.

   *Note*: As described in the introduction, problems in the cloning of monoclonal antibodies can occur if the hybridoma transcribes more than one functional or even nonfunctional heavy or light chain variable region gene. Therefore, it is highly recommended to omit any lambda chain-specific primer in the PCR if the isotyping already indicates that the hybridoma of interest secretes IgGs possessing kappa light chains.
2. For PCR amplification of $V_L$ and $V_H$, use the product of the completed first-strand cDNA reaction and prepare the following mixtures:

| PCR mix for amplification of $V_L$ | PCR mix for amplification of $V_H$ |
|---|---|
| 2 μl cDNA | 2 μl cDNA |
| 1 μl dNTPs (10 mM each) | 1 μl dNTPs (10 mM each) |
| 5 μl 10× ThermoPol buffer (NEB) | 5 μl 10× ThermoPol buffer (NEB) |
| 0.5 μl VL-for primer mix (100 μM) | 0.5 μl VH-for primer mix (100 μM) |
| 0.5 μl VL-rev primer mix (100 μM) | 0.5 μl VH-rev primer mix (100 μM) |
| 2.5 μl DMSO | 2.5 μl DMSO |
| 0.5 μl VentR Polymerase 2 U/μl (NEB) | 0.5 μl VentR Polymerase 2 U/μl (NEB) |
| 38 μl $H_2O$ | 38 μl $H_2O$ |

   *Note*: This standard protocol is optimized for VentR polymerase, a DNA polymerase with a 5–15 fold lower error rate than Taq DNA Polymerase (due to an intrinsic 3′→5′ proofreading exonuclease activity). If using other proofreading polymerases (e.g., Phusion High-Fidelity DNA Polymerase from Finnzymes), reaction and PCR program conditions might have to be adapted. If the proposed PCR mix does not lead to any product, varying the cDNA template amount might be beneficial. If the thermocycler does not have a heated cover, add one drop of mineral oil to the reaction tube to prevent evaporation.

3. Perform the following PCR cycles after an initial denaturation of the DNA template for 3 min at 95°C: 5 cycles of 30 s at 95°C, 30 s initial annealing at 55°C, and 45 s elongation at 72°C, followed by 20 cycles of 30 s at 95°C, 30 s at 63°C, and

45 s at 72°C. After the last cycle is completed, an additional 5 min elongation step at 72°C should be performed before cooling the thermocycler to 4°C.

*Note*: We recommend using a hot start, keeping the PCR tubes on ice and not placing them into the thermocycler until the block has reached 95°C, to minimize unspecific amplification. For successful amplification of $V_L$ and $V_H$, complete annealing of the 3′-ends of the primers with the template DNA is essential. The recommended annealing temperature of 55°C should be suitable for approx. 97% of the sequences found in a reference set of murine germline sequences in the IMGT database. However, as it is not clear a priori which somatic mutations a given monoclonal antibody may carry in the primer regions, we recommend using a gradient PCR program (covering a range between 70° and 50°C in steps of 2°) to determine the optimum annealing temperature and to amplify the antibody genes without unspecific secondary bands. Alternatively, the PCR might also be run in a "touchdown" manner (Don et al. 1991), starting at an annealing temperature of 70°C and ending at 50°C. As after 5 cycles the amplified PCR product will serve itself as template DNA, the annealing temperature of the last 20 cycles can be increased to 63°C.

4. Analyze 1/10 volume of each PCR mixtures by agarose gel electrophoresis, purify the $V_L$ and $V_H$ genes using the PCR clean-up Gel Extraction Kit (Macherey Nagel) according to the manufacturer's instructions and determine the DNA concentration of both genes.

   *Note*: Using the listed primer mixtures, the expected lengths of the PCR products of $V_L$ and $V_H$ are between 375–402 bp and 386–440 bp, respectively. Purification of the PCR products is important to remove any residual primers which might interfere with the subsequent assembly PCR. For the case of multiple bands on the agarose gel, gel-purify the band of correct size using the GenElute Gel Extraction Kit (Sigma-Aldrich). If the final DNA concentration is too low afterward, perform a second PCR using these purified fragments as template for gaining sufficient yields of high-quality DNA.

