Roland Kontermann Stefan Dübel (Eds.)

Antibody Engineering

With 110 Figures, 2 in Color

Springer

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

JÖRG BURMESTER and ANDREAS PLÜCKTHUN

reprinted from:
J. Burmester and A. Plückthun, Construction of scFv fragments from hybridoma or spleen cells by PCR assembly. In: Antibody Engineering, R. Kontermann and S. Dübel, Eds. (Springer-Verlag, Berlin Heidelberg, 2001) pp. 19-40.

Introduction

While 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), very many hybridomas have been generated, and are continuously being made, which produce monoclonal antibodies with very interesting properties. Even when naive and synthetic libraries may be a source of antibodies, often the immune response of an experimental animal may be of interest, requiring an analysis of the antibodies after immunization. Cloning retains and immortalizes these unique and extensively characterized specificities of mAbs, which can be crucial for the rescue of unstable hybridoma cell lines. Molecular cloning and sequencing of antibody variable domains forms the basis of antibody modelling (Rees et al., 1994), antibody engineering (Plückthun 1994, Dall'Acqua and Carter 1998) and experimental structure determination by NMR (Freund et al. 1994) or x-ray crystallography at high resolution (Braden et al. 1998). Moreover, once the variable region genes have been cloned, the antibody domains can be further engineered in a multitude of ways to produce antibody variants with lower immunogenicity (Thompson et al., 1998), higher affinity (Low et al. 1996, Schier et al. 1996, Hanes et al. 1998), altered antigenic specificity (Ohlin et al. 1996, Parsons et al. 1996), or enhanced stability (Martineau and Betton 1999, Wörn and Plückthun 1999). Furthermore, genetic fusions of scFv fragments to effector proteins and toxins are powerful tools for medicine and biotechnology (Hoogenboom et al. 1998).

Jörg Burmester, Universität Zürich, Biochemisches Institut, Winterthurerstr. 190, 8057 Zürich, Switzerland
✉ Andreas Plückthun, Universität Zürich, Biochemisches Institut, Winterthurerstr. 190, 8057 Zürich, Switzerland (*phone* +41-1-6355570; *fax* +41-1-6355712; *e-mail* PLUECKTHUN@BIOCFEBS.UNIZH.CH)

However, a prerequisite for the use of recombinant antibody technologies starting from hybridomas or immune repertoires is the reliable cloning of functional immunoglobulin genes. For this purpose, a standard phage display system was optimized for robustness, vector stability, tight control of the expression of the scFv-geneIII fusion, primer usage for PCR amplification of variable region genes, scFv assembly strategy and subsequent directional cloning using a single rare cutting restriction enzyme. Using this system, a number of variable antibody domains of hybridomas were accessible whose genes could not be cloned in previous experimental setups. This chapter essentially follows our earlier descriptions (Krebber et al. 1997, Plückthun et al. 1996), with some "evolution" in the conditions and primer sequences.

Note that, except for the initial preparation of the cells and the need for high ligation and transformation yields for library cloning, the construction of scFv fragment libraries from immunized mice and that of cloning one specific antibody from hybridomas is essentially the same, as described in this procedure. Why is an enrichment procedure even necessary, when the "monoclonal" antibody mRNA of a hybridoma cell is the starting material? It is not strictly required, but the present procedure provides an easy check that the right sequence has been cloned, and it is especially helpful if the hybridoma expresses more than one chain, as explained under the note of step 7.

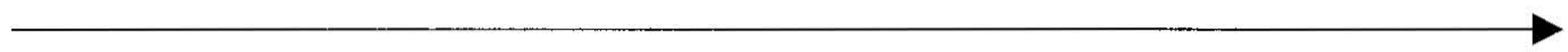


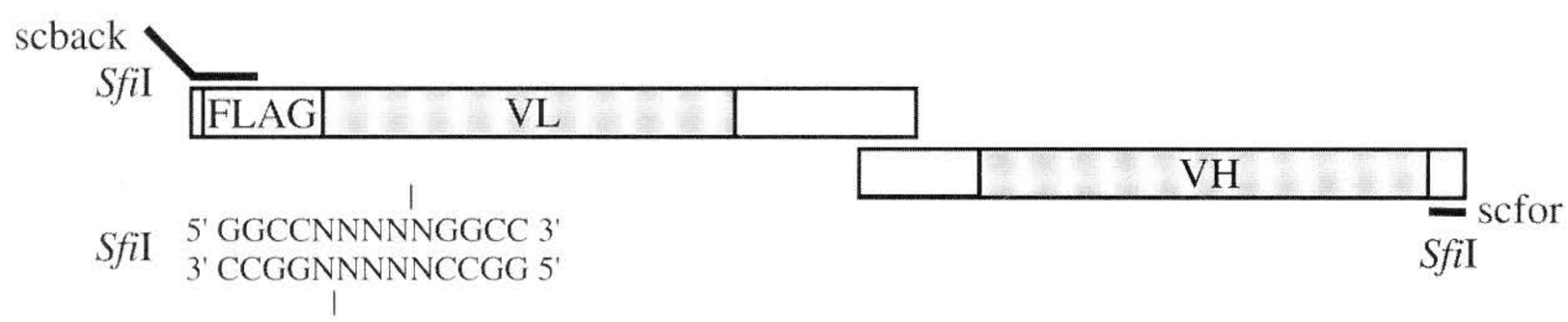
Fig. 1. Scheme of the amplification and cloning procedure. The mRNA is derived from hybridoma or spleen cells and a random hexamer primer mixture (pd$(N)_6$) is used for cDNA synthesis. The cDNA is used as the PCR template for the amplification of VL and VH domains (the primers are listed in Fig. 2) which are assembled (SOE-PCR) into the scFv format by the outer primer pair *scback* and *scfor*. For antibody cloning into the phagemid the rare cutting enzyme *Sfi*I is the only enzyme used. Note that directional cloning of the *Sfi*I inserts is guaranteed because of the different *Sfi*I sites shown. In addition, self-ligation of insert or vector molecules is excluded by the asymmetry of the overhang. The transformed XL1-Blue cells are used for phage production by infection with helper phage. The enrichment of scFv antibody displaying phages by panning against the antigen will allow the detection and selection of functional antibody sequences in library settings or if the hybridoma cell line contained only a small fraction of mRNA specific for this antibody.

