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Construction and use of antibody gene repertoires

A. R. POPE, M. J. EMBLETON, and R. MERNAUGH

1. Introduction

The display of antibody molecules on the surface of bacteriophage (1) provides a powerful method of selecting a specific antibody from a mixed population of antibodies together with the gene encoding it. This ability to co-select proteins and their genes has been exploited to enable the isolation of high-affinity, antigen-specific antibodies derived from an immunized mouse (2) and from unimmunized humans (3). In addition, after selecting such an antibody, a mutant antibody library can be constructed and higher affinity antibody clones selected. Alternatively, a monoclonal antibody can be subcloned from a hybridoma cell line into the bacteriophage and after mutagenesis, antibodies of improved characteristics selected (4).

Bacteriophage fd is a filamentous, single-stranded DNA phage which infects male *Escherichia coli* cells. Adsorption to the host sex pilus is mediated by the gene 3 protein (g3p) displayed at the tip of the virion. The amino-terminal domains of the three g3p molecules on each virion form knob-like structures that are responsible for binding the phage to the F-pilus whilst the C-terminal domain is anchored in the phage coat (5, 6). George Smith found that peptides could be displayed on the surface of phage by fusion to the N-terminus of g3p (7). Phage with binding activities could then be isolated from random peptide libraries after repeated rounds of growth and selection for phage with the desired binding characteristics (8–10).

The range of molecules displayed as gene 3 fusions has now been extended to include folded proteins (1). Antibodies have been displayed as functional binding molecules in the form of single chain Fv fragments (1), Fab fragments (11), and as both bivalent and bispecific dimeric Fv fragments (diabodies) (12). This has allowed the selection of phage from a mixed population according to their binding characteristics, for example single chain Fv antibody fragments with a high affinity for 2-phenyl-5-oxazolone have been selected from a library of antibodies derived from an immunized mouse (2). The enzymes alkaline phosphatase from *E. coli* (13) and *Staphylococcus*

nuclease (14) have catalytic activity when displayed on bacteriophage fd. Further, phage displaying the human receptor molecules, CD4 and platelet-derived growth factor BB receptor (15), specifically bind the appropriate ligand. Bass *et al.* (16) have displayed functional human growth hormone on the surface of the closely related bacteriophage M13. Functional expression of an antibody fragment as a gene 8 protein fusion in M13 (17) has also been described, but this system is, at present, not as well characterized as that using gene 3.

In this chapter we describe in detail (see *Figure 1*) procedures for the cloning and selection of antibody genes using display on bacteriophage as g3p fusions. Many of these techniques will be directly applicable to work with other proteins.

2. Vectors for the display of proteins on the surface of bacteriophage fd

The first vectors for the display of antibodies on the surface of bacteriophage fd were derived from the vector fd-tet (18). However, phage DNA has a low efficiency of transformation when compared to plasmids such as the pUC series of vectors. Phagemid vectors (which contain both a plasmid origin of replication and a filamentous phage origin of replication) allow 100-fold higher efficiencies of transformation to be obtained compared to phage vectors. The higher efficiency of transformation as a result of the use of phagemids enables large libraries to be made. This is important when making a repertoire from an immunized mouse, and more so when making a mouse or human repertoire from a non-immunized source. Gene 3 from bacteriophage fd has therefore been inserted into phagemid vectors with restriction sites allowing insertion of foreign DNA sequences. These vectors were designed to allow the insertion of single chain Fv sequences as fusions at the N-terminus of the gene 3 protein and to be compatible with the PCR primers used by Orlandi *et al.* (19) to amplify antibody variable regions. Super infection with a helper phage, such as M13K07, results in packaging of this phagemid DNA into a phage particle, which displays the protein encoded by the insert as an N-terminal fusion with the gene 3 protein.

The phagemid pCANTAB-5 (*Figure 2*) has been derived from the phagemid pUC119 and provides unique *Sfi*I and *Not*I sites for cloning antibody genes. It allows the cloning of foreign DNA sequences (e.g. scFv fragments) as *Sfi*I/*Not*I fragments at the N-terminus of gene 3. Export of g3p fusions to the periplasm is directed using the synthetic leader sequence. A range of further developments of this vector (pCANTAB-5myc, pCANTAB-5E, and pCANTAB-6) contain an amber codon inserted at the start of the gene 3 segment, allowing expression of the inserted protein from the gene 3 fusion as a soluble fragment in a non-suppressing *E. coli* strain such as HB2151, without recloning the gene. However, in a suppressor strain such as

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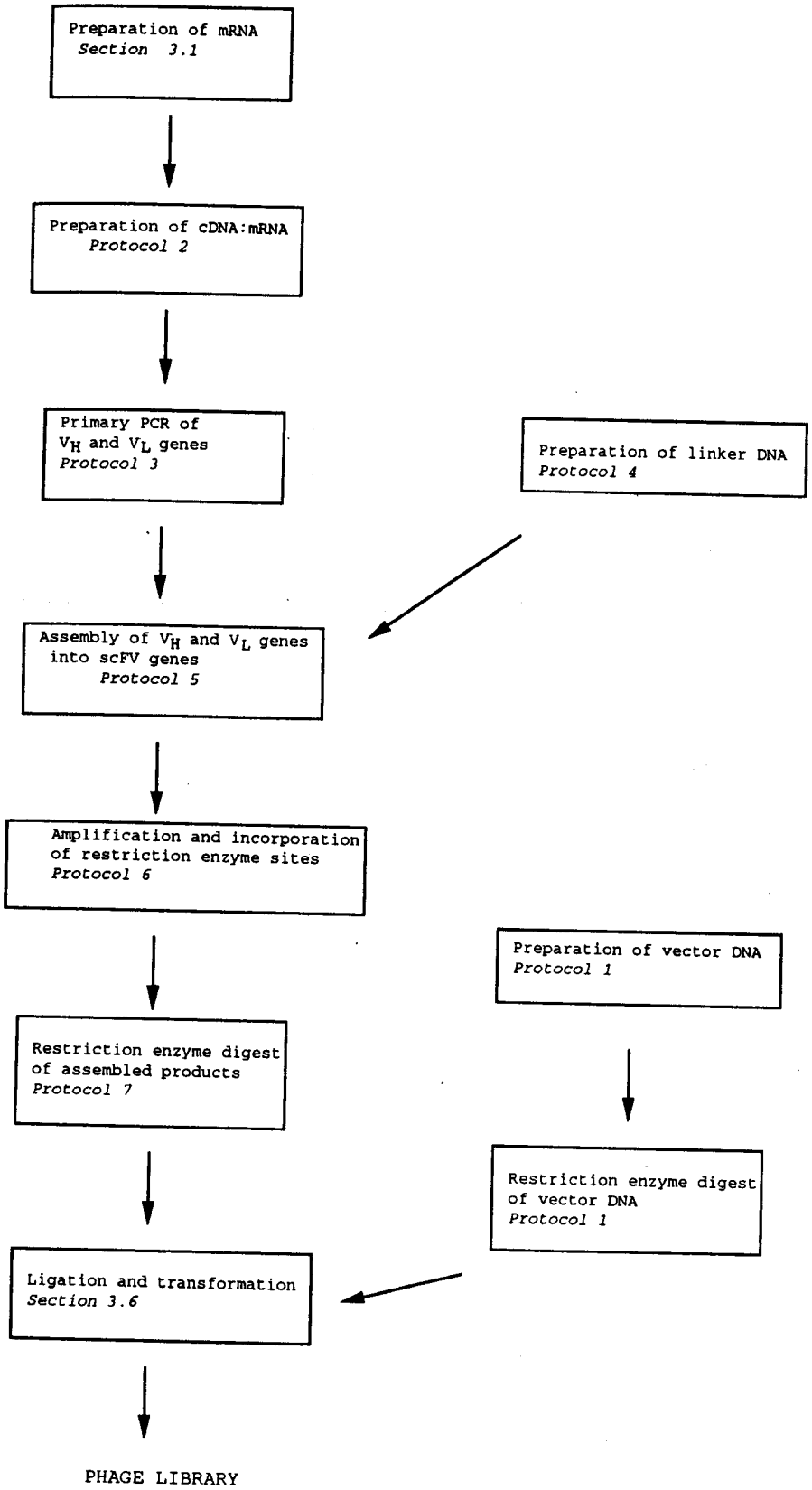
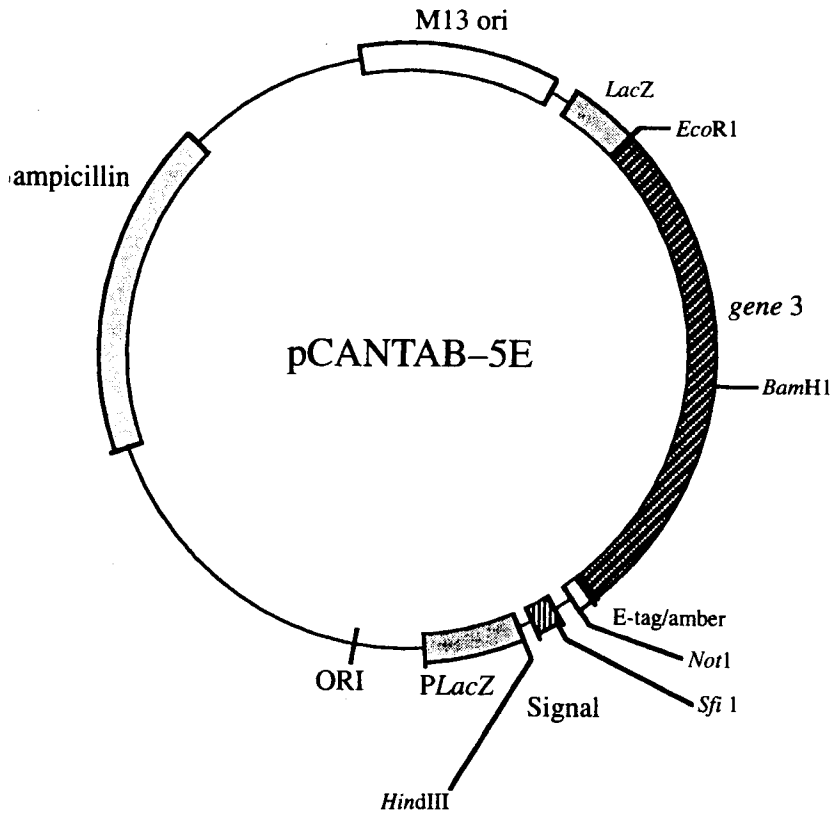


Figure 1. Flow diagram for the cloning of single chain Fv fragments into pCANTAB-5.



*Hin*dIII

AAGCTT TGGAGCCTTT TTTTTGGAGA TTTTCAAC GTG AAA AAA TTA TTA TTC GCA ATT
V K K L L F A I

signal sequence — *Sfi* I

CCT TTA GTT GTT CCT TTC TAT GCG GCC CAG CCG GC C ATG GCC CAG GTC CAA CTG
P L V V P F Y A A Q P A M A Q V Q L

Not I — E-tag

CAG GTC GAC CTC GAG ATC AAA CGG GCG GCC GC A GGT GCG CCG GTG CCG TAT CCG
Q V D L E I K R A A A G A P V P Y P

E-tag — amber — fd-gene 3 sequence

GAT CCG CTG GAA CCG CGT GCC GCA TAG ACT GTT GAA AGT TGT TTA GCA AAA CCT
N P L E P R A A (*) T V E S C L A K P

Figure 2. The phagemid vector pCANTAB-5E is based on pUC119 into which gene 3 of bacteriophage fd has been inserted. The cloning sites *Sfi*I and *Not*I are used for the cloning of scFv sequences. Expression is under control of the *plac* promoter.

TG1 the suppression of the amber codon is far from complete. This has the advantage that both complete gene-3/antibody fusion protein and single chain Fv antibody can be produced in the same cell with a suppressor strain. Induction of expression with IPTG will give single chain Fv antibody only with no gene-3 fusion protein. This can be used for testing the binding specificity and affinity of selected clones. Alternatively, rescue with M13KO7 helper phage will give both phage particles displaying functional antibody (which can be used for selection) and soluble antibody in the same culture.

