

Recombinant Technology

# A two-phagemid system for the creation of non-phage displayed antibody libraries approaching one trillion members

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## Abstract

We have designed a two-phagemid system for the construction of very large non-phage displayed Fab antibody libraries in *E. coli* approaching  $10^{12}$  members. The system can accommodate both periplasmic and cytoplasmic Fab expression and should prove useful for the direct selection of functional antibodies by genetic techniques. We successfully alleviate problems of Fab vector instability and report a set of improved 5' primers for the amplification of mouse Ig V<sub>H</sub> repertoires from mouse spleen. These primers have no more than one mismatch in the last 11 bases for >95% of mouse Ig V<sub>H</sub> genes and minimize the amount of N-terminal amino acid changes while maintaining the flexibility of periplasmic or cytoplasmic antibody expression in *E. coli*. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* Antibody libraries; Combinatorial infection; Fab

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## 1. Introduction

The ability of the immune system to produce tight binding antibodies to most any compound has made them important tools in medical diagnostics and therapy, as well as in the laboratory. Similarly, antibodies have been targeted in the de novo creation of catalysts (catalytic antibodies) in which a mouse is injected with a hapten designed to elicit an antibody-binding site capable of catalysis. The immune reper-

toire is incredibly diverse (e.g.  $>5 \times 10^8$  different antibodies for a mouse); thus, one is challenged to find especially those antibodies that have the desired affinities or catalytic activities. Library sizes much greater than  $5 \times 10^8$  are desirable since the link between heavy and light chains paired in a cell cannot be maintained during library construction. Larger library sizes are desirable so that originally paired heavy and light chains have a reasonable chance of being paired in the library. Accordingly, affinities of antibodies isolated from libraries have been found to be proportional to the size of the library (Griffiths et al., 1994; Vaughan et al., 1997; Sheets et al., 1999). Thus, it is advantageous to screen the largest library size possible to better ensure isolation of rare, but potent antibodies. For the most part, the field of catalytic antibodies has not examined large libraries. It is plausible that the

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*Abbreviations:* Ap, ampicillin; bp, basepairs; cfu, colony forming units; Cm, chloramphenicol; Fab, fragment antigen binding; PCR, polymerase chain reaction; pelB, pectate lyase gene of *Erwinia carotovora*; scFv, single chain Fv fragment, V<sub>H</sub>, heavy chain variable domain; V<sub>L</sub> light chain variable domain

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modest rate enhancements of catalytic antibodies described to date (generally  $10^2$ – $10^4$  over the uncatalyzed reaction) could be ascribed to overlooking rare, but catalytically potent antibodies.

In the construction of an antibody library using a single vector, the library size is limited by the efficiency of transformation of ligated DNA into *Escherichia coli* (generally of  $10^6$ – $10^9$  library members). This has been overcome using 'combinatorial infection' in which heavy and light chain genes were cloned on separate vectors, packaged into phage particles and simultaneously infected into *E. coli* (Waterhouse et al., 1993; Geoffroy et al., 1994; Tsurushita et al., 1996). These systems utilize site-specific recombination in vivo to combine heavy and light chain genes from the two vectors onto one vector. The recombination of chains onto a single vector was necessary since phage display (Parmley and Smith, 1988) was desired and, in one case (Tsurushita et al., 1996), the antibody libraries were designed to be single chain antibody libraries (scFv). Using combinatorial infection and the *lox*-Cre site-specific recombination system from bacteriophage P1, a library of  $6.5 \times 10^{10}$  members has been created (Griffiths et al., 1994). However, several potential drawbacks to these systems exist. (i) Many laboratories have experienced difficulties with antibody display, owing primarily to the selective advantage of clones that do not have complete antibody genes, and the high rate of deletions of antibody genes packaged in phage (Krebber et al., 1997; de Bruin et al., 1999). (ii) Multiple rounds of phage panning and amplification exacerbates selection against antibodies that display inefficiently on the surface of phage. (iii) Very tight binders may not be recovered, or may be recovered at a low frequency during the elution process. (iv) For the selection of catalytic antibodies, an inherent limitation in almost all phage display techniques described to date is that selection of the catalyst is indirect, since the screen selects for those antibodies that have affinity for the hapten.

Recent progress has been made on the selection of enzymatic activity of phage-displayed proteins. However, it should be noted that turnover is not selected for in the use of suicide inhibitors (Soumillion et al., 1994; Janda et al., 1997) and systems in which the enzyme and substrate are displayed on the same phage particle (Pedersen et al., 1998; Demartis

et al., 1999) have not yet been applied to libraries and have other potential limitations (Forrer et al., 1999). Functional selection by genetic means is theoretically a preferable method and generally requires that the desired activity be essential for growth, such as reactions that an auxotrophic strain is unable to catalyze or the cleavage of a substrate designed to release a nutrient (Kast and Hilvert, 1996). However, it is feasible that techniques for coupling substrate/product concentration to a reporter system could significantly expand the number and types of reactions that could employ genetic selection (Firestine et al., 1999).