Outline

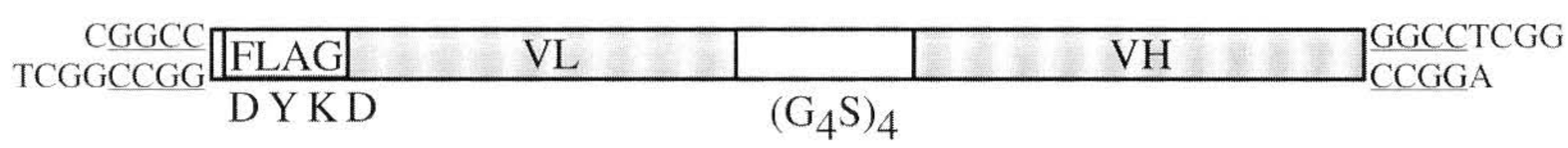
1. Isolation of mRNA (from hybridoma or spleen cells)

2. Synthesis of cDNA

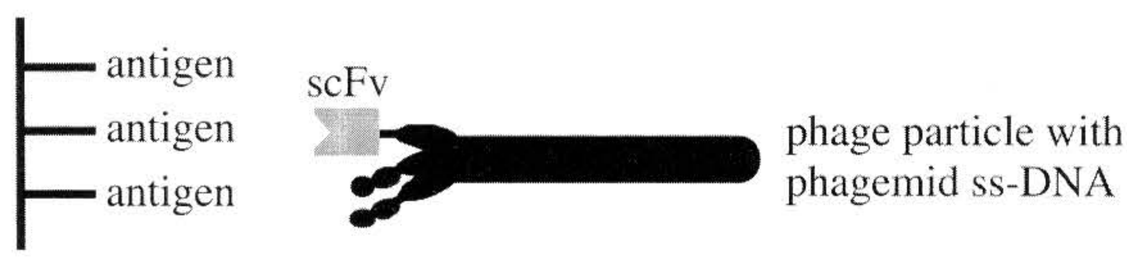
3. PCR amplification of VL and VH domains



4. Assembly of VL and VH by SOE-PCR (splicing by overlap extension)
5. *Sfi*I digestion of the amplified scFv fragment



6. Ligation of *Sfi*I digested insert and phage display vector (pAK100 or pJB12)
7. Transformation into *E. coli* XL1-Blue cells
8. Cell propagation and infection with VSCM13 helper phage



9. Detection and enrichment of binding scFv sequences by phage display

Materials

- 1-5 x 10^6 cells from a growing or frozen hybridoma culture or spleen cells, respectively
- PCR primers (Fig. 2 and Fig. 3) and plasmids (Fig. 4 and Fig. 5)
- Helper phage (e.g. Stratagene VCSM13)
- F^+, *sup*E, *rec*A strain (e.g. Stratagene *E. coli* XL1-Blue)
- Anti M13-HRP conjugate (Pharmacia)
- PEG 6000

Fig. 2. This figure lists the primers used for assembling mouse scFv fragments in the orientation VL-$(G_4S)_4$-VH, which are compatible with the vectors presented in Fig 3. In this nomenclature, "back" refers to "toward the 3' end of the antibody gene" and "for" to "toward the 5' end of the antibody gene". The sequences are given using the IUPAC nomenclature of mixed bases (shown in underlined capital letters, 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), with a column listing the d-fold degeneration encoded in each primer. The "VL-back" primers *LB1-LB17* encode a stretch of 20 bases hybridizing to the mature mouse antibody κ sequences (in capital letters). Underlined is the preceding sequence which encodes the shortened FLAG sequence (Knappik and Plückthun 1994). 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 in turn preceded by the codons specifying the end of the *pelB* signal sequence. The "VL-back" primer *LBλ* for mouse lambda chains is constructed in an analogous manner (the N-terminal glutamate of the mature mouse λ sequence is replaced by aspartate (encoded by GAT) to generate a FLAG tag). The "VL-for" primer sequences are complementary to the J-elements of kappa or lambda chains (capital letters) and also encode three repeats of the Gly_4Ser sequence, the terminal one (bold) of which has a different codon usage so that incorrect overlaps during the PCR assembly reaction are minimized. The "VH-back" primers encode the other part of the linker as well as a *Bam*HI recognition site (underlined), and the overlap with VL for in the sequence shown in bold. The 20 bases given in capital letters hybridize with the mature mouse VH sequences. The last 20 nt at the 3' end of the "VH-for" primers hybridize with the JH region. The first nt shown in capital letters will introduce a silent mutation at the end of VH in order to code for the first nt of the second *Sfi*I recognition site (underlined). The final assembly of the scFv gene by SOE-PCR is carried out with the *scback* and *scfor* primer set. The outer primer *scback* encodes the first *Sfi*I site (underlined).

```
        5'          SfiI          FLAG     3'
scback  ttactcgcggcccagccggccatggcggactacaaaG                          d
                    5'          FLAG     VL →                 3'
LB1                 gccatggcggactacaaaGAYATCCAGCTGACTCAGCC              2
LB2                 gccatggcggactacaaaGAYATTGTTCTCWCCCAGTC              4
LB3                 gccatggcggactacaaaGAYATTGTGMTMACTCAGTC              8
LB4                 gccatggcggactacaaaGAYATTGTGYTRACACAGTC              8
LB5                 gccatggcggactacaaaGAYATTGTRATGACMCAGTC              8
LB6                 gccatggcggactacaaaGAYATTMAGATRAMCCAGTC             16
LB7                 gccatggcggactacaaaGAYATTCAGATGAYDCAGTC             12
LB8                 gccatggcggactacaaaGAYATYCAGATGACACAGAC              4
LB9                 gccatggcggactacaaaGAYATTGTTCTCAWCCAGTC              4
LB10                gccatggcggactacaaaGAYATTGWGCTSACCCAATC              8
LB11                gccatggcggactacaaaGAYATTSTRATGACCCARTC             16
LB12                gccatggcggactacaaaGAYRTTKTGATGACCCARAC             16
LB13                gccatggcggactacaaaGAYATTGTGATGACBCAGKC             12
LB14                gccatggcggactacaaaGAYATTGTGATAACYCAGGA              4
LB15                gccatggcggactacaaaGAYATTGTGATGACCCAGWT              4
LB16                gccatggcggactacaaaGAYATTGTGATGACACAACC              2
LB17                gccatggcggactacaaaGAYATTTTGCTGACTCAGTC              2
LBλ                 gccatggcggactacaaaGATGCTGTTGTGACTCAGGAATC           1
```

Primer VL-for

```
          5'      (Gly4Ser)3-linker          VL →                 3'
LF1'      ggagccgccgccgcc(agaaccaccaccacc)2ACGTTTKATTTCCAGCTTGG         2
LF4       ggagccgccgccgcc(agaaccaccaccacc)2ACGTTTTATTTCCAACTTTG         1
LF5       ggagccgccgccgcc(agaaccaccaccacc)2ACGTTTCAGCTCCAGCTTGG         1
LFλ       ggagccgccgccgcc(agaaccaccaccacc)2ACCTAGGACAGTCAGTTTGG         1
```

