

Standard Protocols for the Construction of Fab Libraries

Michelle A. Clark

1. Introduction

Fab libraries, in which light-chain (LC) and heavy-chain (HC) variable-region genes are cloned into a phagemid vector and subsequently displayed on the surface of the filamentous phage particle, have been widely used for the isolation of antibodies (Abs) with specificity for haptens, foreign antigens (Ags), and self Ags. Immune Fab libraries, in which lymphoid tissue from individuals who, perhaps because of disease, have mounted an immune response to particular Ags, have been used in the recovery of Fabs with binding specificity for a number of clinically relevant Ags including *c-erbB-2* (1) and p53 (2). Fab libraries are thus valuable as a means whereby the genes for Abs of interest can be immortalized and propagated. This enables information to be gathered regarding the Ab, including structural features, V-gene usage, and the nature of the immune response in the individual. Additionally, the isolated Abs can be used to evaluate immunogenic epitope(s) of the Ag. Furthermore, the Abs themselves provide potentially useful diagnostic or therapeutic agents (2). The isolation of Fabs from combinatorial libraries is thus valuable in contributing to the understanding of Ab–Ag interactions, as well as the nature of the *in vivo* immune response.

Technically, the construction of Fab libraries has the advantage of simplicity, compared to the construction of other Ab fragment libraries. The methods described here cover the construction of mouse and human Fab libraries in the phagemid vector, MCO3 (3). This vector has several features, such as different leader sequences for the light and heavy chains, a stop codon that allows easy shuttling between appropriate host strains for the preparation of phage or the

expression of soluble Fab, a *myc* tag for analysis and purification of protein, and a subtilisin cleavage site useful for recovery of bound phage during library screening. Methods included in this chapter are outlined below.

1. RNA is extracted from the tissue of interest (e.g., mouse spleen, human lymph node), and RNA quality is assessed by agarose gel electrophoresis and spectrophotometry (*see Subheading 3.1.*). If DNA is present in the RNA sample, then the sample is digested with DNase I.
2. Reverse transcription (RT) of total RNA is done using immunoglobulin chain specific primers (*see Subheading 3.2.*).
3. The cDNA so generated is used immediately in the polymerase chain reaction (PCR) amplification of immunoglobulin genes using appropriate primers for V-gene families (κ , λ LCs, and γ HCs). PCR reactions are assessed by standard agarose gel electrophoresis. The PCR products from each Ab chain are pooled and precipitated with ethanol. The pooled PCR products are run on a two-concentration agarose gel system to isolate specific product, and are purified using commercial gel purification columns (*see Subheading 3.3.*).
4. Purified PCR products are digested sequentially with *SacI/XbaI* (LC) or *SpeI/XhoI* (HC). Any differences in digestion conditions and subsequent methods are noted (*see Subheadings 3.4.–3.7.*).
5. Phagemid vector, MCO3 (or the LC library in MCO3), is double-cut in preparation for cloning digested PCR products. Vector is cut for insertion of LC (or HC), purified on a two-concentration gel system and double-cut DNA is isolated from the gel using commercial columns. LC or HC PCR product is cloned into the vector and trial ligations done to determine approximate library size and the calculation of vector background (*see Subheadings 3.8. and 3.10.*).
6. Large-scale ligation of double-digested LC PCR product with vector is followed by electroporation into *Escherichia coli* XL1-Blue. DNA carrying the LC libraries is prepared and digested for insertion of HC PCR product. Cloning of digested HC PCR product is done via trial ligation, then large-scale ligation, as for construction of LC library (*see Subheading 3.11.*).
7. Newly constructed Fab libraries are verified by digestion of miniprep DNA with cloning enzymes, PCR analysis from single colonies, and *Bst*NI analysis of diversity and sequencing, and are stored as DNA, bacterial glycerol stocks and phage (*see Subheadings 3.15., 3.17., and 3.18.*).

2. Materials

2.1. RNA Extraction and Analysis

1. Fresh lymphoid tissue or preparation of lymphocytes for library construction.
2. RNase decontamination spray.
3. Autoclaved, precooled (-80°C) mortar and pestle.
4. Guanidine stock solution: 4 M guanidine thiocyanate, 25 mM Na citrate, pH 7.0, 0.5% Sarkosyl. Filter-sterilize through a 0.2- μm filter (*see ref. 4*).

5. Solution D: 54 μ L β -mercaptoethanol mixed with 7 mL guanidine stock solution.
6. 2 M Na acetate, pH 4.1.
7. Buffered, saturated phenol, pH 4.3 (for RNA extraction only).
8. Chloroform:isoamyl alcohol (24:1).
9. Isopropanol.
10. Absolute and 70% (v/v) ethanol.
11. 1% (w/v) Sodium dodecyl sulfate (SDS).
12. RNA sample buffer: 10% (w/v) sucrose, 90% (v/v) formamide, 0.05% (w/v) bromophenol blue.
13. 10 mg/mL Ethidium bromide in H₂O.
14. DNase I (RNase-free) and manufacturer's 10X buffer.
15. Phenol:chloroform:isoamyl alcohol (25:24:1).

2.2. RT and PCR Reactions

1. 10X PCR reaction buffer (commercial).
2. 25 mM MgCl₂.
3. 10 mM Deoxyribonucleoside triphosphate (dNTP) mix (deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, deoxythymine triphosphate).
4. Immunoglobulin 3' primers at 20 μ M (see **Tables 1–4**).
5. Murine leukemia virus RTase (20 U/ μ L).
6. RNasin.
7. *Tth* polymerase (5.5 U/ μ L).
8. LC or HC oligonucleotide primers at 20 μ M (see **Tables 1–4**).

2.3. Digestion and Cloning of PCR Products

1. Appropriate restriction enzymes for cloning PCR products into chosen phage display vector (e.g., *SacI* (100 U/ μ L) *XbaI* (100 U/ μ L), *SpeI* (50 U/ μ L), *XhoI* (40 U/ μ L), and associated 10X buffers).
2. Bovine serum albumin (BSA) (1 mg/mL).
3. 100 mM Tris base.
4. 100 mM Tris-HCl.
5. 350 mM β -mercaptoethanol.
6. 100 mM MgCl₂.
7. Commercial kits for the isolation of DNA from agarose gels and from solution.
8. Appropriate phagemid vector (e.g., MC03) (**Fig. 1**).
9. Low-melting-temperature agarose.
10. Long-wave, hand-held UV lamp.
11. UV-transparent shrink-wrap film.
12. Scalpel blades.
13. Ethidium bromide stock (1 mg/mL).
14. Solution of DNA of known concentration (100 μ g/mL).
15. T4 DNA ligase (400 U/ μ L) and commercial buffer.