We have developed a two-phagemid system for creating very large Fab libraries (approaching  $10^{12}$  members) as a non-phage display alternative for use in the selection of antibody function by genetic means (e.g. selection of catalytic antibodies by auxotrophic complementation (Tang et al., 1991; Lesley et al., 1993; Smiley and Benkovic, 1994)). Like combinatorial infection, the two-phagemid system utilizes heavy and light chain libraries prepared on separate vectors that are packaged into phage and simultaneously infected into *E. coli*. However, since the antibody fragments are Fab and the link between an antibody and its gene is maintained within the *E. coli* cell, a site-specific recombination system is not required. Although a compatible two-vector system for soluble Fab expression has been previously described (Hoogenboom et al., 1991), this system is not suitable for simultaneous infection into *E. coli* using phage as one of the vectors is not a phagemid.

The construction and implementation of the two-phagemid system led to the identification of factors causing the apparent deletion of antibody genes including a recombination prone site in the vector backbone and problems with existing primer systems. Both problems were successfully addressed, the latter by the design of an improved set of primers for the amplification of mouse Ig V<sub>H</sub> genes.

We designed the two-phagemid system to allow for both periplasmic and cytoplasmic expression. Periplasmic expression has generally been viewed as essential for functional expression of antibodies in *E. coli* since the relatively reducing environment of the cytoplasm is not conducive for structural disulfide formation. Thus, the use of genetic means to select for functional antibodies is seemingly limited to

those processes that normally take place in the periplasm or to those reactions in which the reactants and products are free to diffuse across the inner membrane. However, recent studies evidence the feasibility of functional antibody expression in the cytoplasm. Cytoplasmic disulfide bond formation has been demonstrated for several proteins (Derman et al., 1993) including a scFv antibody (Proba et al., 1995) in *trx*B mutants of *E. coli*. In addition, functional expression of antibodies in the eukaryotic cytosol has been demonstrated (Richardson and Marasco, 1995). Furthermore, antibodies can be evolved to be stable in the reducing environment of the cytoplasm (Martineau et al., 1998; Proba et al., 1998).

## 2. Methods

### 2.1. Strains and plasmids

The following strains were used in this work: JS5=(*ara*D139,  $\Delta$ (*ara*, *leu*)7697,  $\Delta$ (*lac*) $\chi$ 74, *gal*U, *gal*K, *hsd*R2( $r_k^- m_k^-$ ), *mcr*A, *mcr*BC, *rps*L(Str<sup>r</sup>) *thi*, *rec*A1/F<sup>r</sup>::Tn10(Tc<sup>r</sup>) *pro*AB *lac*I<sup>q</sup>, *lac*Z $\Delta$ M15 (Bio-Rad, Hercules, CA), DB6507=HB101 *pyr*F74::Tn5(Km<sup>r</sup>) (ATCC#35673), SØ4038=MC4100 *pyr*E::Km<sup>r</sup> and SØ6355=MC1061 *pyr*E::Km<sup>r</sup>. An F' added to a strain name indicates the strain contains this episome: F<sup>r</sup>::Tn10(Tet<sup>r</sup>), *pro*AB, *lac*I<sup>q</sup>, *lac*Z $\Delta$ M15 which was transferred to these strains by mating with X1-1Blue using standard techniques (Provence and Curtiss, 1994).

The plasmids described below are also shown in Fig. 1. The light chain cloning region of pBP107 (Posner et al., 1993) was removed by digestion with *Eco*RI and *Xba*I. The ends were filled in with Klenow and a blunt end ligation resulted in pBPpelB.H. Vector pMOpelb.H is identical to pBPpelb.H except for the change to an *Mfe*I site at amino acid positions 3 and 4 of the heavy chain by changing AAACGT to CAATTG. Vector pMO.H has the pelB leader sequence removed and was created by oligonucleotide replacement into pMOpelB.H.

A large 2.5-kb stuffer was inserted between the *Sac*I/*Xba*I sites of pTC01 (Collet et al., 1992) to create pTC01pelB.L in order to facilitate cloning. Subsequently, a 1.2-kb segment of no described

function which contained a site prone to recombine with the *Sac*I cloning site was removed from pTC01pelB.L by digestion with *Nru*I and *Trh*1111. The ends were filled in using Klenow and a blunt end ligation resulted in pTMOpelB.L. Vector pMO.L has the pelB leader sequence removed from pMOpelB.L.

### 2.2. Cloning of antibody genes

All amplifications of light chain genes (Lc) were performed using Kappa 3' primer and Lc1–Lc7 5' primers described by Kang et al. (1991). For heavy chain (Hc) amplification initial experiments used the primer system described by Kang et al. (1991) with primers IgG1, IgG2a, IgG3 and IgG2b (5'-GAT TGT ACT AGT GGG CCC GCT GGG CTC-3') as 3' primers and Hc2–Hc9 as 5' primers. Amplification of heavy chain genes using the 5' primers described in this paper used the same IgG1, IgG2b and IgG3 3' primers with primer IgG2a(Balb/c) (5'-GGG CTT ACT AGT GGG CCC TCT GGG CTC-3') instead of IgG2a.