#### 3.3.2.2 Assembly of $V_L$ and $V_H$ by SOE-PCR (Splicing by Overlap Extension)

1. For the assembly PCR, use approximately 10 ng of the PCR product of both domains in a total volume of 50 µl, containing 200 µM dNTPs, 3–5% DMSO, 1 µM outer-for, and outer-rev primer (each) and 1 unit VentR DNA Polymerase (NEB). Following a 3 min 95°C step, perform 5 cycles of 1 min at 95°C, 1 min at 63°C, and 1 min at 72°C, followed by another 5 cycles of 1 min at 95°C, 30 s at 56°C, and 1 min at 72°C and finally 25 cycles of 1 min at 95°C, 90 s at 72°C.

   *Note*: Hot start PCR and initial assembly of $V_L$ and $V_H$ in the absence of the primers is usually not necessary but can be performed. It is important to include DMSO in the PCR mix as well as to keep the primer concentration as low as indicated to prevent any risk of primer-dimer formation.

   *Note*: The assembly, as used here, places $V_L$ in front of $V_H$. This has the advantage of placing a shortened FLAG tag, consisting of only four amino acids,

at the *N*-terminus of the construct. Since its last amino acid, Asp, is the same as the first residue of the $V_L$ domain, only three additional amino acids are needed (Knappik and Plückthun 1994) for allowing specific detection using this tag. A slight asymmetry in the $V_H/V_L$ heterodimer with respect to the pseudo two-fold axis (Plückthun et al. 1996) is taken care of with a 20-amino acid linker, leading to monomeric scFv fragments.

### *3.3.3 Digestion and Cloning of scFv Genes*

1. Purify the product of the assembly PCR using the PCR clean-up Gel Extraction Kit (Macherey Nagel) according to the manufacturer's instructions, eluting the product in 30 µl of the recommended buffer. In case there are several bands on the analytical agarose gel, carry out a gel purification of the correct band, as described in 3.3.3.4.
2. Perform a *Sfi*I digest of the amplified scFv gene for 3–4 h at 50°C (At 37°C, the activity of *Sfi*I would be 10 fold-lower). To the 30 µl purified PCR product, add 5 µl 10× NEbuffer 4 (NEB), 5 µl 10× BSA (final concentration, 100 µg/µl), 9 µl $H_2O$, and 1 µl (=20 units) *Sfi*I (NEB).
3. Digest appropriate amounts of vector (pAK100 or pJB12, see Fig. 3.4) with *Sfi*I in the presence of the above-mentioned buffer, including BSA. Use 10 units *Sfi*I for 1 µg vector in 50 µl and incubate 4 h at 50°C. Dephosphorylate the cut vector by adding Calf Intestinal Alkaline Phosphatase (CIP, NEB; 0.5 unit/µg vector) to the digestion mix after 2 h and continue incubation for another 2 h at 50°C.

   *Note*: Dephosphorylation should not be necessary because of the asymmetric overhangs. However, we always include this step to eliminate any risk of religation of single-cut vector.

   *Note*: pAK100 or pJB12 are phage display vectors (Fig. 3.4). When starting from hybridomas, one can also directly clone the $V_L$ and $V_H$ genes into an scFv expression vector with a stronger promoter, such as pAK400, which does not encode a fusion with gIII. However, depending on the number of additional V genes expressed in the hybridoma, a large number of clones may have to be screened from individual colonies.
4. Purify the digested scFv antibody genes and vector by preparative agarose gel electrophoresis in combination with the GenElute Gel Extraction Kit (Sigma-Aldrich).

   *Note*: For obtaining pure preparations of a fully digested vector, it is very important not to overload the agarose gel. Furthermore, the gel electrophoresis has to be run long enough to separate small amounts of undigested vector from the digested vector band. For large-scale vector or insert preparation, electro-elution might be an efficient and convenient alternative. If the concentration of eluted DNA is too low for further applications, Microcon 30 columns (Amicon) can be used for concentration.

5. Ligate 50 ng scFv gene fragment with the vector (molar ratio of vector to insert 1:5) with 5 units T4 DNA ligase (NEB) in the presence of 1× T4 DNA ligase buffer in 10 µl volume. Incubate for 2 h at room temperature or overnight at 16°C.