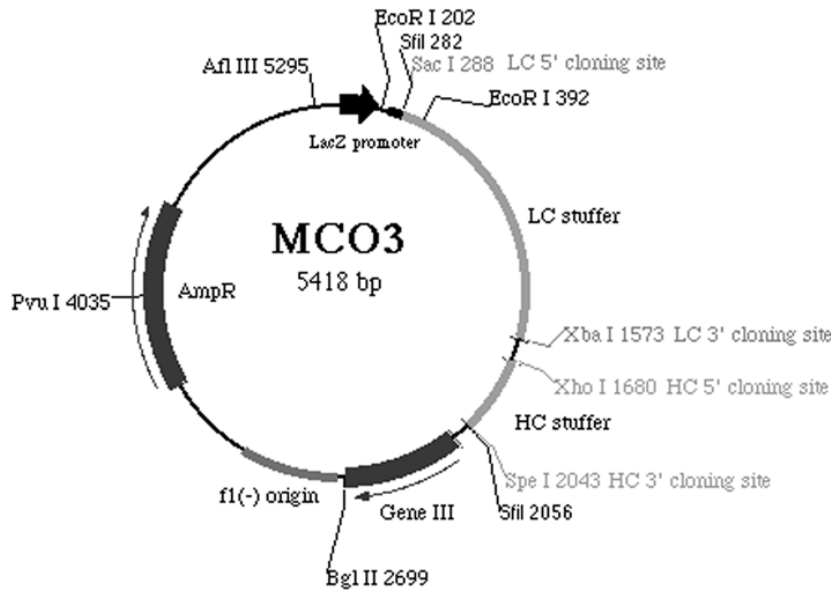


Fig. 1.

Table 1
Murine LC Primer Sequences

	Gene family	Primer sequence 5' to 3'
3' LC primer	C _κ 1	CATGT TCTAG AACACTCATTCCTGTTGAAGCTCTTG
5' LC primers	V _κ A	CATGGAGCTCGATGTTTTGATGACCCAAACTCCA
	V _κ B	GATCGAGCTCGACATTGTGCTCACCCAATCTCC
	V _κ C	CATGGAGCTCGACATTGTGCTRACCCAGTCTTCCA
	V _κ D	CATGGAGCTCGACATCCAGATGACNCAGTCTCAA
	V _κ E	CATGGAGCTCCAAATTGTTCTCACCCAGTCTCCA
	V _κ F	CATGGAGCTCGAAAATGTGCTTCACCCAGTCTCCA

R = A or G; N = A, G, C or T.
Restriction sites are in bold (*Sac*I GAGCTC, *Xba*I TCTAGA).

Table 2
Murine HC Primer Sequences

	Gene family	Primer sequence 5' to 3'
3' HC primers	Igy1	AGGCTT ACTAGT TATGCAAGGCTTACAACC
	Igy2A	AGGCTT ACTAGT ACAGGGCTTGATTGTGGGCC
	Igy2B	AGGCTT ACTAGT ACAGGGGTTCAAGTGTGAAATGG
5' HC primers	IA	TGGAGGCTT CTCGAGG AKGTGCAGCTTCAGGAGTC
	IB	TGGAGGCTT CTCGAGC AGGTGCAGCTGAAGSAGTC
	IIA	TGGAGGCTT CTCGAGS AGGTCCAGCTGCARCAGTC
	IIB	TGGAGGCTT CTCGAGC AGGTCCARCTGCAGCAGYTTGG
	IIC	TGGAGGCTT CTCGAGG AGGTTCAAGCTGCAGCAGTC
	IIIA	TGGAGGCTT CTCGAGG ARGTGAAGCTGGTGGARTCTGG
	IIIB	TGGAGGCTT CTCGAGG AGGTGAAGCTTCTGGAGTCTGG
	IIIC	TGGAGGCTT CTCGAGG AAGTGAAGCTTGAGGAGWCTGG
	IIIDA	TGGAGGCTT CTCGAGG AAGTGCAGCTGGTGGAGTCTGG
	IIIDB	TGGAGGCTT CTCGAGG AAGTGATGCTGGTGGAGTCTGG
	VA	TGGAGGCTT CTCGAGG AGGTYCAGCTKCAGCAG
	C _H 1	GCCAAAACGACACCCCCA

R = A or G; Y = C or T; S = C or G; W = A or T; K = G or T.

Restriction sites are in bold (*Xho*I CTCGAG, *Spe*I ACTAGT).

2.4. Preparation of Electrocompetent Cells and Transformation

1. *E. coli* XL-1 Blue. Cells prepared for electroporation can be obtained commercially or prepared in the laboratory (see **Subheading 3.12.**).
2. Luria-Bertani medium (LB). Composition/L: 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl, pH 7.0. Autoclave.
3. LB agar plates. Composition as for LB, but containing 15 g/L agar.
4. LB–TET50. LB plates containing 50 µg/mL tetracycline, taken from a stock of the antibiotic at 10 mg/mL in 70% ethanol.
5. 2TY. Composition/L: 16 g Bacto-tryptone, 10 g Bacto-yeast extract, 5 g NaCl, pH 7.0. Autoclave.
6. 2TY–TET10. 2TY containing 10 µg/mL tetracycline.
7. Cold, autoclaved 10% glycerol in H₂O.
8. 20% Glucose, filter-sterilized.
9. Appropriate centrifuge rotor and tubes (e.g., Beckman JA14).
10. Cryotubes.
11. Liquid nitrogen.
12. Electroporation cuvettes (0.2 cm gap).
13. SOC. Composition/L: 20 g Bacto-tryptone, 5 g Bacto-yeast extract, 0.5 g NaCl, pH 7.5. Sterilize by autoclaving. Just before use, add 20 mL sterile 1 M MgSO₄ and glucose to a final concentration of 0.4%.

Table 3
Human LC Primer Sequences

	Gene family	Primer name	Primer sequence 5' to 3'
3' LC (κ) primer	C_κ	$C_\kappa 1z$	GCGCCGTCTAGAAATTAACACTCTCCCCTGT TGAAGCT CTTTGTGACGGGCGAACTCAG
5' LC (κ) primers	1	$V_\kappa 1a$	GACATCGAGCTCACCCAGTCTCCA
	2	$V_\kappa 2a$	GATATTGAGCTCACTCAGTCTCCA
	3	$V_\kappa 3a$	GAAATTGAGCTCACGCAGTCTCCA
	κ Constant	CON_κ	ACTGTGGCTGCACCATCTG
3' LC (λ) primer	C_λ	$C_\lambda 2$	CGCCGTCTAGAACTATGAACATTCAAG
5' LC (λ) primers	1,2	$V_\lambda 1,2$	CAGTCTGAGCTCACTCAGCCRCCC
	3	$V_\lambda 3$	CAGCCTGAGCTCACTCAG
	4,5,9	$V_\lambda 4,5,9$	TCTGTGGAGCTCCAGCCGCCCTCAGTG
	6	$V_\lambda 6$	AATTTTGAGCTCACTCAGCCC
	7	$V_\lambda 7$	CAGGCTGAGCTCACTCAGGAG
	8	$V_\lambda 8$	CAGACTGAGCTCACCCAGGAG
	10	$V_\lambda 10$	CAGGCAGAGCTCACTCAGCCA
	λ Constant	$CON_\lambda 2$	AAGGCTGCCCCACGGTCACTCTG

R = A or G.