Extraction of total RNA from a BALB/c mouse spleen was as described (Chomczynski and Sacchi, 1987). The mRNA was purified using Oligotex-dT resin (Qiagen, Santa Clarita, CA) as per the manufacturer's directions. A cDNA copy of the mRNA was used as the template for PCR. PCR amplification using the Kang primers was performed essentially as described (Kang et al., 1991). PCR using the heavy chain primers described in this paper was performed under the following conditions: 200  $\mu$ M each dNTP, 1.5 mM MgCl<sub>2</sub>, 600 nM each primer in a 100- $\mu$ l volume of the buffer recommended by the manufacturer of the thermostable polymerase. After an initial 5-min incubation at 94°C, Taq DNA polymerase (Promega, Madison, WI) was added while the temperature was held at 85°C. Subsequently thermocycling proceeded as follows: two cycles of 94°C for 1 min, 47°C for 2 min, 72°C for 3 min followed by 28 cycles of 94°C for 1 min, 52°C for 2 min, 72°C for 2 min. The PCR products were pooled and purified by ethanol precipitation (sodium acetate) and the approximately 660 bp band was isolated by agarose gel electrophoresis using Wizard PCR Preps (Promega). The antibody PCR product was digested with *Sac*I (Lc) or *Mfe*I (Hc). After purification using Wizard

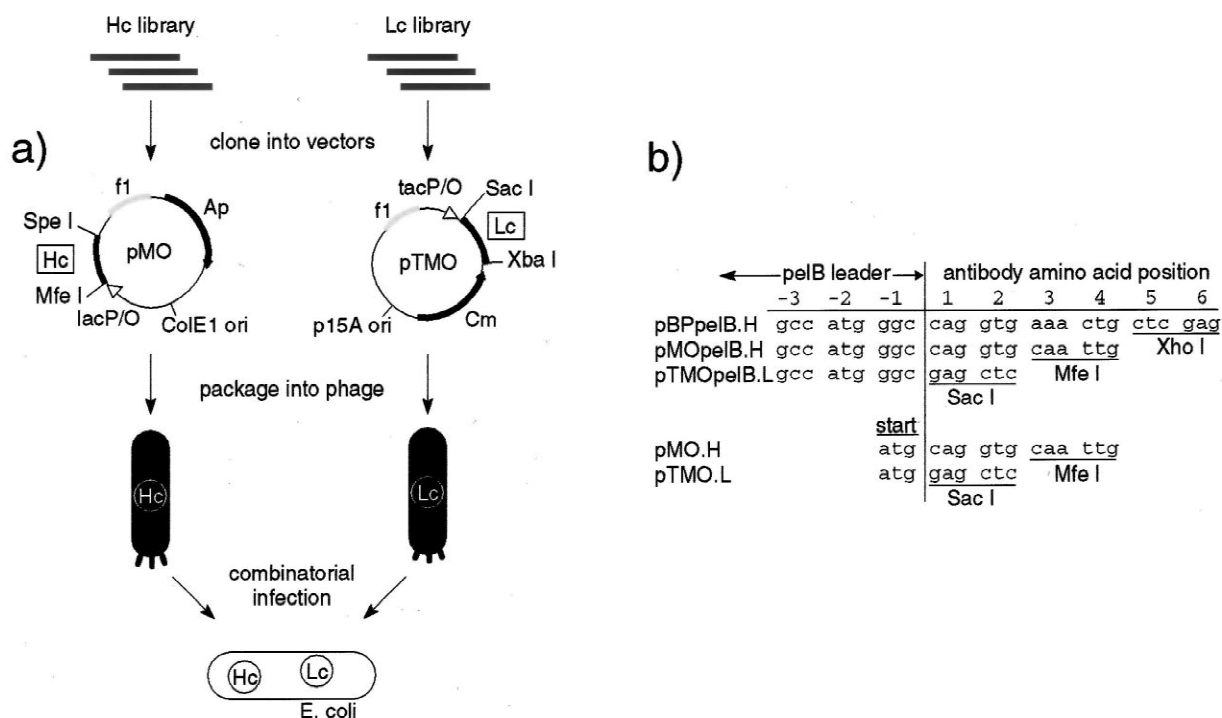


Fig. 1. (a) Schematic of two-phagemid system. Libraries of Hc and Lc genes are created by PCR on mouse spleen cDNA. The Hc gene is cloned between the *MfeI* and *SpeI* sites of phagemid pMO under the control of the lac promoter (*lacP/O*) and the Lc gene is cloned between the *SacI* and *XbaI* sites of phagemid pTMO under the control of the tac promoter (*tacP/O*). The two phagemids are compatible as they have different plasmid origins of replication (*ColE1* and *p15A*) and different antibiotic resistance genes (*Ap* and *Cm*). The phagemids also have the *f1* origin for packaging into filamentous phage. The phagemids containing the Hc or Lc libraries are packaged into phage particles with the use of helper phage. The Hc phage and Lc phage are then used to simultaneously infect a culture of the desired recipient strain. (b) Sequence at beginning of antibody gene in pMO and pTMO derived vectors. For periplasmic expression, the Hc gene is cloned into pMOpelB.H and the Lc is cloned into pTMOpelB.L, both as fusion to the pelB leader sequence. For cytoplasmic expression, the Hc gene is cloned into pMO.H and the Lc is cloned into pTMO.L, both of which contain a start codon instead of the pelB leader. Phagemid pBPpelB.H is an analogous vector to pMOpelB.H used for cloning Hc genes amplified with the primers of Sastry et al. (1989).

PCR preps the antibody genes were digested with *XbaI* (Lc) or *SpeI* (Hc). All digestions were performed for 6 h under the conditions recommended by the manufacturer (Promega) except 5× the recommended amount of enzyme was added. Immediately following each digestion, the enzymes were inactivated by incubation at 65°C for 20 min. The fully digested antibody DNA was purified using Wizard PCR preps except as noted.