Primer VH-back

```
          5'  (Gly4Ser)2-linker   BamHI  VH →                 3'
HB1       ggcggcggcggctccggtggtggtggatccGAKGTRMAGCTTCAGGAGTC            8
HB2       ggcggcggcggctccggtggtggtggatccGAGGTBCAGCTBCAGCAGTC            9
HB3'      ggcggcggcggctccggtggtggtggatccCAGGTGCAGCTGAAGSARTC            4
HB4       ggcggcggcggctccggtggtggtggatccGAGGTCCARCTGCAACARTC            4
HB5       ggcggcggcggctccggtggtggtggatccCAGGTYCAGCTBCAGCARTC           12
HB6'      ggcggcggcggctccggtggtggtggatccCAGGTYCARCTGCAGCARTC            8
HB7'      ggcggcggcggctccggtggtggtggatccCAGGTCCACGTGAAGCARTC            2
HB8'      ggcggcggcggctccggtggtggtggatccGAGGTGAASSTGGTGGARTC            8
HB9'      ggcggcggcggctccggtggtggtggatccGAVGTGAWGSTGGTGGAGTC           12
HB10'     ggcggcggcggctccggtggtggtggatccGAGGTGCAGSTGGTGGARTC            4
HB11'     ggcggcggcggctccggtggtggtggatccGAKGTGCAMCTGGTGGARTC            8
HB12      ggcggcggcggctccggtggtggtggatccGAGGTGAAGCTGATGGARTC            2
HB13'     ggcggcggcggctccggtggtggtggatccGAGGTGCARCTTGTTGARTC            4
HB14'     ggcggcggcggctccggtggtggtggatccGARGTRAAGCTTCTCGARTC            8
HB15'     ggcggcggcggctccggtggtggtggatccGAAGTGAARSTTGAGGARTC            8
HB16'     ggcggcggcggctccggtggtggtggatccCAGGTTACTCTRAAASARTC            8
HB17      ggcggcggcggctccggtggtggtggatccCAGGTCCAACTVCAGCARCC            6
HB18'     ggcggcggcggctccggtggtggtggatccGATGTGAACTTGGAASARTC            4
HB19'     ggcggcggcggctccggtggtggtggatccGAGGTGAAGGTCATCGARTC            2
```

Primer VH-for

```
                  5' EcoRI        3'
scfor             ggaattcggcccccgag
                  5' EcoRI   SfiI    VH →                 3'
HF1               ggaattcggcccccgaggcCGAGGAAACGGTGACCGTGGT              1
HF2               ggaattcggcccccgaggcCGAGGAGACTGTGAGAGTGGT              1
HF3               ggaattcggcccccgaggcCGCAGAGACAGTGACCAGAGT              1
HF4               ggaattcggcccccgaggcCGAGGAGACGGTGACTGAGGT              1
```

VL-κ	1	2	3	4	5	6	7
LB1	D	I	Q	L	T	Q	P
LB2	D	I	V	L	T, S	Q	S
LB3	D	I	V	I, L	T	Q	S
LB4	D	I	V	L	T	Q	S
LB5	D	I	V	M	T	Q	S
LB6	D	I	K, Q	I, M	N, T	Q	S
LB7	D	I	Q	M	I, T	Q	S
LB8	D	I	Q	M	T	Q	T
LB9	D	I	V	L	I, N	Q	S
LB10	D	I	E, V	L	T	Q	S
LB11	D	I	L, V	M	T	Q	S
LB12	D	I, V	L, V	M	T	Q	T
LB13	D	I	V	M	T	Q	A, S
LB14	D	I	V	I	T	Q	D, E
LB15	D	I	V	M	T	Q	I, M L, P
LB16	D	I	V	M	T	Q	P
LB17	D	I	L	L	T	Q	S

102	103	104	105	106	107	108	VL-κ
A, P S, T	K	L	E	I	K	R	*LF1'*
A, P S, T	K	L	E	I	K	R	*LF4*
A, P S, T	K	L	E	L	K	R	*LF5*

102	103	104	105	106	107	108	VL-λ
A, P S, T	K	L	T	V	L	G	*LFλ*

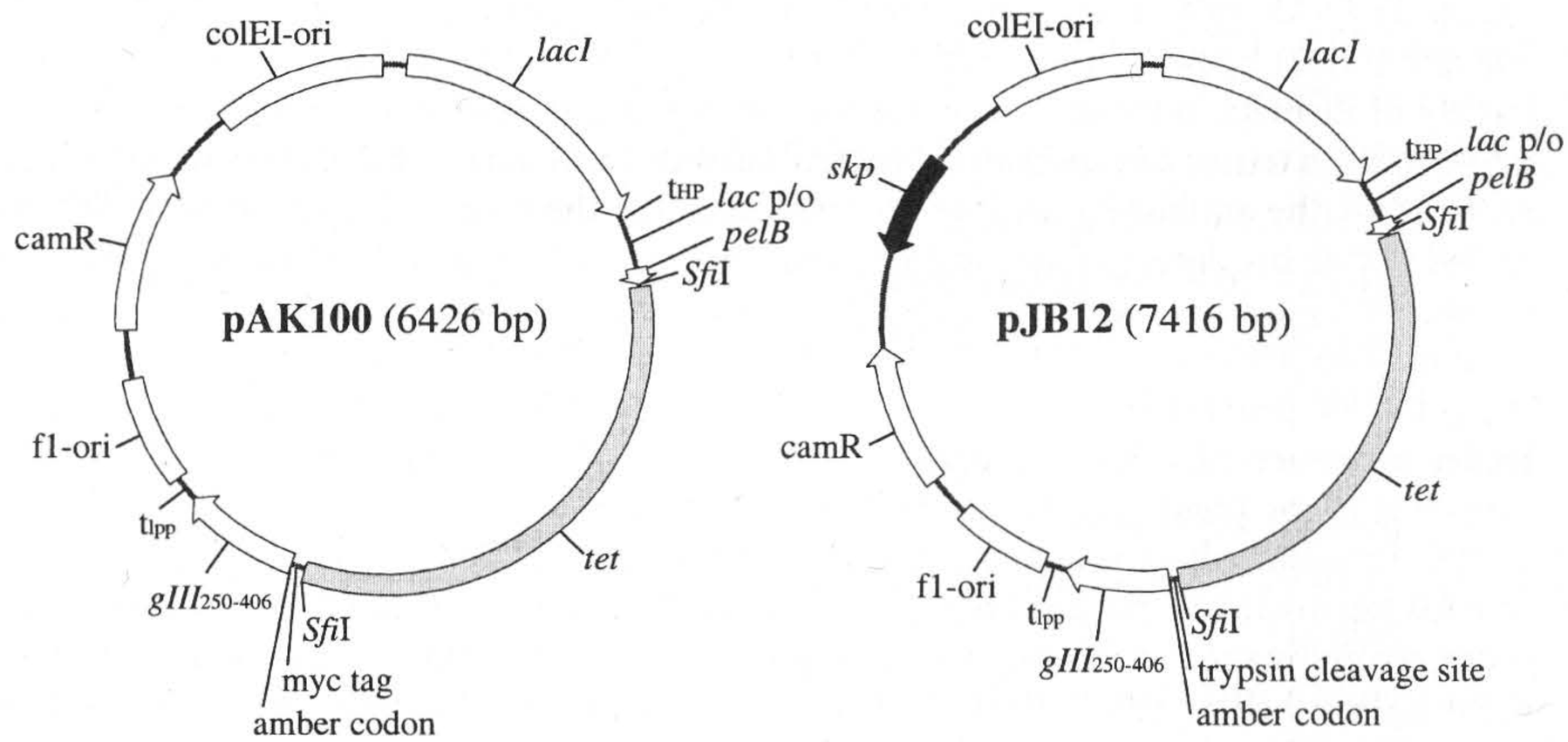
107	108	109	110	111	112	113	VH
T	T	V	T	V	S	S	*HF1*
T	T	L	T	V	S	S	*HF2*
T	L	V	T	V	S	A	*HF3*
T	S	V	T	V	S	S	*HF4*