A derivative of this vector, pCANTAB-5E (Pharmacia, cat. no. 27–9901–01) which has an E tag inserted at the C terminus of the soluble antibody has also been developed. This E tag can be detected in ELISA with the anti E monoclonal antibody which is available commercially (Pharmacia, cat. no. 27–9412–02). It can also be used for purification by affinity chromatography (Pharmacia Recombinant Phage Antibody System purification module 17–1362–01). A similar vector, pCANTAB-5myc, has a c-*myc* tag (a sequence derived from the c-*myc* oncogene) (20) inserted at the C terminus, instead of the E-tag, and can be detected and purified with the anti c-*myc* monoclonal antibody 9E10. A derivative of this, pCANTAB-6 has an additional tag of six histidines that is useful for affinity-chromatography purification (21).

Protocol 1. Preparation of vector DNA

To prepare vector DNA for cloning, RF plasmid DNA is isolated from the bacterial pellet by the plasmid alkaline lysis method (described in ref. 22). This DNA is then purified by centrifugation on a CsCl gradient. For smaller preparations a proprietary mini-prep purification system (Promega or Qiagen) can be used.

Equipment and reagents

For this protocol you will require the following basic equipment and reagents:

- Microcentrifuge
- Sterile H_2O
- Sterile microcentrifuge tubes (Eppendorf)
- Sterile pipette tips
- Agarose (BRL)
- Agarose gel electrophoresis tank (Pharmacia, cat. no. GNA 100)
- TAE buffer: to make a 50 × stock dissolve 242 g Tris base and 57.1 ml of glacial acetic acid in a final volume of 900 ml and then add 100 ml of 0.5 M EDTA (pH 8.0)

In addition to the basic equipment and reagents, the following will also be required:

- *Sfi*I restriction enzyme and 10 × buffer (as supplied by the manufacturer)
- *Not*I restriction enzyme and 10 × buffer (as supplied by the manufacturer)
- Chromospin 1000 (Clontech) columns or a Geneclean II DNA purification kit
- Cold (– 20 °C) ethanol
- Cold (– 20 °C) 70% ethanol
- 3 M sodium acetate buffer, pH 5.2
- Vacuum desiccator
- 65 °C waterbath
- 50 °C waterbath
- 37 °C waterbath

Method

1. Digest 10 µg of vector DNA in a volume of 200 µl with 50 units of the restriction enzyme *Sfi*I for 4 hours at 50 °C in the manufacturer's recommended buffer.

GCA ATT
A I

C CAA CTG
Q L

ag ------
G TAT CCG
Y P

A AAA CCT
K P

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Protocol 1. *Continued*

2. Ethanol precipitate the DNA by adding a 1/10 volume of 3 M Na acetate, pH 5.2, and 2.5 × volumes of cold ethanol. Chill at – 20°C for 15 minutes. Then spin in a microcentrifuge for 5 minutes at 4°C. Remove and discard the supernatant. Wash the pellet with 750 µl of cold (– 20°C) 70% ethanol. Spin briefly (30 seconds). Remove and discard the supernatant. Dry the pellet in a vacuum desiccator for 2–3 minutes. Do not over dry. Dissolve the pellet in the required buffer for the next step in the protocol.
3. Digest the vector DNA in a volume of 200 µl with 50 units of the restriction enzyme *Not*I for 4 hours at 37°C in the manufacturer's recommended buffer. Cloned *Not*I enzyme should be used for the digestion. Heat inactivate at 65°C for 20 minutes.
4. Purify the *Sfi*I–*Not*I double-cut DNA free from the resulting linker fragment. Chroma spin-1000 columns (Clontech) work well for this. Use according to the manufacturer's instructions. Use two columns and load each with 100 µl of cut DNA directly from the final restriction digest. Alternatively, a 0.7–1% low melting point agarose gel can be used to purify the cut vector and the DNA extracted using the 'Wizard PCR preps' DNA Purification System (Promega, cat. no. A7170) Geneclean II kit (BIO 101, cat. no. 3106) or Sephaglass Band Prep kit (Pharmacia, cat. no. 27–9285–01) with the final product dissolved in TE buffer ready for ligation with the DNA insert.

3. Preparation and cloning of antibody DNA

These protocols detail the production of both mouse and human antibody repertoires. These repertoires will be random combinatorial repertoires since the heavy and light chain DNA sequences are amplified separately before being assembled together and cloned. As a result, the original heavy and light chain pairings are lost. Although it is possible to find antibodies to 'self' antigens in the immune system, very often B cells producing such antibodies are eliminated by the body (e.g. by clonal selection and anergy). As a result, random combinatorial libraries make it easier to select human antibodies to human antigens.

These methods can be readily adapted for the generation of hierarchical libraries. A hierarchical library is a repertoire consisting of a single heavy chain or a restricted number of heavy chains combined with a full range of light chains (or vice versa). Such libraries are an important way of improving the affinity of an existing antibody (23) (also see Chapter 2 on affinity maturation by phage display) since they can give new antibodies with a better heavy and light chain pairing than the original.

For those researchers who wish to study the actual heavy and light chain pairings used by the immune system it is possible to preserve these by using 'in-cell PCR'. Protocols describing this new technique are described at the end of this chapter.

The following protocols have been developed for use with material from both immunized and non-immunized individuals. In addition, they can also be used to make single chain Fv clones, expressed on the surface of phage, from monoclonal antibodies. It should be noted that monoclonal antibody cell lines often contain more than a single heavy and single light chain sequence. Up to 10 different scFv clones have previously been obtained from a single monoclonal cell line (personal observation). The screening *Protocols* (*9*, *11*, and *13*) can be used for the phage-display clones derived from a monoclonal antibody. We usually find that approximately 30% of clones are positive (by ELISA) for the antigen of interest. For some monoclonal antibodies the percentage of positive antigen binders can drop well below this and selection with the antigen of interest may be necessary.

An overall scheme for preparing libraries of antibody scFv fragments in pCANTAB-5E is shown in *Figure 1*.

A commercially available system (Pharmacia Recombinant Phage Antibody System Mouse ScFv Module, cat. no. 27–9400–01) contains the necessary reagents for cDNA preparation, primary PCR, and assembly of mouse heavy and light chain sequences preparatory to cloning and expression. An expression module (cat. no. 27–9401–01) contains vector, host cells, and helper phage for the cloning of phage antibodies and for the display and selection of recombinant phage antibodies.

3.1 Preparation of mRNA

It is possible to use total RNA made by phenol/SDS extraction of tissue (22) and this can give excellent results. However, a pure mRNA preparation prepared using an oligo (dT) column has been found to give higher yields in the PCR amplification steps that follow and is therefore generally recommended. Oligo (dT)-affinity purification systems (e.g. Pharmacia QuickPrep^r mRNA purification kit cat. no. 27–9254–01) are commercially available and produce mRNA free of RNase. Poor quality RNA preparations can cause the subsequent PCR steps to fail. In the case of repertoire constructions, even if there are no apparent problems during construction and cloning, the diversity of the repertoire could be greatly reduced and the usefulness of the final library greatly compromised if the RNA is of poor quality.

For non-immunized human repertoires, peripheral blood lymphocytes prepared from whole blood samples are used (3). Generally 500 ml of blood should contain approximately 1×10^8 cells and should give up to 100 μg of total RNA. Use 10 μg per cDNA preparation.

If using a commercial system, such as the Pharmacia QuickPrep^r mRNA kit, then it is important not to overload the oligo (dT) column with cell

extract. (This can cause the column matrix to aggregate and trap contaminants reducing final yield and purity.) The white blood cells from 50 ml of blood (approximately 1×10^7 cells) will give enough mRNA from one oligo (dT) column for four cDNA reactions. The purified mRNA is ethanol precipitated and resuspended in 20 µl of RNase-free water (see *Protocol 2*, Equipment and Reagents list, for RNase-free, DEPC-treated water). For each cDNA reaction 5 µl mRNA is used. The mRNA can be safely stored in ethanol and then precipitated and resuspended prior to use.

Immunized repertoires are prepared with mRNA from the spleen of an immunized mouse. An average mouse spleen should contain approximately 1×10^8 cells. If using purified mRNA it is important not to overload the oligo (dT) column with cell extract. Therefore use 25% of the spleen and freeze the rest. This is also enough material for a total RNA preparation.

In addition, tissue culture cells from a monoclonal antibody cell line can also be used to subclone the monoclonal antibody into the phage-display format. An aliquot of 1×10^7 cells should give up to 100 µg of total RNA, 10 µg of this is used per cDNA preparation. For the Pharmacia QuickPrep^r mRNA kit, Pharmacia recommend using 5×10^6 cells per oligo (dT) column. After ethanol precipitation and resuspending in 20 µl this should be adequate for four cDNA preparations.

An alternative method for mRNA preparation is given in Chapter 6 (*Protocol 2*, step 1).

3.2 cDNA preparation

The mRNA is now used as a template to prepare cDNA. Random hexamer primers are used to prime cDNA synthesis. This generates cDNA:mRNA hybrids. Since this cDNA will be used as a PCR template there is no need to make double-stranded cDNA.

Protocol 2. Preparation of cDNA:mRNA hybrid

Equipment and reagents

In addition to basic equipment and reagents (see *Protocol 1*), the following will also be required for this protocol:

- DEPC-treated H_2O: prepare by adding diethylpyrocarbonate to H_2O (0.1% v/v), and incubate at 37°C for 2 hours. DEPC-treated H_2O should be autoclaved before use to inactivate the diethylpyrocarbonate
- 5 mM dNTP: equimolar mixture of dATP, dCTP, dGTP, and dTTP with a total concentration of 5 mM nucleotide (i.e. 1.25 mM each dNTP)
- 0.1 M dithiothreitol solution
- 10 × first strand buffer: 1.4 M KCl, 0.5 M Tris–HCl, pH 8.1 at 42°C, 80 mM $MgCl_2$
- Random hexamer primers (e.g. pd(N)6 from Pharmacia, cat. no. 27–2166–01)
- RNasin (a ribonuclease inhibitor) (Promega)
- Reverse transcriptase (e.g. murine reverse transcriptase (cloned) Pharmacia, cat. no. 27–0925–01)
- 100°C waterbath
- 65°C waterbath
- 42°C waterbath

Method

1. Set up the following reverse transcription mix:
   - H_2O (DEPC-treated) 19 µl
   - 5 mM dNTP 10 µl
   - 10 × first strand buffer 10 µl
   - 0.1 M dithiothreitol 10 µl
   - Random hexamer primers (10 OD_{260}/ml)[a] 2 µl
   - RNasin (40 U/µl) 4 µl
2. For a total RNA preparation, dilute 10 µg RNA to 40 µl final volume with DEPC-treated water. For oligo (dT) column purified mRNA preparation, add 5 µl (one quarter of the sample) to 35 µl of DEPC-treated water. Heat at 65°C for 3 min and then place on ice for 1 min (to remove secondary structure).
3. Add 40 µl of the diluted RNA to the reverse transcription mix and then add 100 units of reverse transcriptase. Mix and then incubate at 42°C for 1 hour.
4. Boil the reaction mix for 3 minutes, cool on ice for 1 minute and then spin in a microcentrifuge at 13 000 *g* for 5 minutes, to pellet debris. Transfer the supernatant to a new tube. Either store at – 20°C or proceed directly to *Protocol 3*.

[a] Random hexamer primers (e.g. pd(N)6 from Pharmacia) are used to prime cDNA synthesis. Specific primers complementary to the 3′ end of the CH or VL domain can also be used (2, 3), but we have found that random hexamers work better.

3.3 Primary PCR

The cDNA:mRNA hybrid is now used as a template for the 'primary' PCR amplification of the V_H and V_L domains. Specific primers have been designed for the amplification of mouse V_H and V_L genes. These primers, described in *Table 1*, have been shown to generate a diverse library when used to prepare a repertoire of antibodies from an immunized mouse (2). Specific primers have also been designed for the amplification of human V_H and V_L genes (3) and are shown in *Table 2*.

The BACK primer hybridizes to the beginning of framework 1 of the antibody sequence and the FOR primer (or in the case of the mouse kappa light chain the MJKFONX primer) binds to the end of framework 4 (contributed by the J segment during antibody rearrangement). For example, the mouse V_H genes are amplified using the primers VH1BACK and VH1-FOR2. The primers VH1BACK and VH1FOR2 are consensus primers which will cover the majority of mouse heavy chain gene families. Similarly, the primer VK2BACK is used with a mix of four J region primers (MJK1FONX, MJK2FONX, MJK4FONX, and MJK5FONX) to amplify the light chain kappa families. The primer VK2BACK is a consensus primer that will cover

most of the mouse kappa families. However, there are some gaps in the sequences covered by this primer. In the case of the commercially available system from Pharmacia, a mix of five different specific VKBACK primers is used, thus covering the full range of kappa light chains.