The vectors were similarly digested using 2× enzyme for 2 h. After the second digest, the vectors were treated with calf intestinal alkaline phosphatase (CIAP) and the CIAP inactivated as per the manufacturer's recommendations (Promega). The desired fragment was isolated by gel electrophoresis using

QIAEX II (Qiagen). Ligations were performed using 1–2 µg digested vector, 1.5–2-fold (molar) excess antibody insert and 6 Weiss units T4 DNA ligase in a 30-µl volume at 14°C overnight. The ligation mixture was purified by ethanol precipitation (ammonium acetate), resuspended in 30 µl water, and 3-µl samples were electroporated into 40 µl JS5 electrocompetent cells (Bio-Rad) as per manufacturer's instructions. Cells were allowed to recover for 1 h in 0.5 ml SOC (Provence and Curtiss, 1994) which contained 2% glucose and subsequently plated on five 243×243 mm<sup>2</sup> Petri dishes (Nunc, Naperville, IL) with TY medium (8 g tryptone, 5 g yeast extract, and 5 g NaCl per liter) containing 2% glucose and either ampicillin (Ap; 100 µg/ml) or chloram-

phenicol (Cm; 50  $\mu\text{g/ml}$ ). Following incubation overnight at 37°C, colonies from each plate were scraped off into 20 ml 2 $\times$  TY (16 g tryptone, 10 g yeast extract and 5 g NaCl per liter) containing 15% glycerol, 2% glucose, and either Ap or Cm. Cells were spun down and resuspended in 15 ml of same media and frozen at  $-70^\circ\text{C}$  as a library stock. Phagemid were rescued from the libraries and packaged into phage essentially as described (Marks et al., 1991a) using VCSM13 helper phage.

### 2.3. Double phagemid infection of bacteria

For small libraries ( $<5\times 10^9$ ) an overnight culture of the desired strain (DB6507F', SØ4038F' or SØ6355F') was used to inoculate 25 ml 2 $\times$  TY containing 2% glucose and tetracycline (Tc; 12.5  $\mu\text{g/ml}$ ) and kanamycin (Km; 50  $\mu\text{g/ml}$ ) and grown at 37°C until  $\text{OD}_{600\text{ nm}}=0.3\text{--}0.5$ . Lc phage and Hc phage ( $6\times 10^9$  titer units each) were added and the culture incubated at 37°C without shaking for 30 min. For larger libraries ( $>5\times 10^9$ ) an altered protocol was used since Lc phage were limiting. A 100 ml exponential culture ( $\text{OD}_{600\text{ nm}}=0.1$ ) of the desired strain was infected with  $2\times 10^9$  Lc phage and the culture incubated at 37°C without shaking for 30 min. The culture was diluted ten-fold into 900 ml of the same media containing Cm (50  $\mu\text{g/ml}$ ) pre-warmed to 37°C and shaken at 37°C until the  $\text{OD}_{600\text{ nm}}=0.5\text{--}0.8$ . Hc phage ( $10^{12}$  titer units) were added and the culture incubated at 37°C without shaking for 30 min. To determine library size, a small sample was withdrawn and dilution plated in triplicate onto TY plates (to determine the total CFU), or TY plates with either Cm or Ap or both (to quantify the library size).

## 3. Results

### 3.1. Description of the two-phagemid system

In the two-phagemid system (Fig. 1a), libraries of Hc and Lc antibody genes are prepared on separate phagemids. The choice of vectors (Fig. 1b) depends on whether the antibody library is to be targeted for expression in the periplasm or the cytoplasm. In the experiments described herein, these libraries were

derived from PCR amplification of the Hc and Lc genes from cDNA of mRNA isolated from the spleen of a BALB/c mouse. These phagemids have compatible plasmid origins of replication and code for different antibiotic genes and thus can simultaneously be maintained in the same bacterium. Both phagemids also have an origin of replication for f1 filamentous phage and can thus be packaged into phage particles. Since phage infection is a very efficient means of transforming *E. coli* cells, infecting a culture with both Hc and Lc phagemid containing phage particles allows for library sizes approaching the total number of cells in the culture. For example, suppose separate phagemid libraries of  $10^6$  members each are prepared of Hc and Lc genes. If each Hc were matched with each Lc, a library of  $10^{12}$  would be created. Thus, if  $10^{11}$  titer units of phage bearing the  $10^6$  Hc library and  $10^{11}$  titer units of phage bearing the  $10^6$  Lc library are used to infect a culture of  $10^{11}$  bacteria, a library of  $10^{11}$  will have been created (assuming 100% efficiency of infection).

### 3.2. Vector stability

Initial experiments used the primer system described by Kang et al. (1991) to amplify the Lc and Hc antibody genes for cloning into pTC01pelB.L (see Methods) and pBPpelB.H (see Fig. 1a). These experiments were hampered by phagemids with truncated or entirely deleted antibodies being a significant proportion of the libraries. The double infected library only had 30% intact (i.e. correct size) Hc phagemids and  $<10\%$  intact Lc phagemids. Instability of vectors containing antibody genes has been previously noted (Krebber et al., 1997; de Bruin et al., 1999).