VL-λ	1	2	3	4	5	6	7	8
LBλ	D	A	V	V	T	Q	E	S

VH	1	2	3	4	5	6	7
HB1	D, E	V	N, Q	L	Q	E	S
HB2	E	V	Q	L	Q	Q	S
HB3'	Q	V	Q	L	K	E, Q	S
HB4	E	V	Q	L	Q	Q	S
HB5	Q	V	Q	L	Q	Q	S
HB6'	Q	V	Q	L	Q	Q	S
HB7'	Q	V	H	V	K	Q	S
HB8'	E	V	K, N	L, V	V	E	S
HB9'	D, E	V	K, M	L, V	V	E	S
HB10'	E	V	Q	L, V	V	E	S
HB11'	D, E	V	H, Q	L	V	E	S
HB12	E	V	K	L	M	E	S
HB13'	E	V	Q	L	V	E	S
HB14'	E	V	K	L	L	E	S
HB15'	E	V	K	L, V	E	E	S
HB16'	Q	V	T	L	K	E	S
HB17	Q	V	Q	L	Q	Q	P
HB18'	D	V	N	L	E	E, Q	S
HB19'	E	V	K	V	I	E	S

- Standard molecular biology equipment and reagents to:
  - suspend spleen cells (Gibco 10 x EBSS Buffer)
  - determine the isotype of mAbs (Roche IsoStrip Mouse Monoclonal Antibody Isotyping Kit)
  - purify mRNA (Pharmacia QuickPrep mRNA Purification Kit)
  - perform a cDNA synthesis reaction (Pharmacia First Strand cDNA Synthesis Kit)
  - perform PCR reactions
  - cut and gel purify DNA
  - concentrate DNA (Amicon Microcon 30 for volumes less than 500 μl)
  - ligate and transform DNA
  - grow bacteria and phages
  - perform an ELISA
  - perform a Western blot

Fig. 3. Deduced amino acid sequence of the complementary part of primers from Fig. 2. The residues of VL and VH are numbered according to Kabat et al. (1991). The primers *LB1-17* and *HB1-19* determine only the first 2 nucleotides of residue 7, while the *LF* primers determine only the first 2 nucleotides of residue 102, and *LBλ* specifies only the first nucleotide of residue 8. Therefore, the original residues at those positions may not necessarily appear in the PCR amplified antibody gene.



pAK / pJB vector series		phage display	Skp coexpression	enhanced expression	trypsin cleavage site	IMAC purification	C-terminal detection	direct detection	dimerization
pAK100	SfiI SfiI EcoRI HindIII: pelB – tet – myc * gIII 250-406	●							
pAK300	SfiI SfiI HindIII: pelB – tet – 6 his					●	●		
pAK400	SDT7g10 SfiI SfiI HindIII: lac p/o – pelB – tet – 6 his			●		●	●		
pAK500	SfiI SfiI EcoRI HindIII: pelB – tet – dHLX 5 his					●	●		●
pAK600	SfiI SfiI EcoRI HindIII: pelB – tet – alkaline phosphatase							●	●
pJB12	SfiI SfiI EcoRI HindIII: pelB – tet – KDIR * gIII 250-406	●	●		●				
pJB23	SfiI SfiI EcoRI HindIII: pelB – tet – 6 his		●			●	●		
pJB33	SDT7g10 SfiI SfiI EcoRI HindIII: lac p/o – pelB – tet – 6 his		●	●		●	●		

tet-cassette

Fig. 4. The pAK / pJB vector series (see also Fig.5) can be used either for phage display (pAK 100 and pJB12) by the strategy outlined in Fig. 1 or for the expression of the antibody in a variety of formats. All vectors contain a chloramphenicol resistance cassette (camR) and additionally a tetracyclin resistance "stuffer" cassette (*tetA* and *tetR*; 2101 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 cells on tetracycline plates). At the bottom of the figure, the tet-cassette, which is simplified in the vector drawings, is shown in more detail. Furthermore, these vectors contain the *lacI* repressor gene, a strong upstream terminator (t_{HP}), the *lac* promoter/operator and the *pelB* (pectate lyase gene of *Erwinia carotovora*) leader sequence (modified to contain a *Sfi*I site) and a downstream terminator (t_{lpp}). The origins for phage replication and plasmid replication are as described in Ge et al. (1995). The antibody gene is alternatively fused in frame to gene$III_{251-406}$ (for phage display), to a his tag for IMAC purification (Lindner et al. 1992) and C-terminal detection with another recombinant anti-his tag scFv-phosphatase fusion protein (Lindner et al. 1997), to dimerization helices (Pack et al. 1993, see also Chapter 43, Lindner and Plückthun) 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) to act as a detection handle, in addition to the short N-terminal 3-amino acid FLAG tag (Knappik et al. 1994) which is encoded by the primers (Fig. 2). The pJB12 contains a trypsin cleavage site (KDIR) and can therefore conveniently be used for selection of high affinity binders as described by Dziegiel et al. (1995) and Johansen et al. (1995). The asterisk in 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). In the pJB vector series the co-expressed periplasmic protein Skp (Bothmann and Plückthun 1998) or a similarly placed FkpA (Bothmann and Plückthun 2000) can increase the functional yield of antibody fragments expressed in the periplasm (for details, see also Chapter 23).

A

pAK100scFv, pAK300scFv, pAK500scFv, pAK600scFv, pJB12scFv, pJB23scFv

```
                                                  end lacI
...ATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGC
...M  Q  L  A  R  Q  V  S  R  L  E  S  G  Q  *

                        tHP terminator
GGTACCCGATAAAAGCGGCTTCCTGACAGGAGGCCGTTTTGTTTTGCAGC

                     CAP binding site
CCACCTCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGG

  -35                        -10          lac-operator
CTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGA
                                        | → mRNA

                SD1         lacZ
TAACAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTTCTAGA
                            M  T  M  I  T  N  F  *

    SD2          pelB signal sequence
TAACGAGGGCAAATCATGAAATACCTATTGCCTACGGCAGCCGCTGGATT
                M  K  Y  L  L  P  T  A  A  A  G  L

                    SfiI            FLAG      VL
GTTATTACTCGCGGCCCAGCCGGCCATGGCGGACTACAAAGAY...
 L  L  L  A  A  Q  P  A  M  A  D  Y  K  D  ...
```

pAK400scFv, pJB33scFv

```
     SD2            pelB signal sequence
...GAAGGAGATATACATATGAAATACCTATTGCCTACGGCAGCC...
   T7g10            M  K  Y  L  L  P  T  A  A  ...
```