Thus to make a mouse repertoire, two primary PCR reactions are necessary, one for the V_H genes and one for the V_L genes.

In the case of making a human repertoire the situation is more complicated since there is a great deal of sequence diversity which necessitates a large number of primers. Thus for the heavy genes there are six VHBACK primers and four JHFOR primers. For the lambda light chains (V_{lambda}), seven VLBACK and three JLFOR primers are required and for the kappa light chains (V_{kappa}), six VKBACK and five JKFOR primers. This gives a total of 31 primers. For the primary PCR amplification of the heavy genes six reactions are required, each with a separate VHBACK primer, but using the JHFOR primers as a mix. The seven lambda and six kappa light chain gene PCR amplifications are performed in a similar fashion.

After PCR amplification, check the PCR products (2 μl samples on a 2% agarose gel). Problems can be due to either the template (particularly the initial mRNA preparation) or from the PCR amplification itself. Not all thermal cyclers perform identically. The annealing temperature and possibly the ramp rate may need fine tuning. The Pharmacia Recombinant Phage Antibody System (Pharmacia, cat. no. 27–9400–01) contains a control mRNA, and cDNA prepared from this can be used as a suitable control. After PCR, the amplified products are purified ready for the next step in the process. With a human repertoire, the V_H reactions can be pooled prior to purification. The light chain PCR preparations should be processed separately as 13 distinct preparations. At the assembly stage a mix of V_H genes will be linked to each of the separate light chain preparations.

Protocol 3. Primary PCR of antibody genes

Equipment and reagents

In addition to basic equipment and reagents (see *Protocol 1*), the following will also be required for this protocol:

- Taq DNA polymerase[a] (Boehringer, under licence from Perkin Elmer/Cetus)
- 10 × Taq polymerase buffer as provided by the supplier of the Taq polymerase, or: 100 mM Tris–HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, and 0.01% (w/v) gelatin
- 5 mM dNTPs (see *Protocol 2*)
- Hydrochloric acid solution (0.25 M)
- DNA thermal cycler for PCR
- Mineral oil (paraffin oil) (Sigma, cat. no. M-3516)
- Ultraviolet lamp (254 nm) for irradiating contaminating DNA in amplification reaction
- 'Wizard PCR prep' DNA purification kit (Promega)

Method

1. For each PCR the following reaction mix is set up. Suitable controls (no DNA) are also required. All working solutions of primers are at concentrations of 10 pmol/μl.

- H_2O 35 µl
- 10 × Taq buffer 5 µl
- 5 mM dNTP 1.5 µl
- FOR primer[b] (10 pmol/µl) 2.5 µl
- BACK primer[b] (10 pmol/µl) 2.5 µl

2. Irradiate this mix with ultraviolet light at 254 nm for approximately 5 min.
3. Add 2.5 µl cDNA:mRNA hybrid (from *Protocol 2*), and 47.5 µl of the PCR mix to a 0.5 ml microcentrifuge tube. Overlay with 2 drops of mineral oil (Sigma)
4. Place on a cycling heating block preset at 94°C.
5. Add 1 µl Taq DNA polymerase under the paraffin.
6. Amplify using 30 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 2 min. Check each PCR amplification with a 2 µl sample on a 2% gel.
7. Purify on a 2% lmp (low melting point) agarose/Tris–acetate–EDTA gel.[c] Carefully excise the V_H and V_L bands (using a fresh sterile scalpel or razor blade for each) and transfer each band to a separate sterile microcentrifuge tube. The use of suitable molecular weight markers will aid selection of the correct bands. Both the V_H and V_L bands are approximately 350 bases long. (This depends on the germline sequence used and on the length of the CDR3 sequence which is highly variable. Generally, the V_H band has a slightly higher molecular weight than the V_L band.) Use 'Wizard PCR prep DNA purification system' from Promega[d] or the Sephaglass Band Prep kit (cat. no. 27–9285–01) from Pharmacia to purify each DNA band. Recover the DNA in 50 µl H_2O.

For a human repertoire treat each one of the six kappa and the seven lambda PCRs separately. However, it is useful to pool the V_H PCRs and then concentrate them with Promega 'Wizard PCR prep' prior to gel purification. This will reduce losses of material during gel purification.

[a] A proof-reading Taq polymerase can help PCR amplify those monoclonal antibodies which will not amplify with standard Taq polymerase. Amplification can be prevented by somatic mutations (for example in framework 1) causing mismatches with the PCR primers.

[b] *For a mouse repertoire*

FOR and BACK primers shown in *Table 1* are:

Heavy chains: VH1-FOR2
VH1BACK

Light chains: (MJK1FONX, MJK2FONX, MJK4FONX, MJK5FONX)
VK2BACK

[b] *For a human repertoire*

FOR and BACK primers shown in *Table 2* are:

Heavy chains:

FOR: HuJH1–2FOR, HuJH3FOR, HuJH4–5FOR, HuJH6FOR

Protocol 3. *Continued*

BACK: HuVH1aBACK, HuVH2aBACK, HuVH3aBACK, HuVH4aBACK, HuVH5aBACK, HuVH6aBACK
Lambda chains:
FOR: HuJλ1FOR, HuJλ2–3FOR, HuJλ4–5FOR
BACK: Huλ1BACK, Huλ2BACK, Huλ3aBACK, Huλ3bBACK, Huλ4BACK, Huλ5BACK, Huλ6BACK
Kappa chains:
FOR: HuJK1FOR, HuJK2FOR, HuJK3FOR, HuJK4FOR, HuJK5FOR
BACK: HuVK1aBACK, HuVK2aBACK, HuVK3aBACK, HuVK4aBACK, HuVK5aBACK, HuVK6aBACK

[c] To avoid contamination, it is essential to depurinate the electrophoresis apparatus, combs, etc. with 0.25 M HCl overnight before use.

[d] 'Wizard PCR prep' DNA purification kit is from Promega, used according to the manufacturer's instructions. After removal of wash solution (centrifugation step) incubate columns at room temperature to allow isopropanol from wash buffer to evaporate before next step.

Table 1. Oligonucleotides for mouse PCR reactions

Primary PCR oligos (restriction sites underlined):

VH1FOR-2	TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC
VH1BACK	AGG TSM ARC TGC AGS AGT CWGG
MJK1FONX	CCG TTT GAT TTC CAG CTT GGT GCC
MJK2FONX	CCG TTT TAT TTC CAG CTT GGT CCC
MJK4FONX	CCG TTT TAT TTC CAA CTT TGT CCC
MJK5FONX	CCG TTT CAG CTC CAG CTT GGT CCC
VK2BACK	GAC ATT GAGCTC ACC CAG TCT CCA

PCR oligos to make linker:

LINKFOR	TGG AGA CTG GGT GAGCTC AAT GTC
LINKBACK	GGG ACC ACG GTC ACC GTC TCC TCA

Oligos for addition of restriction sites:

VH1BACKSfi	GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTS MAR CTG CAG SAG TCW GG
JK1NOT10	GAG TCA TTC TGC GGC CGC CCG TTT GAT TTC CAG CTT GGT GCC
JK2NOT10	GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAG CTT GGT CCC
JK4NOT10	GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAA CTT TGT CCC
JK5NOT10	GAG TCA TTC TGC GGC CGC CCG TTT CAG CTC CAG CTT GGT CCC

Ambiguity codes

M = A or C
R = A or G
S = G or C
W = A or T

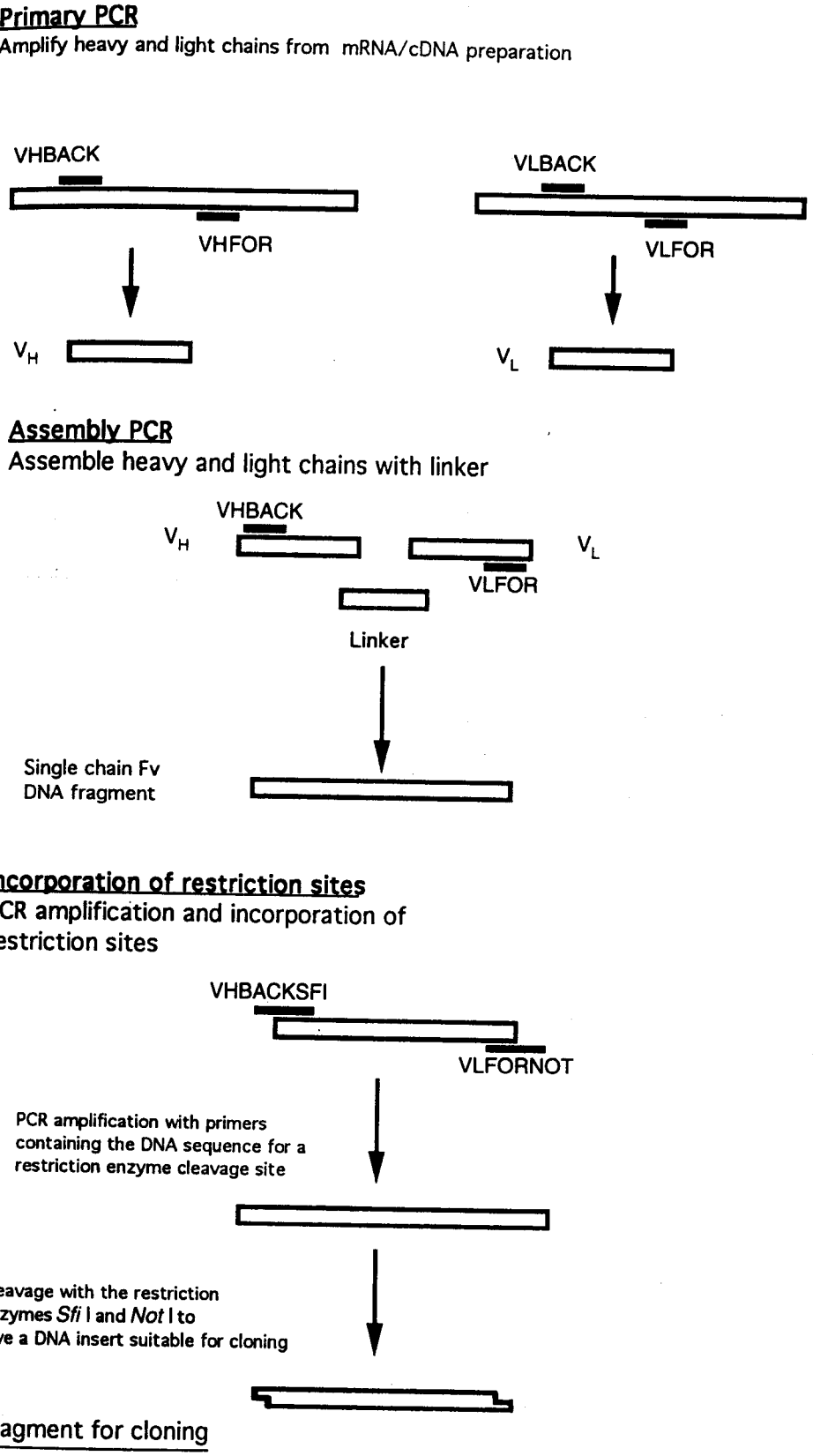

Figure 3. Preparation of a library of inserts encoding single chain Fv fragments.