   *Note*: The ATP-concentration is very crucial for the successful ligation by T4 DNA ligase. Therefore, we recommend using T4 DNA ligase buffer aliquots, which have been properly stored at −20°C and not thawed repeatedly. To allow an easy subcloning of the scFv fragment into vectors for optimized soluble expression and other purposes, compatible vector sets are available (Figs. 3.4 and 3.5).
6. Transform 50 µl chemocompetent XL1-Blue cells (Stratagene) with 5 µl of the ligation mix by heat-shock for 45 s at 42°C, add 500 µl of 2× YT medium after 2 min incubation on ice, and incubate for 45 min, shaking at 37°C.

   *Note*: Make sure not to exceed a ratio of ligation mix/cells of 1:10 (v/v). Chemocompetent *E. coli* are used, if only a very small diversity of clones is expected, e.g., when cloning from a hybridoma. If a larger diversity and thus many clones are required (e.g., when cloning from spleen cells), follow the instructions for electroporation described in steps 3.3.5.1–3.3.5.3.
7. Plate the transformed cells on 2× YT, 1% glucose, chloramphenicol (30 µg/ml) agar plates, and incubate overnight at 37°C.

   *Note*: You may check the ratio of desired ligation product to background by including transformation with "religated" plasmid in the absence of any insert. Alternatively, the background signal can be analyzed by testing for tetracycline resistance after transformation of other *E. coli* strains not possessing an intrinsic *tet* resistance (like Invitrogen's DH5α) with the ligation mix. The portion of vector with unremoved or religated *tet* cassette is typically in the range of 0.01–0.1%.

### 3.3.4 Preparation of Electrocompetent E. coli

1. For preparation of electrocompetent *E. coli* XL1-Blue cells (Stratagene), use 2 ml of a dense overnight pre-culture to inoculate 500 ml medium (2× YT, 15 µg/ml tetracycline). Shake it at 25°C until an $OD_{600}$ of 0.6 is reached, then chill the culture on ice as quickly as possible for 30 min (cool the whole shake flask in a large ice bath).

   *Note*: Sufficient agitation during growth seems to be very important for preparation of electrocompetent cells, reaching reproducible efficiencies of 3–6 × $10^9$ cfu/µg pUC19 DNA. Therefore, use 5 l baffled shake flasks with only 500 ml medium and make sure that the amplitude of the shaker is high enough to vigorously circulate the medium.

   *Note*: The use of electrocompetent bacteria is an alternative to 3.3.3.6 and needed when a large diversity is expected, typically when cloning from spleen cells.