B

pAK100scFv

```
   VH     SfiI     EcoRI            myc tag
...CGGCCTCGGGGGCCGAATTCGAGCAGAAGCTGATCTCTGAGGAAGAC
...  A  S  G  A  E  F  E  Q  K  L  I  S  E  E  D

      geneIII 250-406
CTGTAGGGTGGTGGCTCTGGTTCCGGTGATTTTGATTATGAAAAG...
L  *  G  G  G  S  G  S  G  D  F  D  Y  E  K  ...
```

pJB12scFv

```
   VH     SfiI     EcoRI        trypsin cleavage site
...CGGCCTCGGGGGCCGAATTCGAGCAGAAGGATATCCGTGAGGAAGAC
...  A  S  G  A  E  F  E  Q  K  D  I  R  E  E  D

      geneIII 250-406
CTGTAGGGTGGTGGCTCTGGTTCCGGTGATTTTGATTATGAAAAG...
L  *  G  G  G  S  G  S  G  D  F  D  Y  E  K  ...
```

Fig. 5. A: upstream sequence of pAK100scFv, pAK300scFv, pAK400scFv, pAK500scFv, pAK600scFv, pJB12 and pJB23. The region from the end of the *lacI* repressor gene to the beginning of the antibody VL 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 sequence, N-terminal *SfiI* site, four amino acid FLAG tag and the start of the VL domain (bold) are indicated above the sequence. In pAK400 and pJB33, the 15 bp upstream from the *pelB* start codon are replaced by a sequence including the SD sequence of the phage T7 gene10. **B:** downstream sequence of pAK100scFv and pJB12scFv. The last two bases of VH (bold), *SfiI* and *EcoRI* restriction sites, myc tag or trypsin cleavage site and the start of $geneIII_{251-406}$ are indicated above the sequence.

C

pAK300scFv, pAK400scFv

```
   VH     SfiI        his tag                 HindIII
...CGGCCTCGGGGGCCGATCACCATCATCACCATCATTAGTAAGCTT..
...  A  S  G  A  D  H  H  H  H  H  H  *  *
```

pJB23scFv, pJB33scFv

```
   VH     SfiI     EcoRI  his tag
...CGGCCTCGGGGGCCGAATTCCACCACCATCACCACCATTAATGAAAG
...  A  S  G  A  E  F  H  H  H  H  H  H  *  *

HindIII
CTT...
```

C: downstream sequence of pAK300scFv, pAK400scFv, pJB23scFv and pJB33scFv. The last two bases of VH (bold), *Sfi*I and *Eco*RI restriction sites and his_6 tags are indicated above the sequence.
D: sequences of *Eco*RI/*Hind*III fusion cassettes as used in pAK500 and pAK600. The dHLX dimerization motif 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/*Hind*III cloning cassette the two internal *Eco*RI sites of the AP gene have been removed by silent mutations.

D

pAK500scFv

```
     VH     SfiI     EcoRI                dHLX
  ...CGGCCTCGGGGGCCGAATTCCCCAAACCTAGCACCCCCCCTGGCA
  ...  A  S  G  A  E  F  P  K  P  S  T  P  P  G  S

GCAGTGGTGAACTGGAAGAGCTGCTTAAGCATCTTAAAGAACTTCTGAAG
  S  G  E  L  E  E  L  L  K  H  L  K  E  L  L  K

GGCCCCCGCAAAGGCGAACTCGAGGAACTGCTGAAACATCTGAAGGAGCT
G  P  R  K  G  E  L  E  E  L  L  K  H  L  K  E  L

                                his tag
GCTTAAAGGTGGGAGCGGAGGCGCGCCGCACCATCATCACCATTGACGTC
 L  K  G  G  S  G  G  A  P  H  H  H  H  H  *

 HindIII
TAAGCTT...
```

pAK600scFv

```
   VH     SfiI     EcoRI   alkaline phosphatase (AP)
...CGGCCTCGGGGGCCGAATTCCGGACACCAGAAATGCCTGTTCTG...
     A  S  G  A  E  F  R  T  P  E  M  P  V  L  ...
                       start AP

                                              HindIII
...CTCTTCTACACCATGAAAGCCGCTCTGGGGCTGAAATAAGCTT...
...L  F  Y  T  M  K  A  A  L  G  L  K  *
                                   end AP
```

Procedure

Isolation of mRNA and cDNA synthesis

1. Take 1 to 5 million cells from a frozen or growing hybridoma (for isotype determination use the Roche IsoStrip Mouse Monoclonal Antibody Isotyping Kit) culture or spleen cells, respectively (see note), and perform a mRNA preparation as described in the QuickPrep mRNA Purification Kit (Pharmacia). According to the manufacturer, this kit can be used for up to 5 x 10^7 cells, but in order to get extremely pure mRNA take only 5 x 10^6 cells per oligo(dT)-cellulose column included in the kit, which will yield 1-10 µg of mRNA.

   Note: For mRNA preparation from spleen cells press the organ through a sieve (you may take a sterile strainer), suspend the cells in 10 ml EBSS buffer and separate off the connective tissue by centrifugation (15 s at 200 g). Transfer the supernatant into a fresh tube, centrifuge (7 min at 250 g) and resuspend the cells in 2 ml extraction buffer (Pharmacia QuickPrep mRNA Purification Kit). You typically obtain 5 x 10^7 B-cells (storage at -80°C in extraction buffer) from one mouse spleen.

   EBSS buffer (to suspend spleen cells)
   - 100 ml 10 x EBSS (Gibco)
   - 2.2 g $NaCO_3$
   - 25 ml 1 M HEPES (pH 7.3)
   - Adjust to pH 7.1 with HCl and fill up with water to 1 l

2. Separate the mRNA (step 1 yields 750 µl) in two aliquots and ethanol precipitate each. For an aliquot of 320 µl mRNA in elution buffer take 800 µl ethanol (chilled to -20°C), 10 µl glycogen (10 mg/ml) and 32 µl potassium acetate (2.5 M, pH 5.0).

   Note: The mRNA can be stored in ethanol at -20°C for several months.

3. Collect the precipitated mRNA of one aliquot by centrifugation at 16,000 g, 4°C for 30 min.

4. Wash with 1 ml ice-cold 90% ethanol.

5. Add 20 µl H_2O (diethylpyrocarbonate-treated). The mRNA solution is now ready for cDNA synthesis.

6. For reverse transcription take approximately 0.1-0.5 µg mRNA and 1 µl random hexamer primers (33 µl total reaction volume). The precise

procedure is described in the First Strand cDNA Synthesis Kit (Pharmacia).