Table 2. Oligonucleotides for human PCR reactions

Primary PCR oligos:

HuJH1–2FOR	5′ TGA GGA GAC GGT GAC CAG GGT GCC
HuJH3FOR	5′ TGA AGA GAC GGT GAC CAT TGT CCC
HuJH4–5FOR	5′ TGA GGA GAC GGT GAC CAG GGT TCC
HuJH6FOR	5′ TGA GGA GAC GGT GAC CGT GGT CCC
HuVH1aBACK	5′ CAG GTG CAG CTG GTG CAG TCT GG
HuVH2aBACK	5′ CAG GTC AAC TTA AGG GAG TCT GG
HuVH3aBACK	5′ GAG GTG CAG CTG GTG GAG TCT GG
HuVH4aBACK	5′ CAG GTG CAG CTG CAG GAG TCG GG
HuVH5aBACK	5′ GAG GTG CAG CTG TTG CAG TCT GC
HuVH6aBACK	5′ CAG GTA CAG CTG CAG CAG TCA GG
HuJK1FOR	5′ ACG TTT GAT TTC CAC CTT GGT CCC
HuJK2FOR	5′ ACG TTT GAT CTC CAG CTT GGT CCC
HuJK3FOR	5′ ACG TTT GAT ATC CAC TTT GGT CCC
HuJK4FOR	5′ ACG TTT GAT CTC CAC CTT GGT CCC
HuJK5FOR	5′ ACG TTT AAT CTC CAG TCG TGT CCC
HuVK1aBACK	5′ GAC ATC CAG ATG ACC CAG TCT CC
HuVK2aBACK	5′ GAT GTT GTG ATG ACT CAG TCT CC
HuVK3aBACK	5′ GAA ATT GTG TTG ACG CAG TCT CC
HuVK4aBACK	5′ GAC ATC GTG ATG ACC CAG TCT CC
HuVK5aBACK	5′ GAA ACG ACA CTC ACG CAG TCT CC
HuVK6aBACK	5′ GAA ATT GTG CTG ACT CAG TCT CC
HuJλ1FOR	5′ ACC TAG GAC GGT GAC CTT GGT CCC
HuJλ2–3FOR	5′ ACC TAG GAC GGT CAG CTT GGT CCC
HuJλ4–5FOR	5′ ACC TAA AAC GGT GAG CTG GGT CCC
HuJλ1BACK	5′ CAG TCT GTG TTG ACG CAG CCG CC
HuJλ2BACK	5′ CAG TCT GCC CTG ACT CAG CCT GC

HuJλ1FOR	5′ ACC TAG GAC GGT GAC CTT GGT CCC
HuJλ2–3FOR	5′ ACC TAG GAC GGT CAG CTT GGT CCC
HuJλ4–5FOR	5′ ACC TAA AAC GGT GAG CTG GGT CCC
HuJλ1BACK	5′ CAG TCT GTG TTG ACG CAG CCG CC
HuJλ2BACK	5′ CAG TCT GCC CTG ACT CAG CCT GC

HuJλ3aBACK	5′ TCC TAT GTG CTG ACT CAG CCA CC
HuJλ3bBACK	5′ TCT TCT GAG CTG ACT CAG GAC CC
HuJλ4BACK	5′ CAC GTT ATA CTG ACT CAA CCG CC
HuJλ5BACK	5′ CAG GCT GTG CTC ACT CAG CCG TC
HuJλ6BACK	5′ AAT TTT ATG CTG ACT CAG CCC CA

PCR oligos to make linker:

Reverse JH for scFv linker

RHuJH1–2	5′ GCA CCC TGG TCA CCG TCT CCT CAG GTG G
RHuJH3	5′ GGA CAA TGG TCA CCG TCT CTT CAG GTG G
RHuJH4–5	5′ GAA CCC TGG TCA CCG TCT CCT CAG GTG G
RHuJH6	5′ GGA CCA CGG TCA CCG TCT CCT CAG GTG G

Reverse V_K for scFv linker

RHuVK1aBACKFv	5′ GGA GAC TGG GTC ATC TGG ATG TCC GAT CCG CC
RHuVK2aBACKFv	5′ GGA GAC TGA GTC ATC ACA ACA TCC GAT CCG CC
RHuVK3aBACKFv	5′ GGA GAC TGC GTC AAC ACA ATT TCC GAT CCG CC
RHuVK4aBACKFv	5′ GGA GAC TGG GTC ATC ACG ATG TCC GAT CCG CC
RHuVK5aBACKFv	5′ GGA GAC TGC GTG AGT GTC GTT TCC GAT CCG CC
RHuVK6aBACKFv	5′ GGA GAC TGA GTC AGC ACA ATT TCC GAT CCG CC

Reverse V_λ for scFv linker

RHuVλ1BACKFv	5′ GGC GGC TGC GTC AAC ACA GAC TGC GAT CCG CCA CCG CCA GAG
RHuVλ2BACKFv	5′ GCA GGC TGA GTC AGA GCA GAC TGC GAT CCG CCA CCG CCA GAG
RHuVλ3aBACKFv	5′ GGT GGC TGA GTC AGC ACA TAG GAC GAT CCG CCA CCG CCA GAG
RHuVλ3bBACKFv	5′ GGG TCC TGA GTC AGC TCA GAA GAC GAT CCG CCA CCG CCA GAG
RHuVλ4BACKFv	5′ GGC GGT TGA GTC AGT ATA ACG TGC GAT CCG CCA CCG CCA GAG
RHuVλ5BACKFv	5′ GAC GGC TGA GTC AGC ACA GAC TGC GAT CCG CCA CCG CCA GAG
RHuVλ6BACKFv	5′ TGG GGC TGA GTC AGC ATA AAA TTC GAT CCG CCA CCG CCA GAG

Table 2. Continued

Oligos for addition of restriction sites:

Human V_H Back Primers

HuVH1aBACKSfi	5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG GTG CAG TCT GG
HuVH2aBACKSfi	5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTC AAC TTA AGG GAG TCT GG
HuVH3aBACKSfi	5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTG CAG CTG GTG GAG TCT GG
HuVH4aBACKSfi	5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG CAG GAG TCG GG
HuVH5aBACKSfi	5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG TTG CAG TCT GC
HuVH6aBACKSfi	5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTA CAG CTG CAG CAG TCA GG

Human J_K Forward Primers

HuJK1FORNOT	5′ GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT TTC CAC CTT GGT CCC
HuJK2FORNOT	5′ GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAG CTT GGT CCC
HuJK3FORNOT	5′ GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT ATC CAC TTT GGT CCC
HuJK4FORNOT	5′ GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAC CTT GGT CCC
HuJK5FORNOT	5′ GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT AAT CTC CAG TCG TGT CCC

Human J_λ Forward Primers

HuJλ1FORNOT	5′ GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT GAC CTT GGT CCC
HuJλ2–3FORNOT	5′ GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT CAG CTT GGT CCC
HuJλ4–5FORNOT	5′ GAG TCA TTC TCG ACT TGC GGC CGC ACC TAA AAC GGT GAG CTG GGT CCC

Ambiguity codes

M = A or C
R = A or G
S = G or C
W = A or T

3.4 Assembly of scFv fragments

The heavy and light chain domains are now linked together to give a complete single chain Fv antibody sequence. For this assembly reaction a DNA linker fragment is required. This is generated with *Protocol 4* and used to link the separately amplified V_H and V_L domains. This linker sequence creates a 15 amino acid sequence $(Gly_4Ser)_3$ between the heavy and light chain variable domains. *Figure 3* shows how the products of the primary PCR (*Protocol 3*) and the linker (*Protocol 4*) are assembled (*Protocol 5*).

The linker was originally prepared by oligonucleotide synthesis to generate the sequence: 5′ GGCACCACGGTCACCGTCTCCTCAGGTGGAGGC-GGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACATCG-AGCTCACTCAGTCTCCA 3′ and this was used to construct fd scFvD1.3, a scFv construct of the lysozyme binding antibody D1.3. Having once made this construct and inserted it into a vector, it is more convenient to prepare additional linker material by PCR amplification. Thus for the construction of a mouse repertoire or for creating a phage-display scFv from a monoclonal antibody the primers LINKFOR and LINKBACK are used to make a linker. One end of this PCR product overlaps with the end of the heavy chain (the J region) and the other end with the start of the light chain sequence. Combining the PCR amplified V_H and V_L domains with the linker fragment in a PCR reaction will therefore give an assembled scFv sequence.

A commercially available linker (Pharmacia, cat. no. 27–1588–01) is available. This can be used directly in the assembly reaction for mouse scFvs and can also be used as a template to make either mouse or human linker fragments by PCR. Use 1 µl of the Pharmacia linker as template in *Protocol 4*.

For the construction of a mouse repertoire or for creating a phage-display scFv from a monoclonal antibody only a single linker preparation is required to join the V_H gene J sequence with the VKBACK sequence. In the case of a human repertoire there are seven different Vlambda BACK sequences and six Vkappa BACK sequences. Therefore 13 different linkers are required. For each linker PCR preparation use an equimolar mix of the four RHuJH primers with one of the 13 different reverse light chain primers (RHuVk1aBACKFv or RHuVk2aBACKFv, etc. or RHuVλ1BACKFv or RHuVλ2BACKFv, etc.).

Ambiguity codes
M = A or C
R = A or G
S = G or C
W = A or T

Protocol 4. Preparation of linker[a]

Equipment and reagents

In addition to basic equipment and reagents (see *Protocol 1*), the following will also be required for this protocol:

- Taq DNA polymerase
- 10 × Taq polymerase buffer (see *Protocol 3*)
- 5 mM dNTPs (see *Protocol 2*)
- DNA thermal cycler for PCR
- Mineral oil (Sigma, cat. no. M-3516)
- SPIN-X columns (Costar, USA)
- Loading dye without Bromophenol Blue

All working solutions of primers are at concentrations of 10 pmol/µl.

Protocol 4. *Continued*

Method

1. Set up linker reaction mix. Each linker reaction provides sufficient linker for approximately three assembly reactions:
   - H_2O 36.8 µl
   - 10 × Taq buffer 5.0 µl
   - 5 mM dNTP 2.0 µl
   - FOR primer (10 pmol/µl)[a] 2.5 µl
   - BACK primer (10 pmol/µl)[a] 2.5 µl
   - linker template 1.0 µl
   - Taq DNA polymerase 0.2 µl
2. Cover with mineral oil and place on a cycling heating block at 94°C. Amplify the linker DNA by PCR for 25 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 2 min.
3. Incubate at 60°C for a further 5 min in the heating block.
4. Purify on a 2% low melting point agarose/TAE gel (see *Protocol 1* for TAE buffer) (use loading dye without Bromophenol Blue since the linker DNA sequence required is 93 bp long). Excise the correct (93 bp) gel fragment. Place in the upper chamber of a SPIN-X column and centrifuge in a microcentrifuge for 5 minutes at 13000 *g*. As an alternative to using SPIN-X columns the gel band can be purified using the 'Wizard PCR prep' DNA purification kit from Promega.
5. Ethanol precipitate the DNA (as in *Protocol 1*, step 2) and carefully dry the pellet before resuspending in 5 µl H_2O.

[a] The sequences of LINKFOR and LINKBACK are shown in *Table 1*. The sequences of RHuJH primers, the RHuVkBACKFv primers and the RHuVλBACKFv primers are shown in *Table 2*.

Protocol 5. Assembly of single chain Fv antibody fragments

Equipment and reagents

In addition to basic equipment and reagents (see *Protocol 1*), the following will also be required for this protocol:

- Taq DNA polymerase
- 10 × Taq polymerase buffer (see *Protocol 3*)
- 5 mM dNTPs (see *Protocol 2*)
- DNA thermal cycler for PCR
- Mineral oil (Sigma, cat. no. M-3516)
- Wizard PCR prep DNA purification kit *or*
- Wizard DNA clean-up kit from Promega

Method

1. Estimate the quantities of V_H and V_L DNA prepared by the primary PCR reactions (*Protocol 3*) and the quantity of linker DNA preparation(s) prepared in *Protocol 4* using agarose gel electrophoresis. (Use a molecular weight marker of known concentration in an adjacent

track.) Estimate concentration by the intensity of ethidium bromide staining. Adjust the volumes of V_H, V_L, and linker DNA added in step 2 below to give roughly equal masses of DNA fragments added to the assembly reaction (approximately 50 ng).

2. For a mouse repertoire, set up a single reaction for assembling the products of the primary V_{kappa} PCR reaction with the products of the V_H PCR reaction The assembly reaction is performed in two stages. For each first stage reaction set up the following mixture:

   For a human repertoire, set up six reactions for assembling each of the six primary V_{kappa} PCR reactions with pooled material from the V_H PCR reactions. Also set up seven reactions for assembling each of the seven primary V_{lambda} PCR reactions with the pooled material from the V_H PCR reactions. Each assembly reaction is performed in two stages. For each first stage reaction set up the following mixture:

   - V_H DNA[a] (from *Protocol 3*) x µl (see step 1 above)
   - V_L DNA[a] (from *Protocol 3*) y µl (see step 1 above)
   - Linker DNA (from *Protocol 4*) z µl (see step 1 above)
   - 10 × Taq buffer 2.5 µl
   - 5 mM dNTP (*Protocol 2*) 2.0 µl
   - sterile H_2O up to 25.0 µl
   - Taq DNA polymerase (1 U/µl) 1.0 µl

3. Place on cycling heating block and incubate for 20 cycles of 94°C for 1.5 min and 65°C for 3 min.

4. To each reaction add 25 µl of the following mixture for the second stage.

   - 10 × Taq buffer 2.5 µl
   - 5 mM dNTP 2.0 µl
   - VHBACK[b] (10 pmol/µl) 5.0 µl
   - VLFOR[c] (10 pmol/µl) 5.0 µl
   - sterile H_2O up to 25.0 µl
   - Taq DNA polymerase (1 U/µl) 1.0 µl

5. Amplify the DNA using 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, with a final extension step at 72°C for 10 min.