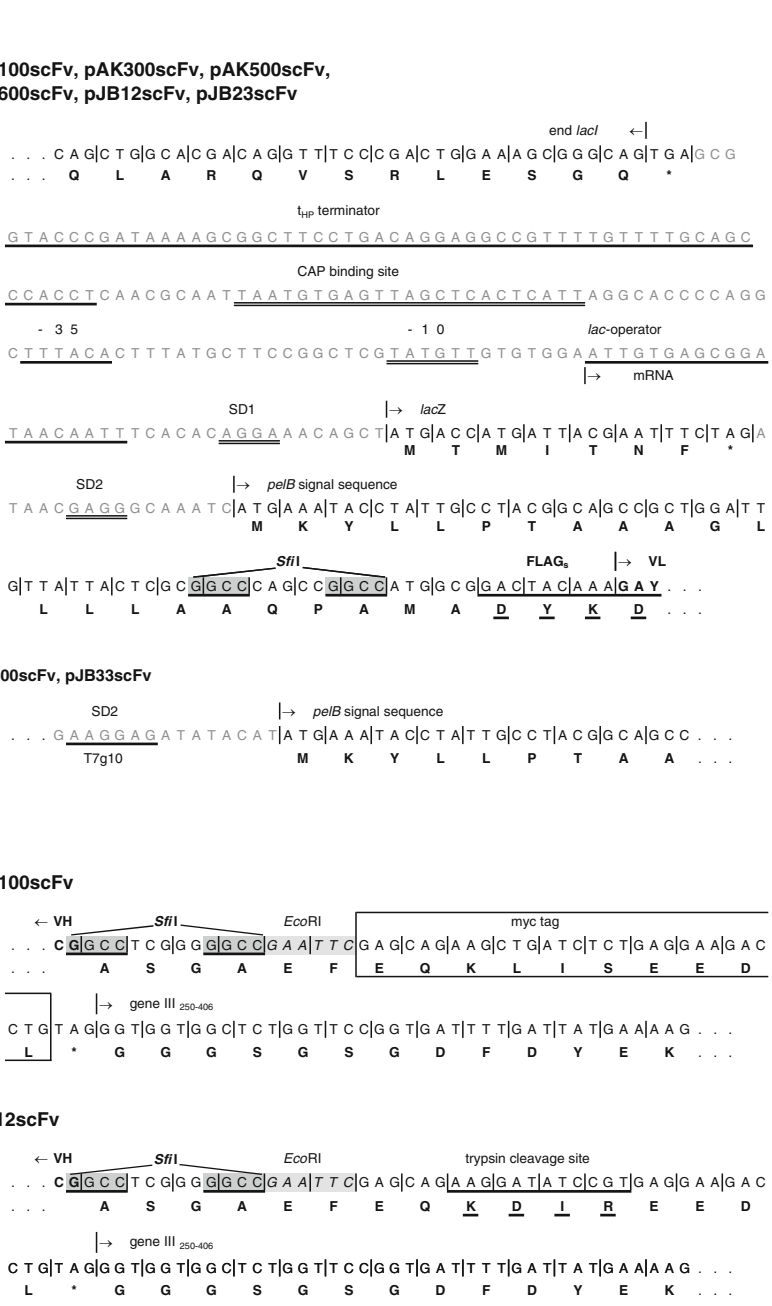



**Fig. 3.5** (Continued)


**Fig. 3.5** *Detailed sequences upstream and downstream of scFv clonig site*. (**a**) Upstream sequence of pAK100scFv, pAK300scFv, pAK400scFv, pAK500scFv, pAK600scFv, pJB12scFv, and pJB23scFv. The symbol scFv indicates that the vectors are shown after an scFv has been introduced, replacing the *tet* stuffer fragment. The region from the end of the *lacI* repressor gene to the beginning of the antibody $V_L$ domain is shown. The *lacI* repressor gene, $t_{HP}$ terminator sequence, CAP binding site, *lac* operator region, including the -35 and -10 sequence, Shine-Dalgarno (SD) sequence of *lacZ* (SD1), *lacZ* peptide, a second SD sequence (SD2), *pelB* signal

2. Centrifuge the bacterial culture in 50 ml aliquots in disposable tubes for 5 min at 5,000 *g*. Remove as much supernatant as possible (leave the tube upside down for 15–30 s on a clean tissue). Then, fill each tube with 1 volume of ice-cold distilled water (i.e., the same volume as the original culture aliquot) and remove the water immediately (the cell pellet is very solid after this first centrifugation step and will not be resuspended by the brief rinsing with distilled water).

   *Note*: All these steps should be carried out using ice-cold solutions and be performed in the cold room. Use only ultra pure water to wash cells and to prepare 10% glycerol, as the presence of impurities such as salts in the water might cause the subsequent transformations to fail.
3. Fill each tube with 1 vol distilled water (i.e., the same volume as the original culture aliquot), resuspend the pellet carefully and incubate for 10 min on ice.

   *Note*: Make sure that the cells are sufficiently solubilized to yield a homogeneous suspension. Cells are best resuspended by swirling rather than pipetting. Never vortex the cell suspension!
4. Transfer the cells into new 50 ml tubes and centrifuge at 5,000 *g* for 10 min. Carefully remove the supernatant and resuspend the pellets each in 50 ml pre-chilled 10% (v/v) glycerol (Fluka). Incubate on ice for 10 min.
5. Centrifuge resuspended cells at 5,000 *g* for 15 min and remove the supernatant (you might lose a small portion of cells – do not put the tubes upside down on tissue in this step!). Carefully resuspend the cells in 1/500 of the original culture volume (= 1 ml) 10% (v/v) glycerol, freeze the cells in 100 µl aliquots by dipping the tubes immediately into liquid nitrogen and store them at −80°C.

   *Note*: Electrocompetent cells can be kept at −80°C for up to 12 months.
6. To determine the transformation efficiency, add 1 µl of 10 pg/µl pUC19 DNA (in water) to 40 µl of barely thawed cells (see step 3.3.5.2). Fifty colonies per 1/1,000 of the transformation volume plated correspond to an efficiency of $5 \times 10^9$ cfu/µg pUC19 DNA.