PCR amplification

7. Take the primers (100 μM) which have been dissolved in TE buffer to prepare appropriate mixtures (LB mix, LF mix, HB mix and HF mix). Mix them according to the degree of degeneration (number of different unique sequences encoded by mixed bases) indicated in the column "d" in Fig 2. Take 1 μl from all non-degenerated primers (d = 1), 2 μl for d = 2-4, 3 μl for d = 6-9 and 4 μl for d = 12-16. To complete the LB mix (see note) add 2.8 μl of *LBλ* (= 5%) to the mixture of *LB1-17* (53 μl). For the LF mix take 19 μl of *LF1'*, 9.5 μl of *LF4*, 9.5 μl of *LF5* and 2 μl of *LFλ* (=5%). The total primer concentration of these mixtures is still 100 μM (ranging from 2.3 to 25 μM of each mixed synthesis primer).

   Note: During the cloning of monoclonal antibodies problems can occur if the hybridoma transcribes more than one functional or even non-functional heavy or light chain variable region gene (Kütemeier et al. 1992). It was found that several kappa chain secreting hybridomas, where X63Ag8.653 myeloma cells were used as a fusion partner, are able to transcribe a functional lambda chain which competes with the kappa VL gene for in-frame scFv antibody assembly (Krebber et al. 1997). Therefore, it is highly recommended to leave out any lambda chain primer in the PCR reactions if the isotyping indicates that the hybridoma of interest secretes a kappa light chain.

8. For PCR amplification of VL and VH use the completed first-strand cDNA reaction and prepare the following mixes.

PCR mix for amplification of VL	PCR mix for amplification of VH
2 μl cDNA	2 μl cDNA
1 μl dNTP's (10 mM each)	1 μl dNTP's (10 mM each)
5 μl 10 x PCR Buffer	5 μl 10 x PCR Buffer
1 μl LB primer mix (100 μM)	1 μl HB primer mix (100 μM)
1 μl LF primer mix (100 μM)	1 μl HF primer mix (100 μM)
40 μl H_2O	40 μl H_2O
prepare 3 tubes of this mix	prepare 3 tubes of this mix

Note: The polymerase is added only after heating these mixes to 92°C. For Taq polymerase a $MgCl_2$ concentration of 2 mM is usually optimal. Although Taq polymerase works generally more robustly than other polymerases, the use of proof-reading polymerases is an alternative, but will require optimization steps such as Mg^{2+} titrations. This might be a problem, when the amount of cDNA template is limited.

9. Heat to 92°C for 3 min (use mineral oil if no lid heating is available), add 1 unit Taq polymerase (see note of step 8) and perform the following cycles (6 independent PCR reactions, 3 each for V_H and V_L with different annealing temperatures): 5 cycles of 1 min at 92°C, 1 min initial annealing at 45°C (alternatively, see note, 50°C and 55°C) and 1 min at 72°C, followed by 20 cycles of 1 min at 92°C, 1 min at 63°C and 1 min at 72°C.

   Note: For amplification of VL and/or VH, complete annealing of the 3'-ends of the primers with the template DNA is essential. Three different initial annealing temperatures (45°C, 50°C, 55°C) are recommended to be able to amplify the great majority of the antibody genes, as it is not clear a priori, which somatic mutations a given monoclonal antibody gene may carry in the primer regions. After 5 cycles the amplified PCR product will serve itself as template DNA. The annealing temperature of the last 20 cycles is therefore 63°C in all 3 cases.

10. Gel-purify the VL and VH genes and determine the DNA concentration of both chains.

    Note: Using the listed primer mixtures, the expected lengths of the PCR products of VL and VH are between 375-402 bp and 386-440 bp, respectively. The 3 different PCR reactions do not necessarily each yield product. However, these PCR products can be pooled if necessary.

11. Use approximately 10 ng of the PCR product of both domains for the assembly PCR (50 µl total volume). Take 200 µM dNTP's, 1 µM (each) *scback* and *scfor* primer. Use 1 unit DNA polymerase (see note of step 8) and perform 7 cycles of 1 min at 92°C, 30 s at 63°C, 50 s at 58°C and 1 min at 72°C, followed by 23 cycles of 1 min at 92°C, 30 s at 63°C and 1 min at 72°C.

    Note: Hot-start PCR and initial assembly of VL and VH in the absence of the primers is usually not necessary, but can be used.

Digestion and cloning of scFv genes

12. Perform a *Sfi*I digest of the amplified scFv for 3-4 hours at 50°C (overlay with mineral oil). In case of Taq polymerase the digestion can be conveniently carried out in the PCR buffer. To 40 µl PCR product add 11 µl 10 x buffer, 13 µl 10 x BSA, 64 µl H_2O and 2 µl (= 20 units) *Sfi*I.

    Note: This procedure might work with other PCR buffers as well but has not been tested yet.

13. Digest appropriate amounts of vector (pAK100 or pJB12, see Fig. 4) with *Sfi*I. Use 10 units *Sfi*I for 1 µg vector in 100 µl volume and incubate overnight at 50°C (overlay with mineral oil).

    Note: For pure preparations of a fully digested vector it is very important to not over-load 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.

14. Purify and concentrate the digested scFv antibody genes and vector by preparative agarose gel (1%) electrophoresis in combination with the QIAEX II Gel Extraction Kit (Qiagen).

    Note: For large scale vector or insert preparation, electroelution is most efficient and convenient. For concentration you may use Microcon 30 columns (Amicon) as described in step 31.

15. Ligate 20 ng scFv gene fragment with the vector (ratio vector to insert 3:2) with 1 unit ligase in 10 µl volume. Incubate overnight at 16°C.

    Note: Compatible vector sets are available which allow an easy recloning of the scFv fragment into vectors for optimized soluble expression, generation of fusion proteins and other purposes (Fig. 4 and Fig. 5).

16. Transform 5-10 µl of the ligation into competent XL1-Blue (Stratagene) cells.

    Note: If many clones are required follow the instructions described in step 23-33.

17. Plate on 2 x 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 testing for tetracycline resistance. The portion of vector with unremoved or religated *tet* cassette (see note of step 13) is typically in the range of 0.01 to 0.1%.

Screening for binders

18. Pick 10 colonies and let them grow separately at 37°C in 2 ml 2 x YT, 1% glucose, chloramphenicol (30 µg/ml), until they reach an $OD_{550} = 0.5$. Add 2 ml 2 xYT, 1% glucose, chloramphenicol (30 µg/ml), 1 mM IPTG, 5 x 10^9 cfu helper phage (Stratagene) and shake overnight at 26°C or 37°C (for some scFv's, growth at 26°C after infection may be necessary). The phage titer after overnight incubation is in the range of 10^{11} cfu per ml supernatant.

    Note: The phage titer should be checked in order to rule out any problems during phage production. To determine the phage titer (in cfu) take a growing culture of XL1-Blue cells ($OD_{550} = 0.4$-0.8) and incubate aliquots of this culture with dilutions of your phage preparation. After 15 min incubation at 37°C, plate appropriate amounts (30-150 cfu/plate) on 2 x YT, 1% glucose, chloramphenicol (30 µg/ml) agar plates.