6. Electrophorese the product of each reaction on a 1.4% low melting point agarose/Tris–acetate–EDTA gel. Excise the band corresponding to the assembled product in each case (about 720 bp). Purify the DNA from the band with a 'Wizard DNA clean-up system' or 'Wizard PCR prep' DNA purification kit (Promega).

[a] For the generation of hierarchical libraries, the V_H and V_L DNA are derived from different sources. Either the V_H or V_L DNA is obtained by PCR amplification of DNA from a single antibody fragment clone or group of clones, by the procedure detailed in *Protocol 3*, where this domain is required to be kept constant. It is important that this DNA is purified as in *Protocol 3*,

Protocol 5. *Continued*

step 7 to avoid contamination with the original partner chain. The DNA encoding the complementary domain is derived as in *Protocol 3* for the primary PCR.

[b] For a mouse repertoire, VHBACK refers to the primer VH1BACK. For a human repertoire, VHBACK refers to an equimolar mix of HuVH1aBACK, HuVH2aBACK, HuVH3aBACK, HuVH4aBACK, HuVH5aBACK, and HuVH6aBACK.

[c] VLFOR is the corresponding light chain forward primer that was used in the primary PCR:
i.e. *for a mouse repertoire,* an equimolar mix of MJK1FONX, MJK2FONX, MJK4FONX, and MJK5FONX.
i.e. *for a human repertoire,* an equimolar mix of HuJK1FOR, HuJK2FOR, HuJK3FOR, HuJK4FOR, and HuJK5FOR for the V_H and V_K assemblies and an equimolar mix of HuJλ1FOR, Huλϑ2–3FOR and HuJλ4–5FOR, for the V_H and V_λ assemblies.

Note. The assembly step can sometimes be difficult. A variation on this assembly protocol has been described by McCafferty *et al.* (21) which can also give good results. For the assembly use equimolar amounts of heavy chain, light chain, and linker. If problems do occur then repeat the assembly using a range of V_H and V_L concentrations (21). It is very important to have good quality heavy and light chain PCR products for the assembly. Repeating the primary PCR with particular attention to the purification step may solve the problem. The annealing temperature and the ramp rate are also important and may need to be fine-tuned if a different thermal cycler is used. We recommend the Perkin Elmer DNA thermal cycler.

3.5 Amplification and digestion

A further amplification is performed using primers which incorporate the restriction sites for the enzymes *Sfi*I and *Not*I (*Protocol 6*). The amplified DNA is then digested with *Sfi*I and *Not*I (*Protocol 7*) to allow cloning into *Sfi*I and *Not*I digested pCANTAB-5 or one of the other pCANTAB series of vectors.

Several identical reactions are usually set up so that there is adequate material, after restriction enzyme digestion and gel purification, to give a large enough repertoire. For a repertoire from an immunized mouse 1×10^6 clones are usually regarded as adequate, whereas for a non-immunized human repertoire at least 1×10^7 clones are required. The larger the library the better the chance of selecting a high-affinity antibody. In addition, for a human repertoire, two sets of reactions are necessary. Pool the material from the six kappa assemblies for a kappa amplification. Similarly, pool the seven lambda assemblies for a lambda amplification. Usually four identical kappa amplifications and four identical lambda amplifications will give enough DNA, but this will depend on the yield and purity of the amplification step.

A suitable control (no DNA) should also be included.

Protocol 6. Amplification and incorporation of restriction enzyme sites

Equipment and reagents

In addition to basic equipment and reagents (see *Protocol 1*), the following will also be required for this protocol:

- Taq DNA polymerase
- 10 × Taq polymerase buffer (see *Protocol 3*)
- 5 mM dNTPs (see *Protocol 2*)
- DNA thermal cycler for PCR
- Mineral oil (Sigma, cat. no. M-3516)

Method

1. Add the following reagents to a 500 µl tube:

• Assembled product from *Protocol 5*	5.0 µl
• 10 × Taq polymerase buffer	5.0 µl
• 5 mM dNTP	2.0 µl
• VHBACKSFI[a] (10 pmol/µl)	2.5 µl
• VLFORNOT[a] (10 pmol/µl)	2.5 µl
• sterile H_2O	up to 50.0 µl
• Taq DNA polymerase (2.5 U/µl)	0.5 µl

2. Cover with mineral oil and place on the block preset at 94°C. Amplify DNA by PCR with 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, followed by an incubation for 10 min at 72°C.
3. Analyse 5 µl of the products by electrophoresis on a 1.4% agarose–Tris–acetate–EDTA gel. If a strong band is seen at approximately 720 bp the products are pooled and digested with *Sfi*I and *Not*I (*Protocol 7*). Some undigested material should always be retained so that, if necessary, this can be reamplified to give more material for the cloning steps.

[a] *For a mouse repertoire*, VHBACKSFI is the primer VH1BACKSfi which incorporates an *Sfi*I restriction site and VLFORNOT is an equimolar mix of the primers JK1NOT10, JK2NOT10, JK4NOT10, and JK5NOT10 (*Table 1*) which incorporate *Not*I restriction sites.
For a human repertoire, two PCR reactions are required, one for the kappa assembled material and one for the lambda assembled material. **VHBACKSFI** is an equimolar mix of the primers VH1aBACKSfi, VH2aBACKSfi, VH3aBACKSfi, VH4aBACKSfi, VH5aBACKSfi, and VH6aBACKSfi, which incorporate *Sfi*I restriction sites.
For the kappa genes reaction, **VLFORNOT** which incorporate a *Not*I restriction site is an equimolar mix of the primers HuJK1FORNOT, HuJK2FORNOT, HuJK3FORNOT, HuJK4FORNOT, and HuJK5FORNOT
For the lambda reaction, **VLFORNOT** is an equimolar mix of the primers HuJλ1FORNOT, HuJλ2–3FORNOT, and HuJλ4–5FORNOT.

Protocol 7. Restriction enzyme digestion of assembled products

Equipment and reagents

In addition to basic equipment and reagents (see *Protocol 1*), the following will also be required for this protocol:

- *Sfi*I restriction enzyme and 10 × buffer
- *Not*I restriction enzyme and 10 × buffer
- Geneclean II DNA purification kit or 'Wizard PCR prep' DNA purification kit *or* Pharmacia Sephaglass Band Prep Kit
- Cold (– 20°C) ethanol
- Cold (– 20°C) 70% ethanol
- 3 M sodium acetate buffer, pH 5.2
- TE buffer: 10 mM Tris–HCl, 1 mM EDTA, pH 8.0
- Phenol (saturated with TE buffer)
- Vacuum desiccator
- 50°C waterbath
- 37°C waterbath

Method

1. Add 200 µl of phenol (TE buffer saturated) to each of the DNA products from the PCR reactions in *Protocol 6*. Mix well. Incubate at room

Protocol 7. *Continued*

temperature for 10 minutes. Mix well. Centrifuge for 5 minutes in a microcentrifuge at 13 000 *g*. Transfer the upper aqueous layer to a fresh tube. Add 200 µl of TE buffer to the phenol in the first tube. Mix well. Centrifuge for 5 minutes at 13 000 *g*. Carefully remove the upper aqueous layer and combine with the first aqueous extract.

2. Ethanol precipitate the DNA contained in the aqueous extract. Wash the pellet twice with 70% EtOH. Dry the DNA pellet but do not over dry. Resuspend in 80 µl H_2O.
3. Digest the DNA product overnight with *Not*I at 37 °C using the reaction mixture below:
   - DNA 80 µl
   - *Not*I buffer × 10 10 µl
   - *Not*I (10 U/µl) 10 µl
4. Ethanol precipitate the *Not*I digested DNA (as in *Protocol 1*, step 2). Resuspend pellet in 80 µl H_2O.
5. Digest with *Sfi*I using the reaction mixture below:
   - DNA (digested with *Not*I) 80 µl
   - *Sfi*I buffer × 10 10 µl
   - *Sfi*I (10 U/µl) 10 µl

   Incubate at 50 °C for 4 h
6. Purify on 1.5% low melting point agarose–TAE (*Protocol 1*) gel. Excise the *Not*I/*Sfi*I digested DNA band and purify the DNA using Geneclean (from BIO 101) following the manufacturer's instructions and recover the DNA in 20 µl of H_2O. Alternatively, use Pharmacia Sephaglass Band Prep kit (cat. no. 27–9285–01) or 'Wizard PCR prep' DNA purification kit (from Promega). Ethanol precipitation can be used but it is very easy to lose the small DNA pellet at this stage.

3.6 Ligation and transformation

Ligation of DNA (e.g. using a DNA ligation kit, Amersham International) and electroporation of *E. coli* cells are performed using standard procedures. Ligate 50 ng of insert with 250 ng of cut vector (*Protocol 1*) per ligation. (The Amersham ligation kit, in some instances, can approximately double the yield of transformants over standard ligation protocols.) Transformation of chemically competent cells can be used instead of electroporation but will give much lower yields of transformants. This is acceptable if constructing a phage-display antibody from a monoclonal antibody but will severely compromise the size of a phage repertoire. A repertoire of 1×10^7 or better can be obtained from 1 µg of insert with 5 µg of cut vector. Chapter 8 gives detailed protocols for ligation (*Protocol 3*) and electrotransformation (*Protocol 4*).

4. Growth and expression of phage antibodies

This section describes the rescue and growth of phagemid as phage particles displaying an antibody as a gene 3 fusion protein on the phage surface. Preparations of phage particles in this way gives material that can be used for antibody selection and can also be used in an ELISA assay for screening and for characterizing the selected antibody. In addition, protocols for soluble antibody expression are described.

4.1 Rescue of phage

The most widely used helper phage for superinfection is M13K07 (25) which will preferentially package phagemid DNA. With phagemid pCANTAB-5 or pCANTAB-6 a yield of around 10^{11} phage per ml is obtained.

Protocol 8. Rescue and PEG precipitation of phagemids by superinfection with helper phage[a]

Equipment and reagents

- Sterile pipette tips
- M13K07 helper phage
- 2TY media: 16 g tryptone, 10 g yeast extract (Difco), 5 g NaCl per litre, adjust pH to 7.0 with NaOH and sterilize by autoclaving.
- Ampicillin 50 mg/ml in H_2O (filter-sterilized)
- Kanamycin 25 mg/ml in H_2O (filter-sterilized)
- 2TYAG: 2TY containing 2% (w/v) filter-sterilized glucose and 100 µg/ml ampicillin
- 2TY/K/A: 2TY (NO GLUCOSE) with kanamycin (25 µg/ml) and 100 µg/ml ampicillin
- 2TY plates: 2TY medium with 15 g/litre Bacto agar (Difco)
- Glucose 20% (w/v) filter-sterilized
- 37 °C shaking incubator
- 30 °C shaking incubator
- Sterile 50 ml polypropylene centrifuge tubes (Falcon, cat. no. 2070)
- Sterile 250 ml flasks
- Benchtop centrifuge
- PEG/NaCl: 20% polyethylene glycol 8000, 2.5 M NaCl
- PBS
- TE buffer: 10 mM Tris, 1 mM EDTA, pH 8.0
- Skimmed milk powder

A. *Rescue*

1. Grow cells containing the phagemid in 2TYAG at 30 °C overnight.
2. Add 250 µl of overnight cell culture to 25 ml 2TYAG in a 250 ml flask and grow at 37 °C with fast shaking for 1–2 hours (to approximately A_{600} = 0.5 to 1.0). Alternatively, inoculate directly from a fresh colony or from a glycerol stock and grow to approximately A_{600} = 0.5 to 1.0. **Note**: never store clones on agar plates as we have found that some scFv clones will not express recombinant protein if stored in this way. Only retransforming into fresh cells will restore expression!)
3. Add M13K07 (or other helper phage) to a final concentration of 10^9 p.f.u./ml.
4. Incubate cells for 30 minutes without shaking at 37 °C. Then grow cells for 30 minutes with moderate shaking (200 r.p.m.) at 37 °C.