Restriction sites are in bold (*SacI* GAGCTC, *XbaI* TCTAGA).

14. LB-CARB50. LB plates containing 50 $\mu\text{g/mL}$ carbenicillin, taken from a stock of the antibiotic at 10 mg/mL in H_2O .

2.5. Library Preparation and Analysis

1. Large (14 cm) 2TY agar plates (*see Subheading 2.4., item 5* containing 15 g/L agar), supplemented with glucose to 2% and carbenicillin to 50 $\mu\text{g/mL}$ (2TY-GLU-CARB).
2. 2TY (*see Subheading 2.4., item 5*).
3. Sterile glycerol.
4. 2TY-GLU. 2TY supplemented with glucose to 2%.
5. Carbenicillin at 10 mg/mL in H_2O .
6. Commercial kit for the isolation of plasmid DNA (maxi/mega-scale).
7. 2TY-GLU-TET-CARB. 2TY supplement with glucose to 2%, tetracycline (5 $\mu\text{g/mL}$) and carbenicillin (20 $\mu\text{g/mL}$).
8. VCS-M13 helper phage.
9. Tetracycline (10 $\mu\text{g/mL}$ in 70% ethanol).
10. 2TY-TET-CARB-KAN. 2TY containing tetracycline (10 $\mu\text{g/mL}$), carbenicillin (50 $\mu\text{g/mL}$), and kanamycin (70 $\mu\text{g/mL}$).

Table 4
Human HC Primer Sequences

	Primer name	Gene family	Primer sequence 5' to 3'
3' HC primers	C _γ 1z	γ1	GCATGT ACTAGT TTTTGTCACAAGATTGGG
	C _γ 2z	γ2	CGGTGG ACTAGT GACACAACATTTGCG
	C _γ 3z	γ3	TGGGCA ACTAGT GCATGTGTGAGTTGTG
	C _γ 4z	γ4	TGGGCA ACTAGT GCATGGGGGACCATATTGGA
	CON _γ a	γ1,2,3,4	TCCACCAAGGGCCCCATCG
5' HC primers	V _H 1a	1 and 4	CAGGTGCAGCT CGAGC AGTCTGGG
	V _H 1f	1 and 4	CAGGTGCAGCTG CTCGAGT CTGG
	V _H 2fN	2	CAGATCAC CTCGAGG AGTCTGGT
	V _H 3a	3	GAGGTGCAGCT CGAGG AGTCTGGG
	V _H 3f	3	GAGGTGCAGCTG CTCGAGT CTGGG
	V _H 5f	5	GAGGTGCAGCT CGAGC AGTCTGGA
	V _H 6f	6	CAGGTACAGCTG CTCGAGT CAGGTCCA
	V _H 7f	7	CAGGTCCAGCT CGAGC AATCTGG

Restriction sites are in bold (*Xho*I CTCGAG, *Spe*I ACTAGT).

11. 2.5 M NaCl, 20% polyethylene glycol (PEG) 6000 in H₂O.
12. Phosphate-buffered saline containing 1% BSA and Na azide at 0.02%.
13. 2TY–TET10.
14. Kanamycin stock (10 mg/mL in H₂O).
15. Dimethylsulfide.
16. Cryotubes.
17. LB agar plates (*see Subheading 2.4., item 3*).
18. Top agar. Prepare LB liquid medium and add agarose to 0.6%. Autoclave.
19. LB liquid medium (*see Subheading 2.4., item 2*).
20. Commercial kits for the isolation of plasmid DNA (miniprep scale).
21. Cracking buffer. 10 mM Tris-HCl, pH 7.0, 1 mM ethylene diamine tetraacetic acid, 50 μg/mL Proteinase K.
22. *Tth* or other thermostable DNA polymerase, commercial buffer, and stock MgCl₂ solution (25 mM).
23. Oligonucleotide primers flanking the sites of insertion of LC and HC in the chosen phage-display vector.
24. Sequencing primers (5' to 3'; redissolve to 20 μM). Before synthesis, check that the suggested sequences will hybridize to the phage display vector selected for library construction.

ompA forward: AAAGACAGCTATCGCGATT

pelB reverse: CAGCGAGTAATAACAATCCA

pelB forward: CTACGGCAGCCGCTGGATTG
gene III: CATCGGCATTTTCGGTCATA

25. *Bst*NI and 10X buffer.

3. Methods

3.1. Preparation of RNA

3.1.1. RNA Extraction from Tissue (see **Note 1**)

1. Wipe down hood, all pipets, and other equipment with 70% ethanol or RNase decontamination spray. Treat an autoclaved mortar and pestle with RNase decontamination spray for 5 min, wipe out with a Kimwipe and keep cold (see **Note 2**).
2. With liquid nitrogen in the mortar, add the tissue and tap with the pestle until the tissue has broken up into small pieces. Let the liquid nitrogen evaporate then grind the tissue into a fine powder. Scrape the powder from the mortar and pestle with a sterile blade and add to fresh solution D. It is best to add approx 1 mL solution D/0.1 g tissue in a 2 mL microcentrifuge tube (see **Notes 2** and **3**).
3. Push the solution through a fine-gauge needle until no lumps are left.
4. Add 66 μ L 2 M Na acetate, pH 4.1, 660 μ L buffered phenol (pH 4.3), and 130 μ L chloroform–isoamyl alcohol (24:1). Mix well after each addition then vortex for 30 s and incubate on ice for 15 min. The solution should be cloudy at this stage (see **Note 3**).
5. Centrifuge for 30 min at 4°C in a microcentrifuge. If the interface between the aqueous (upper) phase and the organic (lower) phase is not well-defined, then extra chloroform should be added until the two phases have separated.
6. Transfer the top, aqueous layer to a fresh tube (avoid the interface because it contains DNA) and back-extract if there was only a small amount of tissue to begin with. To back-extract, add an equal volume of fresh solution D to the organic phase and repeat incubation on ice and centrifugation steps. Pool both aqueous phases.
7. Add an equal volume of isopropanol, mix, and incubate overnight at –20°C.
8. Centrifuge at full speed in a microcentrifuge for 30 min at 4°C to precipitate RNA.
9. Discard supernatant, drain pellet and resuspend RNA in solution D to a total volume of 500 μ L. Pool RNA if there was more than one tube. RNA should be clearly visible as a clean, white pellet at the bottom of the tube.
10. Adjust pH by adding one-tenth vol of 2 M Na acetate, pH 4.1. Add 2 vol of cold 100% ethanol and incubate RNA for 2 h at –20°C. Centrifuge as in **step 8**.
11. Discard supernatant and rinse the pellet with 500 μ L of cold 70% ethanol, followed by 100 μ L of cold 100% ethanol.
12. Air-dry RNA for 15 min. Do not overdry or the RNA will be difficult to resuspend.
13. Resuspend RNA in 20 μ L of sterile H₂O/0.1 g original tissue. Leave on ice, or at 4°C to dissolve. For this and subsequent steps, use the highest quality sterile H₂O available, preferably a commercial batch to reduce the risk of contamination with RNases.