Since this instability seriously limits library size and diversity we sought to examine the cause. After infection of *E. coli*, greater than 90% of the pTC01pelB.L phagemids had a deletion which included the entire Lc antibody gene. Despite the fact that the library was propagated in *recA*<sup>-</sup> strains, this deletion was found to result solely from an uncharacterized recombination event between the *SacI* cloning site (... ggcggagctc ...) and a similar site 1580 bp downstream (... ccggtagctc ...). Removal of the latter recombination prone site (see Methods) to

create pTMOpelB.L eliminated Lc phagemid stability problems. Lc libraries in phagemid pTMOpelB.L had no observable stability problems when grown in 2% glucose in *recA*<sup>-</sup> strains bearing an episomal lacI<sup>q</sup> repressor.

After infection of *E. coli*, approximately 70% of the Hc phagemids had truncated antibody genes. The majority of these were not the result of deletions but were found to result from cloning of truncate antibody fragments of antibodies with an internal *XhoI* site. Hc phagemids with truncated antibody genes were a minor component of the library at first, but would gradually overtake the library. Purifying the correct size digested Hc PCR product by agarose gel electrophoresis before ligation eliminated this problem and greatly decreased the amount of deletions seen in heavy chain libraries to less than 5% of the library after infection of *E. coli*. However, a *XhoI* site which caused the cloning of truncated heavy chain genes was found to reside in the IgG2a heavy chain constant region of BALB/c mice. Since *XhoI* was the 5' cloning site of the heavy chain primers used (originally designed by Sastry et al. (1989)), all IgG2a antibodies will be cut in two and deleted from libraries derived from BALB/c mice. In addition, the IgG2a primer was found to have several mismatches to the IgG2a heavy chain gene of BALB/c mice, thus a correctly matched new primer, IgG2a(Balb/c), was designed.

### 3.3. Design of new mouse Ig V<sub>H</sub> primers

We set out to design new mouse Ig V<sub>H</sub> 5' variable primers compatible with BALB/c mice. In addition, we sought to improve upon the Sastry primers (Sastry et al., 1989) in order to amplify a more diverse library. Specifically, we (a) moved the restriction site farther away from the 3' end of the primer and towards the 5' end of the primer so that fewer antibody residues are vector defined, (b) used a restriction enzyme whose DNA target coded for more common antibody amino acids, and (c) improved matching at 3' end of primers to include a majority of mouse heavy chains in the Kabat database (Johnson et al., 1996).

Initially a V<sub>H</sub> family approach was considered (Marks et al., 1991b; Dattamajumbar et al., 1996). However, it was observed that although 13% of all

mouse Ig V<sub>H</sub> in the Kabat database had proline at position 7, none of the fifteen known mouse Ig V<sub>H</sub> gene families coded for proline at this position. Either mouse heavy chains with proline at position 7 are a variation of one of the existing families or are members of a new family altogether. At any rate, this observation pointed out a danger in designing primers solely on known Ig V<sub>H</sub> gene family sequences.

Instead we chose to define our own 'families' based on amino acid sequences in the N-terminal of mouse Ig V<sub>H</sub> in the Kabat database. By definition, these are families for the purposes of PCR amplification only; but, not too surprisingly, they have a good deal of similarity to known Ig V<sub>H</sub> gene families (Dattamajumbar et al., 1996). First, the frequency of each amino acid at positions 1–8 was determined by searching the Kabat database using Kabatman (Martin, 1996). A restriction enzyme site was sought to fulfill the following criteria: (a) the site must code for common antibody residues at those positions, (b) the site should be located as far from the 3' end of the primer as possible (and close to the beginning of the antibody gene), and (c) the site must be rarely found in mouse Hc genes. Virtually the only restriction enzyme site to meet these criteria was *MfeI*, which codes for Gln and Leu, which are found at positions 3 and 4 in 70 and 98% of all mouse Ig V<sub>H</sub>, respectively. Positions 1 and 2 were set in the vector (pMOpelB.H; Fig. 1) as Gln and Val, respectively (present in 36% of all mouse Ig V<sub>H</sub>).

Since positions 1 and 2 were set in the vector as Gln and Val and positions 3 and 4 were set as Gln and Leu by the *MfeI* cloning site, families were classified according to the amino acid sequences of positions 5–8. To define the families, any amino acid present in >0.5% of mouse Ig V<sub>H</sub> at a given position was selected and designated as 'common' at that position. Next, all combinations of these 'common' amino acids at positions 5–8 were searched for using Kabatman and the number of hits from the 1714 mouse Ig V<sub>H</sub> whose amino acid sequences were complete at positions 5–8 were recorded. Any amino acid sequence which appeared at a frequency greater than 0.5% was considered a separate family. The twelve families that emerged (Table 1) covered 96.0% of all mouse Ig V<sub>H</sub> in the Kabat database. The remaining 4.0% of mouse Ig V<sub>H</sub> in the database were rare sequences of 'common' amino acids or con-

Table 1  
Mouse Ig V<sub>H</sub> families for primer design

Family	Amino acid sequence (5–8)	% of all mouse Ig V <sub>H</sub>
1	GlnGlnSerGly	34.4
2	ValGluSerGly	19.7
3	GlnGlnProGly	13.4
4	GlnGluSerGly	8.2
5	LysGluSerGly	7.5
6	LeuGluSerGly	3.9
7	LysGlnSerGly	1.6
8	ValGlnSerGly	2.9
9	LeuGluThrGly	1.0
10	ValGluThrGly	0.6
11	GluGluSerGly	1.8
12	ValGluSerGlu	1.1
Total		96.0

tained at least one uncommon amino acid at positions 5–8. However, only two of these sequences had more than one uncommon amino acid and thus primers designed to amplify the twelve families would have a reasonable chance of amplifying these ‘uncommon’ sequences as well.