Protocol 8. *Continued*

5. Transfer cells to a polypropylene centrifuge tube. Centrifuge at 2500 *g* for 5 min (benchtop centrifuge) and resuspend the bacterial pellet in 25 ml 2TY/K/A. Transfer to a fresh 250 ml flask.
6. Grow overnight with fast shaking (300 r.p.m.) at 30 °C.
7. Centrifuge at 9000 *g* for 15 min at 4 °C. Transfer supernatant to fresh tube.
8. For ELISA, good signals can be obtained using this supernatant directly (see *Protocol 13*). For phage antibody selection (*Protocol 12*) the phage supernatant can either be used directly or, if required, concentrated by PEG precipitation.

B. *PEG precipitation*

1. Add 1/5 volume PEG/NaCl (20% polyethylene glycol 8000, 2.5 M NaCl). Incubate on ice for 1 h. Centrifuge at 9000 *g* for 15 min at 4 °C.
2. Retain phage pellet. Remove as much supernatant as possible by decanting and allowing tube to drain on to a paper tissue.
3. Resuspend the pellet in 2 ml PBS or TE buffer. Spin at 13 000 r.p.m. for 5 min in a microcentrifuge to remove bacterial debris. Transfer supernatant to fresh tube.
4. For phage selection by panning add 2 ml 4% skimmed milk solution in PBS. This gives a 2.5 × concentration. Adjust the initial volume of phage, as necessary, if a higher concentration is required.

[a] This protocol is for 25 ml cultures, the volumes can be adjusted proportionately for different scale preparations. It is essential that the cultures are vigorously aerated. If the volumes are increased larger vessels are needed (e.g. 25 ml cultures in a 250 ml flask or a 500 ml culture in a 2 litre flask). We also routinely grow clones in 100 µl cultures (*Protocol 9*).

Protocol 9. Rescue and growth of phagemids in microtitre plate format

This protocol is used for screening large numbers of clones for antigen-binding clones.

Equipment and reagents

- As for *Protocol 8*
- Sterile H_2O
- Sterile microcentrifuge tubes (Eppendorf)
- Benchtop centrifuge
- Sterile polypropylene 96-well microtitre plate (Greiner, cat. no. 650201) or 'Cell wells' polystyrene 96-well microtitre plate (Corning, cat. no. 25850)

Method

1. Pick colonies into 100 µl 2TYAG in a sterile microtitre plate. Use a polypropylene 96-well microtitre plate (Greiner) (autoclave before use) or a Corning 'Cell Wells' sterile polystyrene 96-well microtitre plate. Generally polypropylene is preferable for phage growth. Grow clones overnight at 30°C at 150 r.p.m. This is the master plate.
2. Prepare a replica 96-well microtitre plate with 100 µl 2TYAG in each well. Inoculate with 10 µl of cells from the master plate. Then add 40 µl of 50% glycerol to each well of the master plate and store it at –70°C. Grow the second (replica) plate at 30°C at 300 r.p.m. for approximately 5–6 hours to give an A_{600} = 0.5 to 1.0.
3. Add 20 µl of M13K07 (or other helper phage) in 2TYAG (at 10^{10} p.f.u./ml) to each well of the replica plate. Incubate the plate for 30 minutes without shaking at 37°C. Then incubate for 30 minutes with moderate shaking (150 r.p.m.) at 37°C.
4. Centrifuge the plate at 1000 *g* for 5 min (benchtop centrifuge) and resuspend each bacterial pellet in 100 µl 2TY/K/A. Alternatively, a transfer innoculum of 5–10 µl using a multi-prong device (from the plate generated in step 3) can be made into a fresh microtitre plate containing 2TY/K/A.
5. Grow overnight with shaking (150 r.p.m.) at 30°C. For ELISA good signals can be obtained using the supernatant directly, diluted 1:1 with PBS containing 4% skimmed milk powder (see *Protocol 11*).

4.2 Growth and soluble expression

For a detailed study of scFv antibodies derived from phage antibody repertoires it is necessary to express them in soluble form. The pCANTAB-5E, pCANTAB-5myc, pCANTAB-6 vectors (but not pCANTAB-5) have an amber codon between the scFv antibody sequence and gene 3. Growth in a non-suppressor strain such as HB2151 will allow soluble expression of scFv protein. However, in a suppressor strain such as TG1 the suppression of the amber codon is not complete and both complete gene3/antibody fusion protein and soluble antibody protein are made in the same cell.

After the addition of IPTG the expression of antibody is induced. Soluble antibody is transported to the periplasm of the cell. From there it can leak into the growth media. Soluble antibody can then be harvested either from the growth culture or from the periplasmic space. This latter is useful for large-scale production since it reduces the volume of crude extract prior to affinity column purification.

Protocol 10. Soluble expression of antibody[a]

Equipment and reagents

- Sterile pipette tips
- 2TY media (see *Protocol 8*)
- 2TYA: 2TY with 100 µg/ml ampicillin
- 2TYAG: 2TYA with 2% (w/v) filter-sterilized glucose added
- Ampicillin 50 mg/ml in H_2O (filter-sterilized)
- 37 °C shaking incubator
- 30 °C shaking incubator
- Sterile 50 ml polypropylene centrifuge tubes (Falcon, cat. no 2070)
- Sterile 250 ml flasks
- Benchtop centrifuge
- IPTG (isopropyl-β-D-thio-galactopyranoside) 100 mM (23.8 mg/ml) filter-sterilized

Method

1. Grow cells containing the phagemid in 2TYAG at 30 °C overnight or to mid-log phase (A_{600} = 0.5 to 1.0) if inoculation is performed on the same day as rescue.
2. Add 50 µl of cell culture to 5 ml 2TYAG in a 50 ml sterile polypropylene centrifuge tube and grow at 30 °C with fast shaking for 3–4 hours (to A_{600} = 1.0).
3. Centrifuge at 3500 *g* for 5 min (benchtop centrifuge) and resuspend the bacterial pellet in 5 ml 2TYA (2TY with 100 µg/ml ampicillin and NO glucose) and IPTG at 1 mM. (For large-scale preparations this step may be omitted if the cells are grown in 2TYA containing 0.1% glucose. At A_{600} of 1.0 add IPTG to 1 mM. **NB**. The best conditions for induction may vary between different clones.)
4. Antibody may be harvested from the culture supernatant or from the periplasm. If harvesting from the culture supernatant, grow overnight with fast shaking (350 r.p.m.) at 30 °C. For ELISA good signals can be obtained using the supernatant directly, diluted 1 : 1 with PBS containing 4% skimmed milk powder (see *Protocol 13*).

   If harvesting antibody from the periplasm grow for 3 hours at 30 °C after induction with IPTG. Centrifuge at 3500 *g* for 5 min (benchtop centrifuge) and resuspend the bacterial pellet in 1/10 original culture volume of PBS + 1 mM EDTA. Incubate on ice for 15 minutes. Then centrifuge at 3500 *g* for 10 minutes at 4 °C (benchtop centrifuge). Carefully remove and retain supernatant containing soluble antibody.

[a] This protocol is for 5 ml cultures; the volumes can be adjusted proportionately for different scale preparations. It is essential that the cultures are vigorously aerated. If the volumes are increased larger vessels are needed (e.g. 25 ml cultures in a 250 ml flask or a 500 ml culture in a 2 litre flask). We also routinely grow clones in 100 µl cultures in microtitre wells (*Protocol 11*) for screening large numbers of clones for antigen-binding activity.

Protocol 11. Soluble expression of antibody in microtitre plate format

Equipment and reagents

- As for *Protocol 10*
- Sterile microcentrifuge tubes (Eppendorf)
- 2TYG: 2TY with filter-sterilized glucose added to 2% (w/v)
- 37°C shaking incubator
- 30°C shaking incubator
- Sterile polystyrene 96-well microtitre plate (Corning 'Cell Wells', cat. no. 25850) *or* polypropylene 96-well microtitre plate (Greiner, cat. no. 650201)
- Glycerol (50% v/v)
- Benchtop centrifuge

Method

1. Pick colonies into 100 µl 2TYAG in a sterile microtitre plate. Use a sterile polystyrene 96-well microtitre plate (e.g. Corning) or a polypropylene 96-well microtitre plate (Greiner) (autoclave before use). Generally polystyrene is preferable for soluble expression, giving better yields than polypropylene. Grow clones overnight at 30°C at 150 r.p.m. This is the master plate.
2. Prepare a second (replica) 96-well microtitre plate with 100 µl 2TYAG in each well. Inoculate with 10 µl of cells from the master plate. Then add 40 µl of 50% glycerol to each well of the master plate and store at –70°C. Grow the second (replica) plate at 30°C at 150 r.p.m. for approximately 5–6 hours to give an A_{600} of 1.0.
3. Centrifuge at 1000 *g* for 5 min (benchtop centrifuge) and resuspend the bacterial pellet in each well with 100 µl of 2TYA containing 1 mM IPTG.
4. Grow overnight with shaking (150 r.p.m.) at 30°C. Centrifuge at 1000 *g* for 10 min (benchtop centrifuge). For ELISA good signals can be obtained using the supernatant directly, diluted 1:1 with PBS containing 4% skimmed milk powder (see *Protocol 13*).

4.3 Purification of soluble antibody

Several methods are available for purifying soluble antibody. If the vector has a polyhistidine tag then metal chelate affinity purification can be used (21). Ni–NTA (nitrilo-tri-acetic acid) agarose is available from Qiagen and is used according to the manufacturer's recommendations (in the elution buffer it is important to readjust the pH of PBS or saline when imidazole is added). Affinity chromatography with the antigen of interest cross-linked to a Sepharose column (CNBr–Sepharose) is also commonly used. In the case of small haptens, these can be cross-linked to a BSA–Sepharose column. Elution of bound antibody with 250 mM glycine buffer, pH 2.5, followed by neutralization and dialysis is usually suitable. Antibody prepared in this way is normally stable when frozen at –20°C.

Following initial purification it may be necessary to size separate monomeric scFv from dimerized and aggregated material (e.g. with Pharmacia FPLC Superdex 75 small-scale column). This is important for kinetic studies of affinity with the Pharmacia Biacore.

5. Selection of antibody variants displayed on the surface of bacteriophage

Antibody variants expressed on the surface of bacteriophage have been selected on the basis of their affinity for antigen using chromatography, panning, or adsorption to cells. Elution from affinity matrices has been achieved by specific elution using the antigen (or a related compound) or non-specific elution using, for example, 100 mM triethylamine. Washing procedures remove non-specifically bound phage. The phage binds to and is eluted from the surface according to the affinity or the nature of the binding interaction. Eluted phage are then used to infect male *E. coli* cells expressing the F pilus, allowing recovery of phage-encoding antibodies with the desired binding characteristics.

Protocol 12. Selection of antibodies displayed on the surface of bacteriophage

Equipment and reagents

- Sterile H_2O
- Sterile microcentrifuge tubes (Eppendorf)
- Sterile pipette tips
- 1 M Tris–HCl, pH 7.5
- 2TY media (see *Protocol 8*)
- Ampicillin 50 mg/ml in H_2O (filter-sterilized)
- 2TYAG: 2TY with 2% (w/v) filter-sterilized glucose and 100 μg/ml ampicillin
- 2TYAG agar plates: 2TYAG with 1.5% agar (sterilized by autoclaving)
- PBS buffer: 10 mM phosphate buffer, pH 7.2–7.4, with 150 mM NaCl
- Nunc Immuno tube Maxisorp 75 × 12 (cat. no. 4–44202) *or* Falcon 3001 Petri dishes (35 × 10 mm)
- PBS/Tween: PBS buffer with 0.1% Tween-20 (Sigma, cat. no P-2287)
- Sterile glycerol
- 37°C shaking incubator
- 30°C incubator
- Sterile polypropylene centrifuge tubes (Falcon, cat. no. 2070)
- Benchtop centrifuge
- Mid-log TG1 cells

A. *Binding*

1. Antigen is coated on to a large surface area of plastic (for example, Nunc Maxisorp tubes. Similar plastic surfaces such as Falcon 3001 Petri dishes 35 × 10 mm can also be used).

   Coat with 1 ml of 10 μg/ml antigen in PBS. This is analogous to coating an ELISA plate. This is the best coating buffer for many antigens (e.g. BSA or hapten conjugated BSA). For some antigens such as lysozyme which do not coat plastic well in PBS, use 50 mM $NaHCO_3$, pH 9.6. Coating conditions will vary from antigen to antigen. Coating

is overnight (12 + hours). The incubation temperature is antigen dependent. Room temperature or 30°C will give good coating, but for many antigens where correct folding and presentation of epitopes is required then coating at 4°C, to reduce denaturation, is preferred. If a suitable control monoclonal or polyclonal serum is available for the antigen then coating conditions for panning can be determined by ELISA before starting selection.