14. Read A_{260}/A_{280} of 1:100 dilution of the RNA (*see Note 4*).
15. Aliquot and store RNA at -70°C (*see Note 5*).

3.1.2. RNA Analysis

1. RNA can be assessed quickly and easily, using a minigel apparatus. Use a new minigel apparatus or, if this is not possible, treat the gel rig, spacers, and comb with 3–4 washes with 1% SDS. Rinse all apparatus with sterile H_2O . Rinse a spatula with 1% SDS, followed by sterile H_2O , and prepare a standard 1% agarose gel in TBE.
2. Add 0.5–2.0 μg RNA to RNA sample buffer. RNA should be in a volume <50% of the total, which should be <20 μL . Add 1–2 μL 0.1 mg/mL ethidium bromide. Mix well then heat the sample at $60\text{--}65^{\circ}\text{C}$ for 3 min, cool to room temperature, and load onto the gel. Run the gel for about 2 h, room temperature, 50 V.
3. High-quality RNA shows two discrete bands on the gel, representing the 28S and 18S rRNA species. The intensity of the 28S (upper) band is usually twice that of the 18S (lower) band. Any smearing below either of these bands indicates degradation (slight trailing under the bands may be visible if the gel is overloaded). High molecular weight material in the well of the gel is DNA, which needs to be removed by digestion with DNase I.

3.1.3. DNase Treatment of RNA Sample

1. If the RNA is in a volume less than 200 μL , treat as follows, otherwise scale-up to appropriate volume: μL RNA sample, 20 μL 10X DNase digestion buffer, 5 μL DNase I (RNase-free), and sterile H_2O to 200 μL .
2. Incubate for 1 h at 37°C .
3. Add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and mix well (*see Note 3*).
4. Spin 15 min in a microcentrifuge at 4°C . Remove aqueous (upper) layer to a fresh tube.
5. Adjust pH with one-tenth vol 2 M Na acetate, pH 4.1, then precipitate RNA with 2 vol 100% ethanol for 2 h at -80°C .
6. Centrifuge 30 min at 4°C , wash pellet with 200 μL 70% ethanol, then 200 μL 100% ethanol. Air-dry for 15 min.
7. Resuspend the RNA pellet in 40 μL sterile H_2O /0.1 g tissue.
8. Check concentration of RNA (usually about 1 $\mu\text{g}/\mu\text{L}$) and integrity on 1% agarose gel (*see Subheading 3.1.2.*).
9. Aliquot RNA and store at -80°C (*see Note 5*).

3.2. Reverse Transcription of Light Chain (LC) and Heavy Chain (HC) Genes

1. Prepare a reverse transcription (RT) reaction of sufficient volume to supply 1.5 μL reaction mix for each 50 μL PCR reaction at **Subheading 3.3., step 2**. The number of PCR reactions, and hence the volume of the RT mix, will depend on the number of primer combinations required to recover the immunoglobulin

repertoires from the species under study. The RT reaction comprises 5–10 μg RNA in 1X PCR buffer containing 5 mM MgCl_2 , 1 mM dNTP mix, and 1.2 μM 3' LC primer or 3' HC primers (see **Tables 1–4**). A single bulk RT reaction for the LC repertoire and a single bulk RT reaction for the HC repertoire, each RT mix containing all relevant 3' primers is satisfactory.

2. Incubate at 65°C for 5 min, then on ice for 5 min.
3. Add murine leukemia RTase to a final concentration of 1 U/ μL , 1/20 vol RNasin, and incubate at 37°C for 60 min, 95°C for 5 min, then ice for 5 min. cDNA should be used as soon as possible in the PCR.

3.3. PCR of LC and HC Genes

1. Prepare reaction mix sufficient for all combinations of 3' and 5' primers (murine, human, or other species as appropriate), with final concentrations as follows: 1X PCR reaction buffer, 2 mM MgCl_2 , 0.2 mM dNTP mix, 0.03 U/ μL *Tth* polymerase (see **Note 6**).
2. On ice, add 45.5 μL reaction mix/tube, 1.5 μL each 3' and 5' primer (see **Tables 1–4**) from stock concentrations of 20 μM and 1.5 μL RT reaction. Separate reactions should be prepared for each 5' primer with the selected 3' primer. Omit RT reaction from the negative controls. Mix all reactions gently and keep on ice (see **Note 7**).
3. Commence LC PCR with denaturation at 94°C for 4 min, followed by 35 cycles as follows: 94°C for 15 s, 52°C for 50 s, and 72°C for 90 s. Commence HC PCR with denaturation at 94°C for 4 min, then apply touchdown cycling for a total of 35 cycles as in **Table 5** (see **Note 8**):
4. End PCRs with extension at 72°C for 10 min. The samples can be stored at 4°C overnight or frozen if necessary.
5. Run 5 μL of each PCR reaction on standard 1% agarose gels to check the size and yield of the products (see **Subheading 3.9**).
6. Clean up PCR product on two-agarose-gel system as described (see **Subheading 3.8**, steps 2–9).

3.4. Digestion of LC PCR Product with *SacI* for Cloning into MCO3

The LC PCR products (1–3 μg) are digested with *SacI* (50 U/ μg DNA) in a dedicated buffer containing 10 mM Tris base: Tris-HCl (1:3.5, pH 7.3), 0.1 mg/mL BSA, 7 mM β -mercaptoethanol, 20 mM NaCl, 7 mM MgCl_2 , total vol 50 μL . Digest DNA for 2–3 h at 37°C, then heat deactivate *SacI* at 65°C for 15 min.