19. Centrifuge the culture. Take 1.6 ml supernatant and mix it with 0.4 ml 20% PEG 6000, 2.5 M NaCl in a 2 ml Eppendorf cap in order to precipitate the phages (Sambroook et al. 1989).

20. Incubate on ice for 15 min and spin at 16,000 g, 4°C for 20 min.

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

21. Suspend the phage pellet in 400 µl PBS (2% milk) and use 100 µl phage solution per well in an ELISA assay (see step 22) to distinguish functional scFv antibody displaying phages from those which display a non-functional or non-productive antibody fragment.

    Note: Do not (!) centrifuge after suspending the phage pellet because you would spin down phages together with cell debris.

22. If soluble antigen is available include an ELISA control that shows that free antigen is able to compete with bound antigen for phage binding to distinguish non-specific "sticky" phage from specifically binding phage. In principle, the same ELISA protocol which 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 10 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.

Library cloning

23. For preparation of electrocompetent *E. coli* XL1-Blue (Stratagene) cells use 1 ml of a dense overnight pre-culture to inoculate 500 ml medium (2 x YT). Shake it at 37°C until an OD_{550} of 0.7 is reached. Then, chill the culture on ice as quickly as possible (cool the whole shake flask for 10 min in ice-cold water).

    Note: Sufficient agitation and aeration during growth seems to be very important for preparation of electrocompetent cells with reproducible efficiencies of 3-6 x 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 (if not, moderately increase the amount of medium).

24. Centrifuge 400 ml (8 x 50 ml in disposable tubes) for 9 min at 2500 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 50 ml ice-cold distilled water and remove the water immediately (the cell pellet is very solid after this first centrifugation step and will not be resuspended by the brief addition of distilled water).

    Note: It is recommended to carry out steps 24 to 28 in the coldroom.

25. Fill each tube with 25 ml distilled water (200 ml total) and resuspend each pellet carefully by pipetting the solution up and down with a 25 ml wide-gauge sterile plastic pipette (Falcon) which has to be placed very close to the pellet. Make sure that the cells are all taken up in a homogeneous suspension. Incubate for 10 min on ice.

26. Transfer the cells into 4 new 50 ml tubes and centrifuge at 2500 g for 12 min. Carefully remove the supernatant. Resuspend the four pellets in a total volume of 40 ml pre-chilled 10% (v/v) DMSO (Fluka). Incubate for 10 min on ice.

27. Centrifuge (2 x 20 ml) at 2500 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!) and resuspend in 20 ml 10% DMSO. Incubate on ice for 10 min.

28. Centrifuge at 2500 g for 15 min. Carefully resuspend the cells in 2 ml 10% DMSO, freeze the cells (100 µl aliquots) by dipping the tubes in liquid nitrogen and store them at -80°C.

29. To check the transformation efficiency (see also step 32) of the cells add 1 µl of 10 pg/µl pUC19 DNA (in water). Fifty colonies per 1/1000 transformation correspond to an efficiency of 5 x 10^9 cfu/µg pUC19 DNA.

Note: Using 2 mm electroporation cuvettes (2500 V) the time constant for the efficiency test with 10 pg pUC19 DNA should be 5.4-5.6 ms, reflecting properly washed cells.

30. For desalting prior to electroporation fill up the ligation mixture with water to 400 µl and heat it for 10 min at 65°C. Then load it on a Microcon 30 (Amicon) column and centrifuge at 11,000 g for 8 min. Discard the flow-through.

31. Fill up with 400 µl water, centrifuge at 11,000 g and discard the flow-through. Repeat this procedure 3 times. It might be necessary to prolong the last centrifugation step (the residual volume should be between 20 and 50 µl). Finally, turn the columns upside down in clean tubes and centrifuge at 960 g for 3 min.

32. For each transformation use desalted ligation mixture corresponding to 20-100 ng insert. Add the DNA to the barely thawed cells (on ice) and mix by flipping the tube shortly. Now, any further incubation is detrimental. Load it therefore directly into the chilled electroporation cuvette and trigger the pulse. Immediately add the medium (900 µl 2 x YT (room temperature) containing 10 mM $MgCl_2$, 2.5 mM KCl and 0.4% glucose).

Note: For efficient transformation (= 10^8 clones per µg insert DNA) the time constant (using 2 mm cuvettes) should be $\geq$ 5.2 ms.

33. Plate on 2 x YT, 1% glucose, chloramphenicol (30 µg/ml) agar plates (do not exceed 10^8clones per 0.06 m^2) and incubate overnight at 37°C. Scrape the colonies off the plates and subsequently store them at -80°C after addition of 30% glycerol.

Note: Take care that your library is homogeneously mixed.

34. For screening proceed as described in step 18-22. For inoculation take at least 10 fold more viable cells than colonies obtained after transformation. Perform 3 rounds of phage panning (see note of step 22) before testing single clones.

Note: The first panning round is the most crucial because you might lose any desired, but less abundant, antibody sequence by too exten-

sive washing. Therefore, do not exceed 10 washing steps in the first panning round.

Troubleshooting

- In case of low transformation yields it should be analyzed if the problem is the transformation itself or rather the ligation. To check the ligation, an aliquot can be analyzed on an agarose gel which might indicate also any problems with nucleases. Furthermore, it might be instructive to compare the ligation efficiency of *Sfi*I digested PCR product with insert derived from plasmid digestion. In order to check both the ligation and the transformation efficiency, a defined amount of pUC19 DNA can be mixed into the ligation. Due to the chloramphenicol resistance of the cloning vector and the ampicillin resistance of pUC19 DNA it is possible to calculate the ligation efficiency by plating 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 with pUC19 DNA alone.
- If no binders appear from this procedure the primers should be checked since the quality and presence of each of the oligonucleotide is most decisive for reliable cloning of various antibody genes or, in case of library cloning, for broad representation of the immune response. Any sequence absent from a complex mixture will obviously decrease the functional library size and single-base deletions present in any one of the oligonucleotides would be amplified into the final product. Take care, therefore, that the quality of the oligonucleotide primers is satisfactory. Because of the low profit margins, oligonucleotides are today frequently synthesized with a minimum of reagents excess and short reaction times, not reaching the coupling yields that would be possible. A low total yield of synthesized oligonucleotides indicates usually a low coupling yield and is a warning sign that the "full-length" oligonucleotide pool contains a significant portion of molecules with random single-base deletions. Gel purification can ameliorate but not solve the problem, which lies in poor DNA-synthesis quality. It is recommended, especially for library cloning, to sequence the genes of random clones or to check for full-length scFv by Western blot analysis.

Acknowledgements. We wish to thank Anke Krebber, Annemarie Honegger, Lutz Jermutus, Barbara Klinger and Cornelia Rinderknecht for helpful contributions to the reagents and procedures of this article.