2. After coating overnight, rinse the coated Maxisorp tube three times with PBS and then block with 2% (w/v) skimmed milk powder in PBS for 2 h at 37°C. (Completely fill the tube, or cover the surface if using a Petri dish.)
3. Rinse the Maxisorp tube four times with PBS.
4. Prepare phage as in *Protocol 8*. This should give 5×10^{10} to 1×10^{11} per ml. For the first round of selection it may be advantageous to use concentrated phage (prepared as in *Protocol 8*). For subsequent rounds of selection supernatants are routinely used directly, without any need for PEG precipitation and concentration. Add an equal volume of 4% (w/v) skimmed milk powder in PBS buffer and use directly in the selection. Add 1 ml of this phage preparation to the panning tube.
5. Incubate for 1 hour. Incubation can be either stationary or carried out on a turntable mixer with the tube (safely capped) turning slowly end over end. (We normally use the former.) The incubation can be done at room temperature. However, selection at 37°C may help prevent the selection of those antibodies that are less stable.

B. *Washing and eluting*

1. Rinse the Maxisorp tube 20 times with PBS/Tween. This is carried out by filling the tube using a wash bottle and immediately tipping out.
2. Rinse the Maxisorp tube 20 times with PBS.
3. Add 1 ml 100 mM triethylamine to the Maxisorp tube. Incubate (stationary) at room temperature for 10 minutes.

C. *Infection*

1. Remove eluted phage. Add to 500 µl of 1 M Tris–HCl buffer, pH 7.5, in a 15 ml polypropylene centrifuge tube (Falcon). Add half of this (750 µl) to 5 ml of a mid-log culture of *E. coli* TG1 cells. (Store the remaining 750 µl of phage at 4°C.) Shake the cells slowly at 37°C for 1 hour.[a] (If 50 ml of 2 × TY in a 250 ml flask is inoculated with a fresh colony from a minimal media plate at the start of the day (step 2) it will be at approximately mid-log by this stage. It can be stored on ice when it reaches mid-log.)

Protocol 12. *Continued*

2. Centrifuge cells at 3000 *g* for 5 minutes. Carefully discard all of the supernatant and resuspend cells in 500 µl 2TYG. Spread-plate the cells on to 2 × TYAG agar plates (100 µl per plate). Grow overnight at 30°C.
3. After overnight growth add 1.5 ml of 2 × TYAGG (2 × TYAG with 15% glycerol) per plate. Resuspend cells with a glass spreader. Pool the cells from each plate and aliquot into Eppendorf tubes. If a further round of selection is required then inoculate 25 ml of 2 × TYAG with 50 µl of these cells and grow to A_{600} = 1.0 (*Protocol 8A*, step 2) and proceed with phagemid rescue. The remainder of the scraped cells are stored at −70°C.

[a] At this point take a small aliquot of the cells and after serial dilution, plate out on to 2 × TYAG agar plates. Grow overnight at 30°C. This will measure the output of that round of selection. This can be an indication of the success or otherwise of the selection process. Often a rise in the relative number of phage eluted at each round of selection will occur. However, it is not always the case and not too much emphasis should be placed on this. Nevertheless, the colonies from this plating step are of real use for screening the progeny from each round of selection and should be screened for positive binders. Grow and screen as described in *Protocols 8* and *9*.

6. Analysis of phage-derived antibodies by ELISA

Phage antibody clones can be assayed directly for the ability to bind specific antigens by immunoassay techniques such as ELISA. Detection of phage antibodies with antiserum raised in sheep against bacteriophage fd (Pharmacia, cat. no. 27–9402–01) can be used for most antigens, giving very sensitive ELISA assays.

Alternatively, soluble antibody preparations can be used in the ELISA and detected with a monoclonal antibody such as the antibody to the E tag (Pharmacia, cat. no. 27–9412–01) which will bind to the E tag present on antibody clones grown in the vector pCANTAB-5E. Similarly, solubly expressed antibody from clones in the vector pCANTAB-5myc or pCANTAB-6 can be detected with the monoclonal antibody 9E10 which will bind to the c-*myc* tag.

This protocol (*Protocol 13*) should be readily adaptable to the assay of any phage antibody by substituting an appropriate antigen.

Protocol 13. Screening for binders by ELISA

Equipment and reagents

- PBS buffer: 10 mM phosphate buffer, pH 7.2–7.4, with 150 mM NaCl
- PBS/Tween: PBS buffer with 0.1% Tween-20 (Sigma, cat. no P-2287).
- ELISA plates (Falcon, cat. no. 3912)
- ELISA plate reader
- ABTS (2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid); Sigma, cat. no. A-9941) Make up ABTS solution just before use; dissolve each 10 mg tablet in 20 ml of citrate buffer (made up from 10.8 ml of 50 mM citric acid and 9.2 ml 50 mM trisodium citrate stock)

- Anti-fd serum and peroxidase-conjugated rabbit anti-goat immunoglobulin (Sigma, cat. no.) *or* Anti-E-tag antibody (Pharmacia, cat. no. 27–9412–01) and peroxidase-conjugated goat anti-mouse immunoglobulin (Sigma, cat. no.) *or* 9E10 antibody (Cambrige Research Biochemicals) and peroxidase-conjugated anti-mouse immunoglobulin. The 9E10 monoclonal is available from the ATCC (CRL1729, named MYC1-9E10.2)
- Hydrogen peroxide

Method

1. Coat ELISA plate with 10 μg/ml antigen in PBS. This is the best coating buffer for many antigens (e.g. BSA or hapten-conjugated BSA). For some antigens such as lysozyme which do not coat plastic well in PBS use 50 mM $NaHCO_3$, pH 9.6. Coating conditions will vary from antigen to antigen. Coating is overnight (12 + hours). The incubation temperature is antigen-dependent. Room temperature or 30°C will give good coating, but for many antigens where correct folding and presentation of epitopes is required then coating at 4°C is preferred. The normal antigen concentration for coating is 10 μg/ml. With some antigens higher concentrations give improved results (e.g. for ELISA with lysozyme, which coats plastic poorly, we use 1 mg/ml).
2. After coating overnight, rinse the wells three times with PBS and block with 300 μl per well of 2% (w/v) skimmed milk powder in PBS for 2 h at 37°C.
3. Prepare phage supernatants for ELISA. Phage supernatants are routinely used directly, without any need for PEG precipitation and concentration. Add an equal volume of 4% (w/v) skimmed milk powder in PBS and use directly in the assay. Similarly, use soluble antibody from IPTG-induced cultures directly (with an equal volume of 4% (w/v) skimmed milk powder in PBS).
4. Rinse wells three times with PBS and transfer 100 μl supernatants into the wells. Incubate for 1.5 h at room temperature.
5. Wash wells three times for 2 min each time with each of PBS/0.1% Tween-20 and PBS (to remove detergent).
6. For phage supernatants add 100 μl of anti-fd serum raised in sheep (1/1000 dilution) to each well. Incubate for 1 h at room temperature. Proceed to step 7. Alternatively, use peroxidase-conjugated anti-fd (Pharmacia recombinant phage detection system cat. no. 27–9402–01) following the manufacturer's recommended dilution factor. Incubate for 1 h at room temperature. Then proceed directly to step 9. For soluble antibody from culture supernatants of phagemids with an E-tag, add 100 μl of anti E-tag antibody (Pharmacia cat. no. 27–9412–01) (following the manufacturer's recommended dilution factor) to each well. Incubate for 1 h at room temperature.

Protocol 13. *Continued*

For soluble antibody produced from the culture supernatants of phagemids with a *myc* tag, add 100 µl of 9E10 monoclonal antibody (1/1000 dilution) to each well. Incubate for 1 h at room temperature.

7. Wash as in step 5.
8. For phage supernatants add 100 µl peroxidase-conjugated rabbit anti-goat immunoglobulin (1/5000 dilution) to each well. Incubate for 1 h at room temperature. (Anti-goat or anti-sheep antibodies can be used interchangeably.) For soluble antibody from culture supernatants add 100 µl peroxidase-conjugated goat anti-mouse immunoglobulin (1/5000 dilution) to each well. Incubate for 1 h at room temperature.
9. Wash as in step 5.
10. Add 200 µl ABTS containing 1 µl of H_2O_2 per 10 ml to each well and develop until the absorbance at 405 nm is suitable (A_{405} = 0.2 to 1.0). Read absorbance in a plate reader.

Specific primers are used to PCR a sample of DNA derived by picking a colony into 20 µl of distilled water and boiling for 5 min. The diversity of clones generated can be assessed by *Bst*NI digestion of the PCR amplified insert (Chapter 8, *Protocol 6*).

7. In-cell PCR assembly

7.1 Introduction

The protocols given earlier in this chapter describe how to make mouse and human antibody repertoires. In this process, the heavy and light chain DNA sequences are amplified separately before being assembled together, and so the original heavy and light chain pairings are lost. This results in a random combinatorial library. With libraries derived from immunized B-cell donors the selected antibodies have V_H domains that are able to bind to antigen in promiscuous association with a range of V_L domains. Some of these combinations may, by chance, accurately reflect the original host B-cell repertoire, but they will be in the minority and there is currently no way to recognize the original from new antigen-binding V region combinations. Previously, the only way to rescue original repertories has been to exploit hybridoma or other B-cell immortalization technologies (26). In-cell PCR assembly is intended to amplify and link V_H and V_L genes within fixed and permeabilized B cells in such a way that the original V gene combination in each cell is retained. When applied to a population of B cells, this should allow the expression of antibody fragments representative of the host B-cell repertoire. Essentially, the process consists of intracellular first-strand cDNA synthesis,

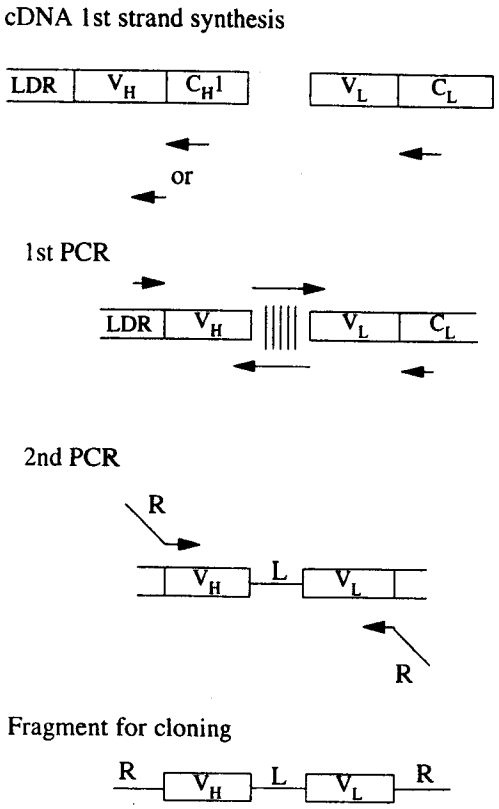

Figure 4. Priming sites are represented by arrows, the position of linker sequences by L, and restriction sites by R. Linkage sites are indicated by parallel vertical lines. LDR = V_H leader region. cDNA 1st strand synthesis can be accomplished using forward primer mixers corresponding to C_L and C_H1 or J_H genes. The 'outer' 1st PCR primer mixes should prime in the V_H leader regions and the C_L genes. (The latter can be the same primers as used for 1st strand synthesis.) 1st PCR linker primer mixes should prime in V_L framework 1 regions and J_H regions, and must have overlapping complementary extensions encoding the polypeptide linker $(G_4S)_3$. A J_H forward linker primer mix could double for V_H cDNA 1st strand synthesis. 2nd PCR primer mixers appending restriction sites should prime in V_H framework 1 regions, and J_L regions.

followed by intracellular amplification and linkage of V_H and V_L cDNA in single-chain Fv (scFv) format by polymerase chain reaction (PCR), then intracellular amplification of the assembled genes in a second PCR. Sufficient assembled DNA is released into the supernatant to allow cloning of the assembled gene into *E. coli*.

In-cell PCR is a novel technique, still at an early stage of development. It has been shown to work by using mixtures of different hybridoma cell lines and demonstrating that the original parental V_H and V_L pairings of each cell line are retained (see Section 7.2). For use of this technique with mouse or human B cells, careful primer design is necessary (see Section 7.3). A range of 'nested' primers are required. In the protocols describing the construction of random combinatorial libraries, the DNA from each PCR amplification

was gel-purified before proceeding to the next stage. For in-cell PCR, this is not possible. The use of 'nested' primers overcomes this problem and allows each stage of the process to be performed inside the cell.