**Fig. 3.5** (continued) sequence, *N*-terminal *Sfi*I site (*underlined and highlighted*), four amino acid $FLAG_s$ tag (*underlined*), and the start of the $V_L$ domain (sequence GAY; *bold*) are indicated above the sequence. In addition, also the corresponding amino acid sequence is shown. In pAK400 and pJB33, the 15 bp upstream from the *pelB* start codon are replaced by another sequence, including the SD sequence of the phage T7 gene10, while everything else is identical. Because of the modularity of the vectors, this feature can be easily introduced into any of the other vectors (see Fig. 3.4) (**b**) Downstream sequence of pAK100scFv and pJB12scFv. The last two bases of $V_H$ (*bold*), the *Sfi*I and *Eco*RI restriction sites, myc tag (*boxed*) or trypsin cleavage site and the start of $geneIII_{250–406}$ are indicated above the sequence. Asterisks indicate amber stop codon, leading to scFv-gene III fusions upon expression in *E. coli* strains with amber suppressor tRNA, such as XL1-Blue. (**c**) Downstream sequence of pAK300scFv, pAK400scFv, pJB23scFv, and pJB33scFv. The last two bases of $V_H$ (*bold*), the *Sfi*I and *Eco*RI restriction sites and $(His)_6$ tag (*boxed*) are indicated above the sequence. (**d**) Sequences of the downstream *Eco*RI/*Hin*dIII fusion cassettes as used in pAK500 and pAK600. The dHLX dimerization motif (*double underlined*) was taken from Pack et al. (1993). The complete sequence of the mature *E. coli* alkaline phosphatase (AP) gene can be found in Shuttleworth et al. (1986). For the *Eco*RI/*Hin*dIII cloning cassette the two internal *Eco*RI sites of the AP gene have been removed by silent mutations. The complete sequences of all vectors are available from the authors upon request

### 3.3.5 Library Preparation/Construction

1. For desalting the DNA prior to electroporation, apply the ligation mix to StrataClean Resin (Stratagene; hydroxylated silica, binding proteins with a high affinity, while having a low affinity for DNA at near neutral pH), followed by precipitation in 70% ethanol. Since most salts and small organic molecules are soluble in 70% ethanol, they can be separated from DNA by centrifugation. Resuspend the precipitated DNA in ultra pure water.
2. For each transformation, use desalted ligation mixtures corresponding to 20–100 ng insert. Add the DNA to 40 µl of barely thawed cells on ice and mix by flipping the tube shortly and gently. Immediately transfer the cell-DNA mix to chilled electroporation cuvette (bubble free), pulse according to the guidelines of the electroporator's manufacturer, and add 1 ml of SOC medium (20 g/l bacto-tryptone, 5 g/l yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM glucose, 10 mM $MgCl_2$, 10 mM $MgSO_4$) to cells immediately after the pulse.

   *Note*: For efficient transformation ($\geq 10^8$ clones per µg insert DNA), the time constant using 2 mm cuvettes should be ≥5 ms, reflecting properly washed cells. Also, make sure that no air-bubbles are trapped in the cell-DNA mix as they will interfere with the electroporation.
3. Resuspend cells completely in SOC medium and shake for 1 h at 37°C. Afterward, plate dilutions on 2× YT, 1% glucose, chloramphenicol (30 µg/ml) agar plates. Use a sterile spreader or sterile glass beads to evenly distribute the culture over the surface of the 12 × 12 cm plate (do not exceed about 5,000 clones per square plate) and incubate overnight at 37°C. The next day, scrape the colonies off the plates in 3–4 ml 2× YT, containing 30% glycerol, and subsequently store them at −80°C.

   *Note*: Take care that your library is homogeneously mixed.
4. For phage panning as described in 3.3.6, inoculate cultures with at least tenfold more viable cells than colonies obtained after transformation, in order to have sufficient oversampling. When starting from spleen cells, perform three rounds of phage panning as described in 3.3.6 before testing single clones. When starting from a hybridoma, one round should be sufficient, and in ideal cases, single clones can be tested right away by phage ELISA.