References

Bothmann H, Plückthun A (1998) Selection for a periplasmic factor improving phage display and functional periplasmic expression. Nature Biotechnol 16:376-380

Bothmann H, Plückthun A (2000) The periplasmic E. Coli peptidylprolyl cis, trans-isomerase FkpA. Increased functional expression of antibody fragments with and without cis-prolines. J Biol Chem 275:17100-17105

Braden BC, Goldman ER, Mariuzza RA, Poljak RJ (1998) Anatomy of an antibody molecule: structure, kinetics, thermodynamics and mutational studies of the antilysozyme antibody D1.3. Immunol Reviews. 163:45-57

Dall'Acqua W, Carter P (1998) Antibody engineering. Curr Opin Struct Biol 8:443-450

Dziegiel M, Nielson LK, Anderson PS, Blancher A, Dickmeiss E, Engberg J (1995) Phage display used for gene cloning of human recombinant antibody against the erythrocyte surface antigen, rhesus D. J Immmunol Meth 182:7-19

Freund C, Ross A, Plückthun A, Holak TA (1994) Structural and dynamic properties of the Fv fragment and the single-chain Fv fragment of an antibody in solution investigated by heteronuclear three-dimensional NMR spectroscopy. Biochemistry 33:3296-3303

Ge L, Knappik A, Pack P, Freund C, Plückthun A (1995) Expressing antibodies in *Escherichia coli.* In: Borrebaeck CAK (ed) Antibody Engineering, 2nd edition. Oxford University Press, pp 229-236

Hanes J, Jermutus L, Weber-Bornhauser S, Bosshard HR, Plückthun A (1998) Ribosome display efficiently selects and evolves high-affinity antibodies *in vitro* from immune libraries. Proc Natl Acad Sci USA 95:14130-14135

Hoogenboom HR, de Bruine AP, Hufton SE, Hoet RM, Arends JW, Roovers RC (1998) Antibody phage display technology and its applications. Immunotechnology 4:1-20

Johansen LK, Albrechtsen B, Andersen HW, Engberg J (1995) pFab60: a new, efficient vector for expression of antibody Fab fragments displayed on phage. Prot Eng 10:1063-1067

Kabat EA, Wu TT, Reid-Miller M, Perry HM, Gottesman KS, Foeller C (1991) Sequences of Proteins of Immunological Interest. 5th edn. US Department of Health and Human Services, Public Service, NIH

Knappik A, Ge L, Honegger A, Pack P, Fischer M, Wellnhofer G, Hoess A, Wölle J, Plückthun A, 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

Knappik A, Plückthun A (1994) An improved affinity tag based on the FLAG peptide for the detection and purification of recombinant antibody fragments. BioTechniques 17:754-761

Krebber A, Bornhauser S, Burmester J, Honegger A, Willuda J, Bosshard HR, Plückthun A (1997) Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. J Immunol Meth 201:35-55

Kütemeier G, Harloff C, Mocikat R (1992) Rapid isolation of immunoglobulin variable genes from cell lysates of rat hybridomas by polymerase chain reaction. Hybridoma 11:23-32

Lindner P, Guth B, Wülfing C, Krebber C, Steipe B, Müller F, Plückthun A (1992) Purification of native proteins from the cytoplasm and periplasm of *Escherichia coli* using IMAC and histidine tails: a comparison of proteins and protocols. Methods 4:41-56

Lindner P, Bauer K, Krebber A, Nieba L, Kremmer E, Krebber C, Honegger A, Klinger B, Mocikat R, Plückthun A (1997) Specific detection of his-tagged proteins with recombinant anti-his tag scFv-phosphatase or scFv-phage fusions. BioTechniques 22:140-149

Low NM, Holliger PH, Winter G (1996) Mimicking somatic hypermutation: affinity maturation of antibodies displayed on bacteriophage using a bacterial mutator strain. J Mol Biol 260:359-368

Martineau P, Betton JM (1999) In vitro folding and thermodynamic stability of an antibody fragment selected *in vivo* for high expression levels in *Escherichia coli* cytoplasm. J Mol Biol 292:921-929

Munro S, Pelham HR (1986) An Hsp70-like protein in the ER: Identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell 46:291-300

Ohlin M, Owman H, Mach M, Borrebaeck CAK (1996) Light chain shuffling of a high affinity antibody results in a drift in epitope recognition. Mol Immunol 33:47-56

Pack P, Kujau M, Schroeckh V, Knüpfer U, Wenderoth R, Riesenberg D, Plückthun A (1993) Improved bivalent miniantibodies, with identical avidity as whole antibodies, produced by high cell density fermentation of *Escherichia coli.* Biotechnology 11:1271-1277

Parsons HL, Earnshaw JC, Wilton J, Johnson KS, Schueler PA, Mahoney W, McCafferty J (1996) Directing phage selections towards specific epitopes. Prot Eng 9:1043-1049

Plückthun A (1994) Recombinant antibodies. In: Van Oss CJ, Van Regenmortel MHV (eds) Immunochemistry. Marcel Dekker, New York, pp 201-236

Plückthun A, Krebber A, Krebber C, Horn U, Knüpfer U, Wenderoth R, Nieba L, Proba K, Riesenberg D (1996) Producing antibodies in *Escherichia coli*: From PCR to fermentation. In: McCafferty J, Hoogenboom HR (eds) Antibody Engineering: A Practical approach. IRL press, Oxford, pp 203-252

Rees AR, Staunton D, Webster DM, Searle SJ, Henry AH, Pederson JT (1994) Antibody design: beyond the natural limits. Trends Biotechnol 12:199-206

Sambrook J, Fritsch E, Maniatis T (1989) Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schier R, Bye, J, Apell G, McCall A, Adams GP, Malmqvist M, Weiner LM, Marks JD (1996) Isolation of high-affinity monomeric human anti-c-erbB-2 single chain Fv using affinity-driven selection. J Mol Biol 255:28-43

Shuttleworth H, Taylor J, Minton N (1986) Sequence of the gene for alkaline phosphatase from *Escherichia coli* JM83. Nucleic Acids Res 14:8689

Thompson JE, Vaughan TJ, Williams AJ, Wilton J, Johnson KS, Bacon L, Green JA, Field R, Ruddock S; Martins S, Pope AR, Tempest PR, Jackson RH (1999) A fully human antibody neutralising biologically active human TGFbeta2 for use in therapy. J Immunol Meth 227:17-29

Vaughan TJ, Williams AJ, Pritchard K, Osbourn JK, Pope AR, Earnshaw JC, McCafferty J, Hodits RA, Wilton J, Johnson KS (1996) Human antibodies with sub-nanomolar

affinities isolated from a large non-immunized phage display library. Nature Biotechnol 14:309-314
Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR (1994) Making antibodies by phage display technology. Ann Rev Immunol 12:433-455
Wörn A, Plückthun A (1999) Different equilibrium stability behavior of scFv fragments: identification, classification, and improvement by protein engineering. Biochemistry 38:8739-8750

Abbreviations

BSA	bovine serum albumin
DMSO	dimethylsulfoxide
EBSS	Earle's buffered salt solution
HRP	horse radish peroxidase
IPTG	isopropylthiogalactoside
PEG	polyethylene glycol