

# Chapter 8

## Production of Antibody Fab Fragments in *Escherichia coli*

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**Abstract** A phage-display library is the most broadly used platform for preparation of recombinant human monoclonal antibody Fab fragments. Panning is effective for the selection of immunoglobulin genes from naïve and immune libraries. However, it is possible to bypass the phage display system if human peripheral lymphocytes are obtained from seropositive patients with infectious diseases as a source of immunoglobulin genes. Direct screening of bacterial colonies producing Fab fragments by colony blotting using filter membranes is practical for the isolation of human Fab fragments to major antigens of pathogens. An oligoclonal culture can also be used, and is a partial application of Epstein-Barr virus transformation of peripheral lymphocytes. Using these procedures, neutralizing antibody Fab fragments to various antigens can be obtained with a sufficient level of cloning efficacy. Chain shuffling and site-directed mutagenesis are also useful ways to improve the quality of the cloned antibody Fab fragments.

### 8.1 Introduction

The immunoglobulin molecule is a complex structure of four polypeptide chains organized as a homodimer of a heterodimer comprising heavy and light chains. The proteolytic enzyme papain cleaves the molecules into two identical Fab (fragment, antigen binding) fragments and one Fc (fragment, crystallization) fragment, since the enzyme cuts above hinge disulfide bond(s) in the heavy chains. Thus, the Fab fragment is composed of a light chain consisting of a variable region (VL) and a constant region (CL) and the Fd region of the heavy chain containing a variable region (VH) and a constant region (CH1). The domains formed by the two variable regions bind the epitope as a specific antigen.

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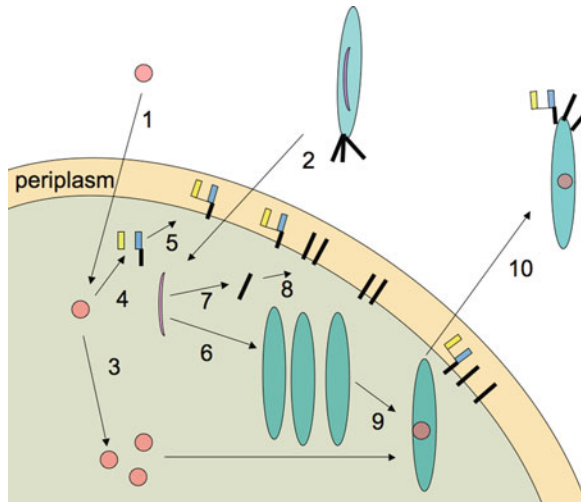
Several methods for preparation of human monoclonal and polyclonal antibodies have been developed through advances in molecular biology, with the goal of therapeutic use of antibodies (Hoet et al., 2005; Kuroiwa et al., 2002; Mendez et al., 1997; Tomizuka et al., 1997). Application of a phage display system (Smith, 1985) is an effective method for expression of a huge repertoire of immunoglobulin genes (Barbas et al., 1991; Thie et al., 2008). Using this system, valuable Fab fragments can be selected from the gene library (Barbas et al., 1992; Burton et al., 1991). The methods and protocols for phage display have been described in detail in reviews and books (Barbas et al., 2001). An antibody Fv fragment composed of only the variable regions (VH-VL), and especially a single chain Fv (scFv), can also be prepared using phage display technology (Bird et al., 1988) and is frequently used because of its better tolerance and expression in bacteria in comparison with a Fab fragment (Mondon et al., 2008). However, Fab fragments are more stable than scFv due to the association between the CH1 and CL regions.

In this chapter, we describe the production of recombinant human Fab fragments in *Escherichia coli*. In addition to phage display followed by screening using panning, a method that allows bypassing of the panning process is introduced.

## 8.2 Phage Display System

Phagemid-based systems are most commonly used among a variety of phage display systems. Phagemids contain origins for replication of *E. coli* and filamentous phage (M13, fl or fd), a group of viruses that infect *E. coli* (Mead and Kemper, 1988). A phage particle consists of a single-stranded DNA (ssDNA) genome. The phage surface has 3–5 copies of phage coat protein-3 (pIII), which is involved in host cell recognition and infection. The major coat protein is phage coat protein-8 (pVIII), which covers the length of the particle with approximately 2,700 copies that contribute to the structural stability of the phage particle (Russel, 1991). Thus, phagemid combines the characteristics of a plasmid (antibiotic resistance and facilitation of replication of double-stranded DNA (dsDNA)) with those of a phage (production and packaging of ssDNA into a phage particle). Light chain and Fd region genes are cloned into appropriate sites. Secretion signal sequences (e.g. pelB, ompA or PhoA) are coupled to both genes, which allow secretion of Fd and light chain fragments into the periplasm fraction of *E. coli*. The Fab fragment is fused to pIII or its C-terminal domain to allow display on the phage surface.