For each family a list of all possible combinations of codons were made for positions 5–8, not including the third base of the triplet of position 8. These were searched for in the Kabat database using SeqhuntII (Johnson et al., 1995). Of the 394 possibilities only 39 were found at all, 21 of which coded for 93.3% of all mouse Ig V<sub>H</sub>. Searches of the Kabat database using BLAST (Altschul et al., 1990) were used to find the most common triplets coding for positions 1 and 2 for each of the potential 21 primers. Since positions 1 and 2 are at the extreme 5′ end of the primers, mismatches there will have a minimal effect on amplification. The primer list was trimmed to 11 by eliminating those that only differed by one base in the final eleven 3′ bases and were also a good match at positions 1 and 2. Two other relatively rare sequences, which had only two mismatches to other primers, were also eliminated to give the final nine primers as shown in Table 2. The eliminated primers, which may be useful in some instances, are also shown.

### 3.4. Evaluation of the mouse Ig V<sub>H</sub> primers

The nine primers were then compared to all mouse

Ig V<sub>H</sub> sequences in the Kabat database. Outside the *MfeI* cloning site, these nine primers were an exact match for >48% of all mouse Ig V<sub>H</sub> genes and had no more than three mismatches for >91% of all mouse Ig V<sub>H</sub> genes. Since the 3′ end of the primer is most important for primer performance, we designed these primers such that there was no mismatch in the last eleven bases of the primers for >75% and no more than one mismatch for >95% of all mouse Ig V<sub>H</sub>. The thermodynamics of these nine primers were inspected using the Primer Select program of DNAs-tar and were found to have a  $T_m$  between 50.2 and 55.5°C, and no secondary structure under the conditions used in PCR. These primers, when compared to those of Sastry et al. (1989), were almost universally found to have less secondary structure (at 25°C), lower primer free energy ( $\Delta G$ ) and a larger  $\Delta G$  profile for representative sequences of each of our defined families. All nine primers were effective at amplification of mouse Ig V<sub>H</sub> genes from spleens from BALB/c mice (Fig. 2).

To substantiate the diversity of libraries constructed using our primers, 40 individual clones from two separate mouse Ig V<sub>H</sub> libraries in pMOpelB.H were selected at random and sequenced. All of the nine 5′ primers and all four of the 3′ primers were represented. No duplicate sequences were found indicating that the libraries are as diverse as can be characterized by sequencing methods. These sequences were found to be in eight of the 11 subgroups defined by Kabat et al. (1991). Three clones contained partial deletions of the heavy chain gene. Two of these sequences were found to be missing the first seven amino acids apparently due to primer moHc8 mispriming to an internal site of the antibody. However, two other moHc8 derived Hc genes were complete. These results compare quite favorably with individual clone sequencing of libraries constructed with other primers (Sastry et al., 1989; Kettleborough et al., 1993).

### 3.5. Demonstration of the two-phagemid system

Once the individual Hc and Lc libraries were constructed and their integrity and diversity was established, the phagemid libraries were packaged into filamentous phage. There is a great deal of variation in the yield of packaged phage depending

Table 2  
5' Primers for PCR amplification of mouse Hc genes

Primer name	Sequence (5'–3'; <i>Mfe</i> I site underlined)	% of all mouse Ig V <sub>H</sub> with exact match outside <i>Mfe</i> I site	% of all mouse Ig V <sub>H</sub> with exact match for last 11 bases of primer
moHc1a	gaggttcaattgcagcagtctgg	13.2	26.7
moHc1b	gaggtccaattgcaacagtctgg	3.2	4.2
moHc2a	gaggtgcaattggtggagtctgg	5.6	14.1
moHc3	caggtccaattgcagcagcctgg	12.6	12.8
moHc4a	gaggtgcaattgcaggagtcagg	2.8	5.5
moHc5a	caggtgcaattgaaggagtcagg	5.1	5.4
moHc6a	gaggtgcaattgctcagtcctgg	2.5	3.3
moHc8a	cagatccaattggtgcagtctgg	2.5	2.5
moHc9	gaagtgcaattgttgagactgg	1.0	1.0
Subtotal		48.5	75.5
<i>Not used<sup>a</sup></i>			
moHc1c	gaggtccaattgcaacaatctgg	1.7	1.7
moHc1d	gaggtccaattgcagcagtcagg	0.4	0.9
moHc2b	gaggtgcaattggtggaatctgg	4.9	4.9
moHc4b	gatgtgcaattgcaggagtcggg	1.3	1.4
moHc4c	caggtgcaattgcaggagtcctgg	0.8	1.3
moHc5b	caggttcaattgaaagagtcctgg	1.1	1.9
moHc6b <sup>b</sup>	caattgctcgagtcagg	–	0.6
moHc7	caggtgcaattgaagcagtcagg	1.3	1.3
moHc8b	cagatccaattggtacagtcctgg	0.4	0.4
moHc10	gaggtgcaattggttgagactgg	0.6	0.6
moHc11	gaagtgcaattggaggagtcctgg	1.8	1.8
moHc12	gaagtgcaattggtggagtcctga	0.9	1.0
Total		63.7	93.3

<sup>a</sup> These primers have not been synthesized nor their utility demonstrated.