3.5. Digestion of LC PCR Product with *XbaI*

1. Clean-up of the *SacI* cut DNA prior to digestion with *XbaI* is not necessary (see **Note 9**). Increase the volume to 100 μL with a dedicated buffer containing

Table 5
Touchdown Conditions for HC PCR

Denaturation	Annealing	Extension	Cycles
94°C, 30 s	65°C, 1 min	72°C, 90 s	2
94°C, 30 s	64°C, 1 min	72°C, 90 s	2
94°C, 30 s	63°C, 1 min	72°C, 90 s	2
94°C, 30 s	62°C, 1 min	72°C, 90 s	2
94°C, 30 s	61°C, 1 min	72°C, 90 s	2
94°C, 30 s	60°C, 1 min	72°C, 90 s	2
94°C, 30 s	59°C, 1 min	72°C, 90 s	2
94°C, 30 s	58°C, 1 min	72°C, 90 s	2
94°C, 30 s	57°C, 1 min	72°C, 90 s	2
94°C, 30 s	56°C, 1 min	72°C, 90 s	2
94°C, 30 s	55°C, 1 min	72°C, 90 s	15

100 mM NaCl, 10 mM Tris base: Tris-HCl (5:1, pH 7.9), 0.1 mg/mL BSA, 7 mM β -mercaptoethanol, 7 mM MgCl₂ and 50 U/ μ g DNA *Xba*I. Digest for 2–3 h at 37°C, then heat-deactivate at 65°C for 15 min.

2. Clean up *Sac*I/*Xba*I digested LC DNA with commercial DNA purification columns. We have found Qiagen columns to be quick and reliable, but phenol extraction (pH 8.0) followed by ethanol precipitation will work just as well. Resuspend DNA in H₂O and calculate concentration of DNA as described below (see **Subheading 3.9.**) or by reading A₂₆₀.

3.6. Digestion of HC PCR Product with *Spe*I

The HC PCR products (1–3 μ g) are digested with *Spe*I (25 U/ μ g DNA) in buffer containing 10 mM Tris base: Tris-HCl (1:4.5, pH 7.3), 0.1 mg/mL BSA, 7 mM β -mercaptoethanol, 80 mM NaCl, 7 mM MgCl₂ in a total volume of 50 μ L. Digest DNA for 2–3 h at 37°C, then heat-deactivate at 65°C for 15 min.

3.7. Digestion of HC PCR Product with *Xho*I

1. As in **Subheading 3.5.**, *Spe*I-cut DNA can be digested with *Xho*I without prior cleanup by adapting the buffer composition (see **Notes 9** and **10**). Add a dedicated buffer containing 100 mM NaCl, 10 mM Tris base: Tris-HCl (5:1), 0.1 mg/mL BSA, 7 mM β -mercaptoethanol, 100 mM NaCl, 7 mM MgCl₂, and *Xho*I to 20 U/ μ g DNA, raising the total volume of the reaction mix to 100 μ L. Incubate for 2–3 h at 37°C, then heat-deactivate at 65°C for 15 min.
2. Clean up *Spe*I/*Xho*I digested HC DNA and determine concentration of DNA as described (see **Subheading 3.9.**).

3.8. Preparation of Double-Cut MCO3 for Cloning of Digested LC and HC PCR Products, and Cleanup of PCR Products (see Note 11)

1. MCO3 vector (20–40 μg) (**Fig. 1**) is digested in a commercial buffer with *SacI* (50 U/ μL) and *XbaI* (50 U/ μL) for 2–3 h at 37°C. For cloning of digested HC PCR product, the LC library in MCO3 is similarly digested with *SpeI* (25 U/ μL) and *XhoI* (20 U/ μL). Double-digestion of vector DNA is efficient because of the length of intervening sequence between the restriction sites (compare digestion of PCR products).
2. Pour a thick 0.8% agarose gel in TBE containing 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide and load the wells with <5 μg DNA/well. A thick-spaced well-former in a minigel apparatus is suitable.
3. Run the gel at 20–25 V overnight at room temperature so that there is good separation of vector (4.1 kb for MCO3) from the LC stuffer fragment (1.3 kb) and uncut and single cut vector (5.4 kb). When LC products have been cloned into the vector, double-digestion should give a vector fragment of 4.4 kb plus a 0.3-kb stuffer fragment; single-cut vector yields a band of 4.7 kb. Electrophoresis of PCR product (660 bp) need only be for a few hours.
4. Under long-wave UV, cut away the agarose around the vector or PCR products with a clean scalpel. Long-wave UV is used so that nicking of DNA is minimized because it can affect subsequent cloning steps.
5. Prepare low-melting-temperature agarose at a concentration of 0.4–0.6% in TBE buffer and pour around the agarose gel containing the double-cut vector. Allow this to set at 4°C.
6. Run the gel at 4°C until the double-cut vector band (or PCR product) has run into the low-melting-temperature agarose, typically a further 2 h at 50 V.
7. Under long-wave UV, excise the band from the low-melting-temperature gel and purify the DNA using a commercial DNA purification kit.
8. Estimate the DNA concentration by DNA spotting (see **Subheading 3.9.**; see **Note 12**) and/or measurement of the A_{260} .
9. Digested vector DNA should be purified again on the two-gel system as described in **steps 2–6** to ensure a low background of contaminating single-cut vector. Specific PCR product may only need one purification in this manner unless a large number of non-specific products are present.

3.9. Determination of DNA Concentration by Spot Testing (see Note 12)

1. Stretch a piece of UV transparent plastic wrap over a UV transilluminator.
2. Spot several aliquots of 1–5 μL of ethidium bromide (2 $\mu\text{g}/\text{mL}$). There should be enough for the series of standards and the unknown DNA samples.
3. Add an equal volume (1–5 μL) of unknown DNA sample and standard DNA solutions (0, 1, 2.5, 5, 10, and 20 $\mu\text{g}/\text{mL}$) onto the wrap. Mix with spotted ethidium bromide by carefully pipeting up and down.

4. Photograph the spots under short-wave UV and estimate the concentration of the unknown by comparing with the intensity of fluorescence in the standards.

3.10. Small-Scale Ligation of PCR Product into MCO3 Vector

1. Prepare the ligation reaction as follows: double-digested MCO3 vector (for LC insertion) or LC library in MCO3 (for HC insertion) (50–100 ng), double-digested, purified LC or HC PCR products (16–32 ng; 1:2 or 1:5, vector:insert), T4 DNA ligase (0.15 U/ μ L) in 1X ligase buffer. Ligation controls should be set up to include only the vector to check for presence of single-cut DNA and vector only with no ligase to check for the presence of uncut DNA. A control insert (such as Fab LC or HC DNA cut out of a vector with *SacI/XbaI* or *SpeI/XhoI*, respectively) can be used also to check the efficiency of ligation of PCR product.
2. Incubate the ligation reactions at 15°C overnight or 1–3 h at room temperature.
3. Ligation reactions can be used without further purification for electroporation into XL1-Blue cells (see **Subheadings 3.12.**, **3.13.**) to check vector background and library size (see **Notes 13** and **16**).