   *Note*: The first panning round is the most crucial, as you might lose any desired, but less abundant antibody sequence by too extensive washing. Therefore, do not exceed ten washing steps in this first panning round. The panning procedure is analogous to the phage ELISA (3.3.6), except that a pool of phages is grown and that phages are eluted from antigen (at the end of 3.3.6.4), which are afterward added again to exponentially growing bacteria. This is described in detail elsewhere (Barbas et al. 2001; Lee et al. 2007) and also in this volume.

   *Note*: The screening of single clones can be performed in three ways. First, at the level of phages (phage ELISA), as described in Sect. 3.3.3.6, second, after retransforming of an suppressor tRNA-deficient strain such as, e.g., *E. coli* strain JM83 (Yanisch-Perron et al. 1985), still with the amber codon, containing

pAK100 derived plasmids, or third, after recloning into a more efficient expression vector such as pAK400 (Fig. 3.5), which carries no gene III. In second and third option, the soluble scFv is screened by ELISA.

*Note*: This protocol does not describe the periplasmic expression of scFv fragments in *E. coli* (Glockshuber et al. 1990) and their subsequent purification (reviewed in Plückthun et al. 1996). More details can be found in chapter 27 "Improving expression of scFv fragments by coexpression of periplasmic chaperones" in this volume.

### 3.3.6 Screening for Binders by Phage ELISA

1. When starting from hybridoma, pick 10 colonies (from spleen, as many as you can handle) and grow them separately at 37°C in 2 ml 2× YT, 1% glucose, chloramphenicol (30 μg/ml) until they reach an $OD_{600}$ of 0.5. This level of glucose fully represses expression, and, thus, the growth temperature can be 37°C. Dilute 1:10 in 2× YT, 1% glucose, chloramphenicol (30 μg/ml), containing 1 mM IPTG, and $1 \times 10^{10}$ pfu VCSM13 helper phage (Stratagene) per ml, and grow overnight at 26°C or 37°C (for some murine scFvs with aggregation tendencies, growth at 26°C after infection may be necessary). The phage titer after overnight incubation is in the range of $10^{11}$–$10^{12}$ cfu per ml supernatant.

   *Note*: XL1-Blue should be grown on agar plates and in media containing tetracycline (*tet*) as the F′-plasmid encoding for the F-pili required for infection of bacteria also carries the *tet* resistance gene. The phage titer (in cfu) should be determined in order to rule out any problems during phage production. To do so, take a log-phase culture of XL1-Blue cells ($OD_{600}$ = 0.4–0.6) and incubate aliquots of this culture with serial dilutions of your phage preparation. After 15 min incubation at 37°C, plate appropriate amounts (30–150 cfu/plate) on 2× YT, 1% glucose, chloramphenicol (30 μg/ml) agar plates.

   *Note*: We are aware of the fact that the presence of such a high level of glucose during IPTG-induction is rather unusual. However, for phage display, the induction level does not have to be very high. Based on our experiences with this vector series described here, a combination of IPTG addition and the presence of glucose seems to be crucial for the successful expression of some scFv fragments, notably those with nonideal biophysical properties, and appropriate for most, but may have to be checked for each scFv individually in case of unusual properties.
2. Centrifuge the culture 10 min at 16,000 *g* and 4°C. Take 1.6 ml supernatant and mix it with 0.4 ml 20% PEG 6000 (Fluka), 2.5 M NaCl in a 2 ml Eppendorf tube in order to precipitate the phages (Sambrook and Russell 2001).

   *Note*: We recommend that the PEG solution be freshly prepared.
3. Incubate on ice for 30–60 min and centrifuge for 15 min at 5,600 *g* and 4°C.

   *Note*: It is important not to centrifuge phages at too high a *g* force, as otherwise, it will be difficult to resuspend them homogeneously, resulting in a

decreased phage titer. The size of the white pellet does not necessarily reflect a high or low phage titer.

4. Resuspend the phage pellet in 400 µl PBS (with 10% (v/v) glycerol). For complete resuspension, incubate the phage solution on an orbital shaker at 800 rpm for 15 min at 4°C. Pellet insoluble matter (cell debris) by centrifugation for 10 min at 11,000 *g* and 4°C and transfer the phage solution to a fresh tube. Use 100 µl phage solution per well in an ELISA assay to distinguish phages displaying functional scFv antibody from those which display nonfunctional or nonproductive antibody fragments.
5. If soluble antigen is available, include a competition ELISA control showing that free antigen is able to compete with bound antigen for phage binding to distinguish nonspecific "sticky" from specifically binding phages. In principle, the same ELISA protocol that was used for the hybridoma screening procedure can be used.