<sup>b</sup> Kabat database had no sequence information for position 1 and 2 for subfamily 6b.

on the type of plasmid and the propagating strain used (Vieira and Messing, 1987). Using an established method for phagemid rescue (Marks et al., 1991a), the yield of phage bearing pMOpelB.H from JS5 cells was typical ( $\sim 10^{13}$  per liter). However, the yield of phage pTMOpelB.L from JS5 was much lower ( $< 3 \times 10^{10}$  per liter) perhaps owing to this phagemid being derived from pACYC184 (a low copy number plasmid). Still, the amount of phage was sufficient to create large libraries by first infecting  $10^9$  *E. coli* cells with the Lc phage, growing the cells in the presence of Cm until the culture had approximately  $10^{12}$  cells and then infecting with  $10^{12}$  Hc phage. For libraries of less than  $10^{10}$ , simultaneous infection with both phagemids was a more convenient methodology.

We evaluated the efficacy of the two-phagemid

system (utilizing pTMOpelB.L and pMOpelb.H) to create large antibody libraries. Individual Hc and Lc library sizes typically ranged from  $10^5$  to  $10^7$ . In one experiment, phage bearing a  $1.3 \times 10^7$  member Lc phagemid library and phage bearing a  $5.4 \times 10^6$  member Hc phagemid library were used to infect a liter of  $\sim 10^{12}$  SØ6355F' cells. After infection, the culture was found to have  $3.0 \times 10^{11}$  Ap<sup>R</sup> Cm<sup>R</sup> cfu by dilution plating. Since this number was less than the maximum library size ( $7.0 \times 10^{13}$ ) that would be achieved by matching each Hc with each Lc, the library was determined to have  $3.0 \times 10^{11}$  members. In a random sample of ten Ap<sup>R</sup> Cm<sup>R</sup> colonies from this library, all ten were found to have Lc and Hc phagemids of the correct size demonstrating the fidelity of the process and the stability of the libraries.

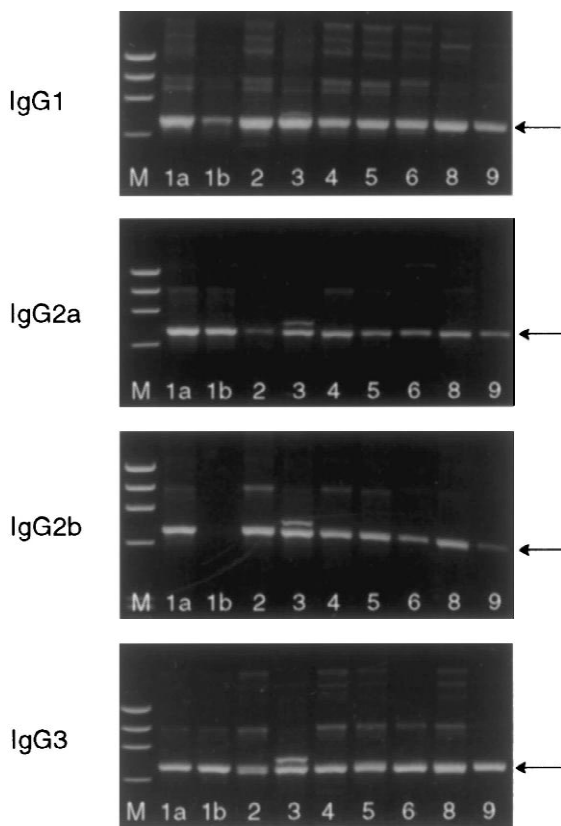


Fig. 2. PCR amplification of mouse Ig  $V_H$  genes. An aliquot of cDNA derived from the spleens of three BALB/c mice were amplified by PCR using the following primers. 5' primers: the nine primers listed in Table 2. 3' primers: IgG1, IgG2a(Balb/c), IgG2b and IgG3. The four panels correspond to the four IgG primers. The first lane of each panel (M) contained molecular weight markers (prominent bands 1353, 1078, 872, and 603 bp). The number (X) below each lane corresponds to the 5' primer used (moHcX). The major band at the arrows of approximately 650 bp is the Hc antibody gene. Conditions for PCR were as described in Methods.

#### 4. Discussion

We have developed a two-phagemid system for the creation of antibody libraries approaching  $10^{12}$  members. Our initial experiments utilized the primer system originally described by Sastry et al. (1989) for Hc amplification. This system was found to be incompatible with amplifying Hc genes from

BALB/c mice due to an internal *XhoI* site in all BALB/c mice IgG2a heavy chains. *XhoI* is the restriction enzyme used in the Sastry 5' primers, thus all IgG2a antibodies will be cut in two and lost. Upon searching for a new restriction enzyme site to integrate into the Sastry primers, it became clear that new primers could be designed which would (a) alter fewer residues at the N-terminus of Hc, (b) provide a better match at the 3' end of the primer, and (c) amplify a more diverse repertoire of mouse Ig  $V_H$ .