3.11. Large-Scale Ligation of PCR Product with Vector

1. Prepare ligation reaction as follows: double-digested, purified MCO3 vector (for LC insertion) or LC library in MCO3 (for HC insertion) (2–3 μ g), double-digested, purified LC or HC PCR products (1 μ g; 1:2, vector:insert), T4 ligase (20 U/ μ L) in 1X ligase buffer.
2. Incubate reactions at 15°C overnight (see **Note 14**).
3. Clean up either by using a commercial DNA cleanup kit or by phenol extraction and ethanol precipitation (see **Note 3**).
4. Elute or resuspend the DNA in 50 μ L H₂O.
5. Electroporate the ligated material into *E. coli* XL1 Blue (see **Subheadings 3.12.** and **3.13.**) to calculate the size of the library (see **Note 17**).

3.12. Preparation of Electrocompetent Cells (see Note 15)

1. Sample XL1 Blue cells from a fresh culture on LB–TET50 plates and grow an overnight starter culture in 2TY–TET10.
2. Prepare 1 L 10% glycerol, sterilize, and chill to 4°C overnight.
3. Add 10 mL overnight culture to 1 L 2TY and divide between four large (e.g., 1-L) flasks. Add to each flask 25 mL 20% D-glucose and tetracycline to a final concentration of 10 μ g/mL.
4. Chill a high-speed rotor and centrifuge tubes to 4°C.
5. Grow cultures at 37°C until OD₆₀₀ reaches 0.8–1.0 (typically about 4.5 h).
6. Transfer the contents of the flasks into four 250-mL chilled, centrifuge bottles and allow to stand on ice until cold (typically about 30 min).
7. Centrifuge at 1200g, 15 min, 4°C.
8. Resuspend the pellets with a 10-mL pipet in cold 10% glycerol to a total volume of 500 mL. Transfer to two centrifuge bottles. Keep cells on ice while resuspending.

9. Centrifuge at 1200g, 15 min, 4°C.
10. Resuspend the pellets with a 10-mL pipet in cold 10% glycerol to a total volume of 250 mL and transfer to one centrifuge bottle. Keep cells on ice while resuspending.
11. Centrifuge at 1200g, 15 min, 4°C.
12. Remove supernatant with a pipet. Be careful because the pellet is soft here. Resuspend the pellet with a 10-mL pipet in 10% glycerol to a total volume of 50 mL. Keep cells on ice while resuspending.
13. Centrifuge at 1200g, 15 min, 4°C.
14. Remove supernatant with a pipet. Be careful because the pellet is very soft here. Resuspend the pellet with a 10-mL pipet in residual glycerol. Keep cells on ice while resuspending. The final volume should be 5–10 mL/L original culture.
15. Aliquot 200 μ L/cryotube and quick-freeze in liquid nitrogen (wear safety glasses when doing this). Store cells at -80°C .
16. Transformation efficiency of cells should be determined by electroporating 100 pg control (uncut) vector DNA and plating onto appropriate selective medium (*see Subheading 3.13.*). Cells should yield 10^9 transformants/ μ g DNA.

3.13. Electroporation into *E. coli* XL1 Blue

1. Use new cuvetts for each library. The cuvetts should be kept at -20°C .
2. For small-scale ligation, add 50 μ L electrocompetent XL1 Blue to each cuvet and 1 μ L ligation mixture (*see Subheading 3.10., step 3*). Use one cuvet for each ligation reaction. For large-scale library construction, add 100 μ L competent XL1 Blue to each cuvet and 3 μ L ligation products dissolved in H_2O (*see Subheading 3.11., step 5*). Between 15 and 20 cuvetts will be needed for each large-scale ligation. Electroporation conditions are according to manufacturer's recommendations.
3. After electroporation, rescue cells by quickly adding to each cuvet 1 mL SOC. Transfer to a 5-mL tube and incubate at 37°C for 60 min (*see Note 15*). After rescue, preparation of a large-scale library should yield a total volume of cells of 15–20 mL.
4. Plate out 100 and 10 μ L from each transformation onto LB–CARB50 plates and incubate overnight at 37°C . For large-scale library, pool remaining transformations after rescue and proceed to **Subheading 3.14.**
5. From the number of colonies that appear on the plates, calculate the library size (*see Notes 16 and 17*).

3.14. Large-Scale Preparation of LC Library DNA and Bacterial Glycerol Stock

1. The library pool should comprise 15–20 mL in SOC after transformation (*see Subheading 3.13., step 3*). Divide ~ 5 mL from this between 10 large 2TY–GLU–CARB plates, spread, and incubate overnight at 30°C .

2. Scrape cells off plate into 2TY, add glycerol to a final concentration of 25%, and store the LC library at -70°C as a bacterial glycerol stock.
3. Make up the volume of the remaining pool to 100 mL with 2TY–GLU and incubate 60 min, 37°C .
4. Add carbenicillin to $20\text{ }\mu\text{g/mL}$ and incubate a further 60 min at 37°C .
5. Increase volume to 500 mL with 2TY–GLU, increase carbenicillin to $50\text{ }\mu\text{g/mL}$, and incubate overnight at 37°C .
6. Recover cells by centrifugation and prepare DNA by CsCl gradient or other reliable method (*see Note 18*).
7. Analyze library to confirm insert size and diversity (*see Subheading 3.17.*; *see Note 19*).

3.15. Preparation of HC Library DNA, Bacterial Glycerol Stock, and Library Phage

1. After ligation of HC genes into the LC library and electroporation into XL1 Blue, prepare the glycerol stock of the final Fab library (*see Subheading 3.14., steps 1 and 2*).
2. Make up the volume of the remaining pool of cells to 100 mL with 2TY–GLU–TET–CARB and incubate 60 min, 37°C .
3. Add 2.5×10^{12} VCSM13 helper phage (*see Subheading 3.16.*; *see Note 20*) to each 100 mL culture. Increase the concentration of tetracycline to $10\text{ }\mu\text{g/mL}$ and carbenicillin to $50\text{ }\mu\text{g/mL}$. Incubate with shaking for 2 h, 37°C .
4. Centrifuge the cells at $1500g$, 10 min. Resuspend the pellet in 500 mL 2TY–TET–CARB–KAN and incubate overnight at 30°C with shaking.
5. Centrifuge to collect bacteria (**step 4**) and collect the supernatant. Precipitate phage from the supernatant by adding 1/5 vol 2.5 M NaCl, 20% PEG, and incubating on ice for 60 min.
6. Pellet the phage by centrifuging at $6200g$, 4°C , 20 min. The phage should appear as a large white pellet. Resuspend the pellet in 1 mL PBS–1% BSA–Na azide. Spin the phage for 1 min in a microcentrifuge to remove bacterial debris. Recover the clarified supernatant to a fresh microcentrifuge tube and reprecipitate phage by adding 1/5 vol 2.5 M NaCl–20% PEG. Spin again (the phage will precipitate immediately) and resuspend in 1 mL fresh PBS–1% BSA–Na azide. The phage can be stored at 4°C for up to 12 mo.
7. If required, DNA can be prepared using standard procedures from the bacterial pellet (**step 5**).