   *Note*: For weak binders, it might be important to use more phages for ELISA analysis. In this case, the culture volume should be increased ten times. If no functional clone shows up in ELISA of single clones, perform one round of phage panning in order to enrich the functional binders. The enrichment should be checked by comparison of eluted phages from a specific surface versus a surface without antigen. In addition, it is recommended to analyze the phage solution by immunoblot, using an anti-M13 HRP-conjugated antibody (GE Healthcare), to ensure the correct fusion of the scFv to the gIII-protein as well as its correct display on the phage surface.

## 3.4 Troubleshooting

This part of the protocol contains general comment about potential pitfalls of the recommended standard method. The most critical steps were already highlighted directly following the instructions in the different subsections.

(a) In case of low transformation yields, check whether the problem is the transformation itself or rather the ligation. To investigate the quality of ligation, analyzing an aliquot by agarose gel electrophoresis might indicate any problems caused by nucleases. Furthermore, it might be informative to compare the ligation efficiency of *Sfi*I digested PCR product with inserts derived from plasmid digestion. In order to check both the ligation and the transformation efficiency, a defined amount of pUC19 DNA can be added to the ligation mixture. Because of the chloramphenicol resistance of the cloning vector and the ampicillin resistance of pUC19 DNA, it is possible to calculate the ligation efficiency by plating double transformed cells on ampicillin or chloramphenicol plates, respectively, and comparing the number of clones. The transformation efficiency (in presence of the ligation mixture) can be judged by comparison of the colony number after transformation with pUC19 DNA alone.

(b) The quality of the oligonucleotides used in this procedure is crucial for the successful and reliable amplification of various antibody genes as well as their subsequent assembly into scFvs. The number of proposed primers is important for a broad representation of the immune response, as any sequence absent from the complex mixture will obviously decrease the functional library size. We also strongly recommend using primers that have been accurately purified after their synthesis (either by HPLC or, for longer primers, by PAGE) to ensure that no single-base deletions are present in any of the oligonucleotides. These deletions as well as any insertions would cause frameshifts in the final gene assembly, resulting in a number of nonfunctional library members. Therefore, we also suggest – especially for library cloning – to sequence the genes of several random clones as well as to check for full-length scFv by western blot analysis detecting its fusion partner gene III (see note at 3.3.6.5).

(c) In case of severe problems in the PCR amplification of the $V_H$ and $V_L$ genes (steps 3.3.2), it might be worth considering to divide this reaction into two separate ones. Using the proposed primers without any overhang at their 5′-end (which either codes for the $FLAG_s$-tag and the *Sfi*I cleavage site, or the $(Gly_4Ser)_4$-linker), the pure antibody DNA should be amplified in a first PCR reaction, and, subsequently, a second PCR should be performed for reamplification and introduction of the appropriate overhangs with the original full-length primers. This procedure increases the degree of matching in both reactions, and might therefore help in the annealing step of the primers.

(d) Whenever expression of the scFv gene is not required, the bacteria should be grown in the presence of 1% glucose. Glucose will cause a tight suppression of the *lac* promoter, thereby ensuring the genetic stability of the inserted scFv genes. Likewise, we suggest growing XL1-Blue always on agar plates and in media containing tetracycline (*tet*) to keep the bacteria infective, as the *tet* resistance is located on the F′-plasmid that also contains the genes encoding F-pilus formation. Always use fresh XL1-Blue colonies, as subcloning might occasionally lead to the formation of *tet* resistant cells, which are no longer infectable. As the F-pilus expression is reduced when the bacteria are past log phase as well as when grown at temperatures below 34°C, we also recommend growing them at 37°C to $OD_{600}$ = 0.4–0.6.

(e) When working with libraries, double transformants can and will occur (Goldsmith et al. 2007). It is thus highly recommended that the scFv fragments of interest be recloned into a new vector (e.g., from pAK100 to pAK400), when they are analyzed at the level of pure unfused protein, thereby also introducing a stronger translation initiation region. It should be noted that diluted retransformation cannot resolve plasmid mixtures, as in *E. coli,* plasmids can form reversible concatamers.

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