Numerous primer systems have been devised, but only a few are suitable for the amplification and cloning of large, diverse Fab libraries. Universal primers (LeBoeuf et al., 1989; Chaudhary et al., 1990; Dattamajumbar et al., 1996) are useful for attempting amplification of a single clone of unknown sequence but their lack of diversity impairs their ability to uniformly amplify a diverse library. To account for diversity, some systems have used degeneracy in a universal primer (Orlandi et al., 1989; Gavilondo-Cowley et al., 1990; Kettleborough et al., 1993; Dübel et al., 1994). However degeneracy may diminish amplification (Jones and Bendig, 1991), create biased libraries (Ørum et al., 1993) as well as result in PCR products which are difficult to digest with restriction enzymes if the degeneracy is near the restriction enzyme site (M. Ostermeier, unpublished observation).

Most primer systems have cloning sites within the antibody itself and hence a certain number of antibody N-terminal amino acids are fixed by the vector and cloning site. The number of 'vector fixed' amino acids depends on how close the cloning site is to the N-terminus of the antibody gene. In addition, the primers themselves may alter the amino acids of the antibody on the 3' side of the cloning site. For example, the Kettleborough (Kettleborough et al., 1993) and Sastry (Sastry et al., 1989) primer systems, if they were to amplify all mouse Ig  $V_H$  genes in the Kabat database, would alter >92.2 and 100% of the Hc antibodies, respectively. Since crystal structures show that the N-terminal of heavy chains is near the CDR3, changes in the N-terminal could significantly effect binding.

To minimize this potential problem, primer systems have been developed in which the cloning site is outside the mature antibody gene. However, we

desired a versatile primer system capable of amplifying antibody genes for cloning into either periplasmic or cytoplasmic expression phagemids. Thus, primer systems designed to anneal to natural leader sequences of mouse Ig V<sub>H</sub> (Jones and Bendig, 1991) were not feasible as they would not allow periplasmic expression without additional N-terminal amino acids. Similarly, neither the system of Zhou et al. (1994), which utilizes an introduced *Sfi*I site within the pelB leader sequence, nor systems for the creation of scFv libraries (Krebber et al., 1997) are compatible with cytoplasmic expression without additional N-terminal amino acids. In addition, the Zhou system does not avoid N-terminal changes since the N-terminal amino acids are still primer defined. Thus 45% of mouse Ig V<sub>H</sub> in the Kabat database, if amplified by the Zhou primers, will have at least one altered residue.

Our nine 5' primers, using vector pMOpelB.H, will alter 73% of all mouse Ig V<sub>H</sub> N-termini. The number of antibodies with altered residues could be minimized to only 44% by using both pMOpelB.H and a vector identical to pMOpelB.H except it would code for Glu and Val at positions 1 and 2 instead of Gln and Val. The use of two plasmids minimally increases the amount of work, since one can use a mixture of both plasmids together (which would only differ by one nucleotide) for constructing the library. Our nine 5' primers have less than three mismatches outside the *Mfe*I cloning site for >91% and only one mismatch in the last 11 bases of the primer for >95% of mouse Ig V<sub>H</sub> genes in the Kabat data base. They efficiently amplify a diverse repertoire of mouse Ig V<sub>H</sub> genes independent of the heavy chain IgG subtype.

The two-phagemid system was designed to create very large antibody libraries. The size of antibody libraries is primarily limited by the transformation efficiency of ligated vectors into *E. coli* and thus library size is typically limited to 10<sup>6</sup>–10<sup>8</sup> per µg of vector. Because phage infection is a much more efficient means to introduce vectors into cells, Griffiths et al. (1994) were able to achieve library size of 6.5×10<sup>10</sup> members through 'combinatorial infection' using a two vector plasmid/phagemid system with the Hc and Lc genes on separate vectors. Since this system is for phage display, the two antibody genes

(light and heavy) had to be combined on the same phagemid in vivo using Cre catalyzed recombination so that the antibody genes and their corresponding phage-displayed antibody product remained linked.

We have developed a non-phage display, two-phagemid system capable of equally large antibody libraries for use when phage display is not needed or desired. The two-phagemid system allows for periplasmic or cytoplasmic expression. In either case, the two phagemids and their antibody product remain within an intact cell, so there is no need for a site-specific recombination system. The library size is limited only by the volume of culture medium used. The largest library we have constructed using the two-phagemid system had 3.0×10<sup>11</sup> members. To the best of our knowledge, this is the largest antibody library described in the literature to date.

The two-phagemid system has the potential to be useful for the isolation of antigen binders as well as for our intended use, the isolation of catalytic antibodies by auxotrophic complementation. In order to examine large libraries, the two-phagemid system requires that genetic methods exist for selecting the desired antibody. This significantly limits the number of antigen-binders or reactions that can be examined. However, if progress in production of antibody fragments under non-reducing conditions (i.e. in the cytoplasm) continues, a two-hybrid system can be envisioned for the isolation of antigen binders. Additionally, it is feasible that techniques for coupling substrate/product concentration to a reporter system could significantly expand the number and types of antibody catalyzed reactions that could employ genetic selection (Firestine et al., 1999). Finally, we note that it should be possible to create similarly large bacterial cell surface displayed antibody libraries (Georgiou et al., 1997) using the two-phagemid system.

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