3.16. Preparation of Helper Phage (*see Note 20*)

1. Inoculate an overnight plate culture of XL1–Blue (*see Subheading 3.12., step 1*) into 2TY–TET10. Grow overnight at 37°C .
2. Prepare two flasks, each containing 100 mL 2TY–TET10, and inoculate with 2 mL of overnight culture. Incubate 2 h, 37°C with shaking.

3. Add 2×10^{11} pfu VCSM13 helper phage from a commercial source to each 100 mL culture.
4. Incubate 1 h, 37°C with shaking.
5. Add kanamycin to 70 µg/mL and incubate further 3–4 h, 37°C with shaking.
6. Centrifuge the culture at 2500g for 15 min. Transfer the supernatant to a fresh container. Discard the pellet.
7. Add dimethylsulfoxide to supernatant to 7% final concentration and aliquot helper phage in 1–2-mL lots. Store the tubes at –70°C.
8. Titer the phage by inoculating XL1 Blue into 10 mL 2TY–TET 10, and growing until the A_{600} reaches a value of 1 (late log phase). Prewarm fresh, dry LB plates to 37°C (see **Note 21**), melt a stock of top agar, and maintain it at 50°C until required. Prepare serial dilutions of helper phage (10^{-6} , 10^{-8} , 10^{-10} , 10^{-12}) in LB and add 1 µL of each dilution to 100 µL of log-phase XL1 Blue cells. Add the mixture to 3-mL aliquots of top agar that has been cooled to about 42°C (see **Note 21**). Pour quickly to a prewarmed LB plate and swirl to distribute the mixture evenly over the surface. Incubate overnight at 37°C and count plaques the following day. Calculate titer of phage as pfu/mL.

3.17. Analysis of Library Clones

1. After construction of the phage library containing LC (see **Note 19**) and HC, prepare plasmid DNA from individual colonies on a miniprep scale. Plates used for titrating the LC and the complete libraries are a convenient source of clones. The analysis aims to verify the presence of LC and HC inserts, which is done by simply digesting plasmid DNA with the enzymes used for cloning (*SacI/XbaI* for the LC and *SpeI/XhoI* for the HC). LC and HC inserts should be 660 bp in size and at least 90% of the clones should contain a full-length insert.
2. An alternative to digestion of miniprep DNA with cloning enzymes is to amplify the DNA directly from bacterial colonies (see **Subheading 3.18.**), then digest with the enzyme, *Bst*NI, to provide a fingerprint of the LC and HC inserts. This can be used to assess the diversity of LC and HC sequences. DNA prepared in this way can also be used in sequencing reactions.
3. The ultimate test of the library is the sequencing of random clones. This should be done on DNA from 10–20 clones to verify that the inserts are immunoglobulin, that they are derived from the species of interest, and to confirm that they are full-length with no widespread cloning errors, such as deletion of restriction sites. Sequencing is not covered in this protocol but is amply described in many general methods books and contract sequencing services are widely available. Sequencing primers are suggested (see **Subheading 2.5., item 24**).

3.18. Crack PCR and *Bst*NI Digestion

1. Transfer a single colony to a 1.5-mL microcentrifuge tube containing 20 µL cracking buffer (see **Note 22**).
2. Incubate for 15 min at 55°C, 15 min at 80°C, then in ice for 1 min.

3. Centrifuge for 3 min in a microcentrifuge and transfer the supernatant to a fresh tube.
4. Prepare a PCR mix containing 2 mM MgCl₂, 2 U/μL *Tth* polymerase, and 25 pmol of each flanking primer in 1X PCR buffer. Prepare sufficient for 48 μL for each PCR reaction to be carried out (*see* **Notes 6** and **7**). Add 2 μL of each colony supernatant.
5. Commence PCR at 94°C for 3 min, then carry out 30 cycles as follows: 50°C for 30 s, 72°C for 60 s, 95°C for 30 s. Finish by incubating at 72°C for 10 min.
6. Confirm that the reactions have been successful by analyzing 5 μL on standard 1% agarose gels before sampling 10 μL of each PCR product for digestion with *Bst*NI at 50°C for 3 h.
7. Analyze the profiles of restriction fragments on 8 and 12% polyacrylamide gels in TBE buffer (*see* **Note 22**). Details for preparing these gels are not covered here but are amply described in many general protocol books.

4. Notes

1. The RNA method described takes 2 d and gives high-quality RNA. It is suitable for the purification of RNA from small quantities of tissue (>0.05 g) in 2 mL microcentrifuge tubes. For the construction of human libraries, we have found lymph nodes to be a source of RNA superior to peripheral blood lymphocytes (**5**).
2. Aseptic technique should be used throughout the procedure and gloves should be worn and changed frequently to minimize RNase contamination. It is best to use disposable plasticware and fresh, sterile solutions. For details on how to treat glassware and nonsterile solutions to prevent contamination with RNases, consult a general laboratory manual.
3. Grinding of sample tissue should be done in a class II Biohazard tissue culture hood. Phenol/chloroform extractions should be performed in a fume hood.
4. A₂₆₀ of 1 is equivalent to 40 μg/mL RNA. Good-quality RNA yields A₂₆₀/A₂₈₀ of 1.8–2.0.
5. RNA is best stored long-term at –70°C in 100% ethanol. However, we have successfully stored RNA at –80°C for more than 5 yr in sterile H₂O without apparent degradation.
6. cDNA product can be stored short term in a dedicated box at –80°C, although the preparation of fresh reactions is recommended. Reagents for cDNA synthesis and PCR should be dispensed into small aliquots and stored at –20°C. Discard unused portions after use.
7. Precautions should be taken when doing PCR to minimize contamination from external sources of template DNA. These are listed as follows: keep cDNA reagents separate from PCR product; PCR reactions are to be set up in a hood or designated bench space; use only PCR-dedicated pipets for setup; wipe down pipets with 0.1 M NaOH, followed by 70% ethanol before use; always use plugged tips and sterile technique so as not to create aerosols that could contaminate other reactions; use sterile disposable plasticware for preparation of reagents and solutions and for PCR reactions; keep caps tightly closed on all

tubes not in immediate use; the cDNA should be the last component added to the PCR reaction; pipet PCR product separately, away from reaction assembly area; store PCR products in a dedicated box at -20°C ; negative controls in PCRs are imperative and all primer combinations should be covered. If there are many combinations, then it is advisable to pool a few 5' primers with a single 3' primer.