Protocol 14. In-cell PCR assembly

Equipment and reagents

- Benchtop centrifuge
- Microcentrifuge
- Haemocytometer and microscope
- Rotator
- Sterile pipetters and tips
- 1 ml syringes, and 21- and 26-gauge needles
- Waterbaths or heat blocks at 42°C and 65°C
- Cycling heat block, preferably with hot lid, and compatible tubes
- 2 ml Eppendorf tubes and 500 µl microcentrifuge tubes
- Gel electrophoresis apparatus for DNA agarose gels
- Viable cell suspension; may be hybridoma or other immortalized cells (cloned or uncloned), spleen cells from immunized mice, etc.
- 10% formal saline: 0.15 M NaCl containing 10% (v/v) formalin solution (100% formalin = 40% formaldehyde), equivalent to 4% formaldehyde in final concentration
- 0.5% v/v Nonidet P-40 (NP-40) in distilled water
- PBS (see *Protocol 12*)
- PBS–glycine, pH 7.2–7.4 PBS containing 0.1 M glycine
- AMV reverse transcriptase
- RNase inhibitor (e.g. Promega RNasin)
- Sterile water
- 5 mM dNTPs (see *Protocol 2*)
- 10 × 1st strand buffer (see *Protocol 2*)
- Dithiothreitol (DTT): 100 mM solution, diluted in distilled water from a 1 M stock frozen in 0.01 M sodium acetate
- Oligonucleotide primers, diluted in water to 10 pmol/µl
- Taq polymerase
- 10 × Taq polymerase buffer (see *Protocol 3*)
- Low melting point agarose
- TAE buffer (see *Protocol 1*)
- Ethidium bromide (stock solution 10 mg/ml in water)

A. *Preparation of B cells for in-cell PCR*

1. Wash the cells three times in PBS, centrifuging at 50–100 *g* for 5 min between washes.
2. Count the cells in a haemocytometer and aliquot up to 10^7 cells into a 2 ml Eppendorf tube. Spin at 12 000–14 000 *g* in a microcentrifuge for 2.5 min.
3. Remove the supernatant and suspend the cell pellet in 1 ml ice-cold 10% formal saline, using a syringe and 21-gauge needle. To minimize cell losses, retain the syringe and needle for re-use. Incubate the tube at 4°C on a rotator, or on ice with frequent agitation for 1 hour.
4. Spin the tube in a microcentrifuge for 2.5 min and remove the supernatant. Suspend the cells in ice-cold PBS/glycine, using the syringe and needle, and repeat the centrifugation for a total of three washes.
5. Resuspend the cells in 1 ml ice-cold 0.5% NP-40 using the same syringe and needle, and incubate for 1 hour on a rotator at 4°C or on ice with frequent agitation.

6. Spin down and wash three times in PBS/glycine as in step 4.
7. Finally resuspend the cells in PBS/glycine, using the syringe and a 26-gauge needle.
8. Count the cells, checking that they are free of microscopic clumps. If clumps are present, re-syringe the cells. Adjust the concentration to 2×10^6 per ml. Store in aliquots at –70°C or proceed directly to part B.

B. *cDNA first-strand synthesis*

1. Prepare a '1st strand' mix as follows:
   - V_H primer 2.5 µl
   - $V_L$primer 2.5 µl
   - dNTP mix 5.0 µl
   - DTT 5.0 µl
   - 10 × 1st strand buffer 5.0 µl
   - Cells[a] 26 µl
2. Heat the mix at 65°C for 3 min. Allow to cool to ambient temperature for 10 min, then place on ice.
3. Add 2 µl (40 units) AMV reverse transcriptase and 2 µl (80 units) RNase inhibitor. Incubate at 42°C for 1 hour, with occasional gentle shaking or pipetting to suspend the cells.
4. Transfer the cell suspension to a 500 µl tube and spin down at 12 000–14 000 r.p.m. for 2.5 min.
5. Discard all of the supernatant using a pipette with a fine tip. When withdrawing the last few microlitres of supernatant, slightly invert the tube and drag the pipette tip along the side of the tube to the top, while applying suction. This procedure should leave a cell pellet free of residual liquid, rendering more than a single wash unnecessary. Washes are mandatory, but must be kept to a minimum to avoid undue loss of cells; stringency is thus very important.
6. Suspend the pellet in 200 µl PBS/glycine and spin again.
7. Remove all the supernatant and resuspend the cells in 100 µl PBS/glycine. The cells can be used immediately in PCR, or frozen in aliquots at –70°C.

C. *In-cell PCR assembly*

1. Prepare the 1st PCR mix in a 500 µl tube as follows:
   - V_H back primer mix[b] 2.5 µl
   - V_L forward primer mix[b] 2.5 µl
   - V_H forward linker primer mix 2.5 µl
   - V_L back linker primer mix 2.5 µl

Protocol 14. *Continued*

- dNTP mix 2.0 µl
- Taq DNA polymerase (2.5 units) 0.5 µl
- 10 × Taq polymerase buffer 5.0 µl
- Cells[c] 10.0 µl
- water 22.5 µl
- (total volume of PCR 50 µl)

If using a hot-lid temperature cycler, do not use oil. A hot-lid temperature cycler will give optimal results and avoid problems due to oil coating the cells. If using a basic cycler, add 1 drop of mineral oil to the tube.

2. Give the tube 30 cycles as follows:

   95 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min.

   The reaction works more efficiently at stringent annealing temperatures of 65 °C or more than at lower temperatures.

3. After cycling, spin down the cells, remove (but keep) all of the supernatant and suspend the cells in 200 µl PBS/glycine. If an oil overlay is used, first remove the cells from the PCR tube by inserting a pipette tip through the oil to the bottom of the tube, and withdrawing the cells from the bottom so that the supernatant washes them into the pipette tip. Do not allow oil to enter the tip. Transfer the cell suspension to a clean tube for centrifugation and resuspension.

4. Spin the cells down again, discard all of the wash supernatant and resuspend the pellet in 10 µl PBS/glycine and use immediately as template for the 2nd PCR.

5. Prepare the 2nd PCR mix in a 500 µl tube as follows:

   - V_H back primer mix[b] 2.5 µl
   - V_L forward primer mix[b] 2.5 µl
   - dNTP mix 2.5 µl
   - Taq DNA polymerase (2.5 units) 0.5 µl
   - 10 × Taq polymerase buffer 5.0 µl
   - washed cells (from the 1st PCR) 10.0 µl
   - water 27.5 µl

   Add 1 drop of oil if necessary.

6. Give the tube 30 cycles as follows:

   95 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min.

7. After cycling spin down the cells as step 3, and discard them. Keep the supernatant.

8. Prepare a 1.5% low melting point agarose gel in TAE buffer, incorporating 0.5 µg/ml ethidium bromide. Electrophorese the supernatants of the 1st and 2nd PCRs, using a suitable size marker. The 1st PCR

should give bands around 300–350 bp and the 2nd PCR around 650 bp.

9. Excise the approximately 650 bp bands from the 2nd PCR lane(s) and purify the DNA. A number of proprietary kits are available for this purpose. (see *Protocol 3*, step 7).

10. Digest the purified DNA with appropriate restriction endonucleases and clone into a suitable expression or selection vector (plasmid, phage, or phagemid) (*Protocol 7*).

[a] Fixed and permeabilized cells (from *Protocol 12A*) thawed, spun down, and resuspended in water to a final volume of 26 µl.
[b] See Section 7.2.
[c] 10^5 template cells in 10 µl PBS/glycine (it is not necessary to wash and suspend the cells in water).

7.2 Application

The fidelity of this process has been demonstrated (27) using 1:1 and 1:9 mixtures of two hybridoma cell lines, B1-8 (28) and NQ10/12.5 (29), and mixes of primers matched to each cell line. Results for two separate experiments with 1:9 B1-8:NQ10/12.5 mixtures are shown in Table 3. The purpose of this model was solely to identify and compare V_H and V_L gene combinations in clones prepared by in-cell assembly and random combinatorial assembly (i.e. with soluble cDNA templates), the genes being identified by hybridization with internal oligonucleotide probes and by PCR screening (30). The primers were not designed to produce full-length V_H and V_L sequences in the the second PCR. For expression of full-length scFv fragments the scheme outlined below is suggested. In the examples in *Table 3*, Experiment 1 used primers exactly as described in ref. 27. For Experiment 2, the same V_H back and V_L forward primers as in Experiment 1 were used in each PCR, but the four linker primers in the 1st PCR incorporated a loxP site (31). Linkage by the loxP site appeared to be more efficient than linkage by overlap extension as described in (27) (MJE, unpublished), but is not described here in detail because it requires the cells to be incubated in *cre* recombinase between the first and second PCRs. It is understood that this enzyme is no longer commercially available.

The results of the experiment shown in *Table 3* indicate that with both forms of linkage, in-cell PCR assembly resulted in clones containing only the parental hybridoma V_H and V_L combinations, while random combinatorial assembly using identical primers resulted in crossover combinations in which B1-8 V_H was linked to NQ10/12.5 V_L.

Assembly of full-length scFv requires that the 2nd PCR primers must prime at the 5′ end of the V_H genes (framework 1) and the 3′ end of the V_L genes (J_L). They must also be fully nested with respect to the 1st PCR primer for efficient amplification of the assembled genes. These requirements can be

Table 3. Assembly of immunoglobulin V genes from 1:9 mixes of B1-8:NQ10/12.5 murine hybridoma cells

Assembly method	Expt no.	No. of V186.2 +ve clones[a]	Fraction of V186.2 +ve vc clones with:	
			B1-8 V_H + B1-8 V^b	B1-8 V_H + NQ10/12.5 Vk^b
Random combination	1	34/450	3/34	31/34
	2	29/250	5/29	24/29
In-cell PCR	1	27/450	27/27	0/27
	2	34/250	34/34	0/34

[a] Bacterial colonies were hybridized with ^{32}P-labelled V186/2 probe specific for the B1-8 V_H gene (27).
[b] V186.2 +ve replica-plated colonies were PCR-screened using 2nd PCR primers specific for B1-8 V_H (back primer) and either B1-8 V or NQ10/12.5 V_k (forward primers) (27).

achieved by using back primers in the V_H leader regions and forward primers in the C_L regions for the 1st PCR, as indicated in *Figure 4*. Primers for cDNA 1st strand synthesis should prime in the C_L and C_H1 (or J_H) regions. In addition to priming in the V_H framework 1 and J_L regions, the 2nd PCR primers should incorporate restriction sites external to the genes in order to facilitate cloning into an appropriate vector, as suggested in *Figure 4*. Although, as yet, untested for assembly and expression of murine scFv genes, the suggested scheme should be applicable for scFv cloning from spleen cells of immunized mice. A list of purpose-designed primers is not available, but their design may be based on primers shown in *Table 3* and ref. 32 and the linker strategy employed in *Table 3*, Experiment 1 (27).

8. Conclusions

Display of proteins on the surface of bacteriophage gains its power as a method by linking proteins, presented for binding to ligand, with the DNA encoding them. The phage system mimics the natural processes by which the immune system produces molecules which bind tightly to ligands, allowing large numbers of variants to be surveyed and leading to the more rapid isolation of improved protein molecules. The monoclonal antibody technique of Kohler and Milstein (33) has enabled isolation of highly specific antibodies of importance in research, diagnosis, and therapy. Phage antibody technology extends this technology. For example, a monoclonal antibody can be cloned into the phage-display system and mutagenesis or other genetic manipulation techniques used to generate a large variant population from the original antibody. Phage antibody selection can then be used to isolate those antibodies with improved affinities or specificities.

In addition, with phage-display technology, the immune system can be bypassed and human antibodies selected directly from a non-immunized source (3). The ability to make human antibodies to a wide range of targets (e.g. parasites, bacteria, viruses, and other pathogens) is of obvious benefit. In addition, the ability to make human antibodies to human ('self') antigens also has important consequences for therapeutic use, especially for cancer treatment and for the regulation of disease states.

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Antibody engineering

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Edited by

JOHN McCAFFERTY

Cambridge Antibody Technology Ltd
The Science Park
Melbourn
Cambridgeshire SG8 6JJ

HENNIE HOOGENBOOM

CESAME at Department of Pathology
Academisch Ziekenhuis
Maastricht PO Box 5800
6202 AZ Maastricht
The Netherlands

and

DAVE CHISWELL

Cambridge Antibody Technology Ltd,
The Science Park
Melbourn
Cambridgeshire SG8 6JJ

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