8. Touchdown PCR has been found to give the best yield of specific product in the PCR of murine HC genes.
9. The efficiency of ligation of the LC or HC genes into MCO3 is substantially reduced when incomplete digestion has occurred. To increase the efficiency of digestion of the PCR product with both restriction enzymes, a two-step buffer system was developed. In order to achieve the optimal conditions for digestion of DNA with *Xba*I, the pH and the salt concentration have to be increased. To do this, the volume of the reaction is doubled as it is for the digestion with *Xho*I/*Spe*I of the HC.
10. Note that in some murine HCs, there is an internal *Xho*I site, which results in additional, smaller products. These need to be gel-purified away from the full-length HC amplicon prior to ligation so that only complete HC genes are cloned into the library.
11. Extensive purification of the vector after digestion with *Sac*I/*Xba*I (for cloning of LC PCR product) or *Spe*I/*Xho*I (for cloning of HC PCR product into the LC library) is carried out to minimize contamination of the preparation with uncut vector, single-cut vector, or stuffer fragments. We have found it to be important that contaminating material (i.e., any form of the vector or insert that is not desirable in the final library) be removed so that only full-length PCR products are cloned into appropriately cleaved vector, thus ensuring that the final library size is correctly estimated and the library will only express full length Fab. Approximately 5 μg double-cut vector is recovered from every 20 μg MCO3 digested. For ligation of LC PCR product, 2–3 μg double-digested, purified vector is required. Similar amounts are required for ligation of the HC PCR product into the LC library.
12. The method of determining DNA concentration by spotting onto a transilluminator is used when there is insufficient DNA ($<250\text{ ng/mL}$) to assay spectrophotometrically, or there are other substances present that will interfere with UV quantification. It gives adequate concentration estimates for subsequent double digestions and ligations. Standards should contain a single species of DNA, about the same size as the expected size of the unknown DNA. DNA standards are stable for several months at -20°C .
13. A trial ligation of double-digested vector with double-digested LC or HC DNA will determine the optimal vector:insert ratio (molar ratio) for large-scale ligation of vector with insert to create the library. An optimal vector:insert ratio greater than 2:1 is generally an indication that only partial digestion of the PCR product has occurred.

14. For the large-scale ligation, incubation overnight works best rather than a few hours at room temperature. Cleanup of the large-scale ligation is essential to remove buffer, to have the ligation reaction in a smaller volume and for the DNA to be in H₂O for the electroporation.
15. A high transformation efficiency of XL1-Blue is required for the construction of Fab libraries. In order to obtain highly electrocompetent cells, work as quickly as possible during resuspension of cells, and do not leave cells on ice any longer than necessary. Cells made competent by chemical means are not of sufficiently high quality for library construction. If electrocompetent cells are not available in-house, a commercial source can be used, although the cost of these can be high.
16. An indication of library size can be obtained from the small-scale ligation by calculating colony-forming units (cfu)/ μ g DNA, which should be $>10^7$. The value for ligation of the vector alone should be $<1 \times 10^4$ cfu/ μ g, which indicates a low proportion of single-cut vector. This is essential to ensure a good library size in the large-scale ligation.
17. Library size is calculated from the test plates after large-scale ligation of LC products and after ligation of HC products into LC library as cfu/ μ L, then multiplied by the total volume (15–20 mL) of the library after electroporation and rescue. Libraries of $2\text{--}8 \times 10^7$ LC and HC are standard.
18. Generally, the LC library is stored as DNA at -20°C and as a bacterial glycerol stock from which phage can be prepared if required. The LC DNA library is then subjected to digestion, cleanup (*see Subheading 3.8.*), trial ligation (*see Subheading 3.10.*), large-scale ligation with HC (*see Subheading 3.11.*) and electroporation (*see Subheading 3.13.*) to create the final Fab library.
19. It is best to proceed with the LC library by electroporating into *E. coli*, preparing DNA, verifying that the library size is adequate, and that the inserts are full-length (*see Subheading 3.17.*) prior to cloning of the HC. Once these analyses have been carried out, the HC can be cloned into the LC library for completion of the Fab library.
20. Initial stocks of helper phage may need to be purchased, but, afterwards, stocks made in-house (*see Subheading 3.16.*) are generally of high quality and can be stored for several years at -70°C without loss of infectivity. Helper phage are stable for a few months at 4°C .
21. When titrating helper phage, it is critical that the plates be warm and dry because the top agar sets quickly. It is also important to cool the molten top agar a little after removing from the water bath because the *E. coli* will be killed if the temperature is too high.
22. For crack PCR, colonies need to be fresh (no more than 1 wk old). Do not use excess ($>30 \mu\text{L}$) cracking buffer as the ethylene diamine tetraacetic acid will inhibit the PCR. Analysis of 10 clones is usually enough to get an estimate of the quality of the library. *Bst*NI digestion provides a fingerprint of the clones, which gives an estimate of the diversity of the library. A range of banding patterns is

seen, if the clones are different. Similarity, or otherwise, of banding patterns should be scored (e.g., 3/10 same, 7/10 different profiles).

References

1. Clark, M. A., Papaioannou, A., Hawkins, N. J., Fiddes, R. J., and Ward, R. L. (1997). Isolation of human anti-c-erbB-2 Fabs from a lymph node-derived phage display library. *Clin. Exp. Immunol.* **109**, 166–174.
2. Coomber, D. W. J., Clark, M. A., Hawkins, N. J., and Ward, R. L. (1999) Generation of anti-p53 Fab fragments from individuals with colorectal cancer using phage display. *J. Immunol.* **163**, 2276–2283.
3. Ward, R. L., Clark, M. A., Lees, J., and Hawkins, N. J. (1996) Retrieval of human antibodies from phage-display libraries using enzymatic cleavage. *J. Immunol. Meth.* **189**, 73–82.
4. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.
5. Yip, Y. L., Hawkins, N. J., Clark, M. A., and Ward, R. L. (1997) Evaluation of different lymphoid tissue sources for the construction of human immunoglobulin gene libraries. *Immunotechnology* **3**, 195–203.



<http://www.springer.com/978-0-89603-906-3>

Antibody Phage Display
Methods and Protocols
O'Brien, P.M.; Aitken, R. (Eds.)
2002, XV, 401 p., Hardcover
ISBN: 978-0-89603-906-3
A product of Humana Press