Recombinant phagemids are introduced into competent *E. coli* by electroporation (Fig. 8.1). Transformation efficiency is critical because it has a direct influence on library size; therefore, electrocompetent cells are better than normal competent cells. After transformation, *E. coli* is superinfected with helper phage (e.g. VCS M13) to give a whole phage particle. Helper phages have a slightly defective origin of replication and phagemid DNA is dominantly packaged into the phage particle. Synthesized Fd and light chain fragments are transported to the periplasm and a



**Fig. 8.1** Replication of recombinant phage. 1, Phagemid is transformed to *E. coli*. 2, Infection with helper phage. 3, Phagemid replicates. 4, Phagemid produces Fd heavy and light chains, one of which is fused to pIII coat protein. 5, Fd heavy and light chains are transferred to the periplasm and form an inter-chain disulfide bond. 6, Helper phage produces a virion package. 7, Normal pIII. 8, pIII is transferred to the periplasm. 9, Phagemid is packaged in a virion. 10, Phagemid moves out of cells with Fab and normal pIII

S-S bond forms between the fragments in the reducing environment. Fab fragments with fused pIII are displayed on the phage surface concomitant with wild-type pIII provided by helper phages. As a result, recombinant phage can infect *E. coli*. Some phagemids utilize pVIII as a Fab fusion protein. However, high valency display libraries generally lead to low affinity Fab fragments due to avidity effects.

Fab-pIII fusion proteins may be toxic to cells, resulting in gene deletion and plasmid instability. Stringent control of fusion protein expression during the propagation steps is critical. In general, *lac* promoter is used to drive gene expression in phagemid vectors; thus, accommodation of leaky expression is needed. Then glucose is added, or *lac* repressor is overexpressed to elevate metabolic activator-regulated repression. A robust terminator upstream of the *lac* promoter also reduces the background expression (Krebber et al., 1996). The *tet* promoter (tetracycline inducible promoter) can be used as an alternative approach (Zahn et al., 1999). With this promoter, gene expression is tightly repressed in the absence of an inducer such as doxycycline or tetracycline.

A phage display system connects the genotype (antibody genes packaged in phage particles) and phenotype (Fab on the surface of phage particles). Affinity selection or biopanning is then needed to isolate Fab fragments against a range of antigens.

### 8.3 Construction of Human Fab Antibody Gene Libraries

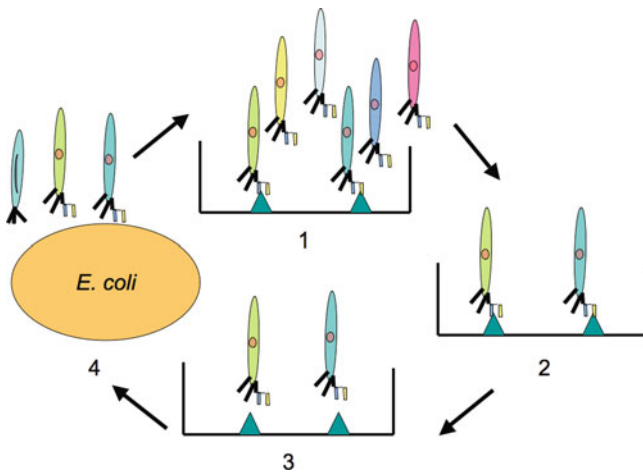
Immunoglobulin genes encoding Fab fragments can be isolated from immunized (immune library) or nonimmunized (naïve library) donors. An immune library is suitable for generation of Fab fragments for targeting with high affinity and specificity. The sources of immunoglobulin genes may be bone marrow, spleen, tonsils or peripheral blood lymphocytes (PBLs). Bone marrow is the ideal source, but is difficult to obtain. PBLs are most commonly used and one-step RT-PCR enables construction of libraries with sufficient amounts of heavy ( $\gamma$  and  $\mu$ ) and light-chain ( $\kappa$  and  $\lambda$ ) genes using pairs of Fab specific primers. A naïve library is constructed from IgM mRNA of B cells taken from a nonimmunized donor. Antibodies from naïve libraries generally show weaker affinity, but the library size is larger and the chance of obtaining specific antibodies with affinity against the target is increased (de Haard et al., 1999). A large library is constructed through combination of heavy and light chain genes cloned individually in each library.

Semi-synthetic libraries have been used to merge natural and synthetic diversity. For example, the VH region of the library has been created from semi-synthetic complementarity determining regions (CDRs) 1 and 2 with natural CDR3 from 35 donors with autoimmune diseases and 10 normal donors (Hoet et al., 2005). Synthetic libraries composed of 6 oligonucleotide-derived CDRs for heavy and light chains have diversity that is almost the same as that obtained naturally (Rothe et al., 2008; Shi et al., 2010). Affinities of up to 100 pM have been shown for Fab fragments from these libraries, and semi-synthetic and synthetic libraries are commercially available.

### 8.4 Screening Procedure by Panning

Selection of phages with Fab fragments reactive to antigen is performed by panning (Fig. 8.2). Phage particles presenting Fab fragments are incubated over an immobilized antigen of choice in ELISA plates or immunotubes (Kang et al., 1991; Marks et al., 1991). Non-binding phage are removed by washing. Phages that bind to the antigen are eluted by changing the binding conditions (e.g. pH change or competitive elution) and amplified by re-infection of *E. coli* cells. The amplified phages are then subjected to further rounds of panning with gradual increases in the frequency and intensity of the washing conditions. The panning process has a combination of positive selection (affinity and specificity) and negative selection (toxicity to bacterial cells and a tendency to delete unnecessary DNA), and 2–3 selection rounds may be preferable. To recover Fabs that are tightly bind to antigen, proteolytic enzyme digestion is used to cut a protease site between the Fab and pIII (Ward et al., 1996).

Some membrane proteins are difficult to produce in soluble form, and protein folding is critical to obtain the epitope conformation. In cells displaying antigen, protein is produced in mammalian cells and displayed on the cell surface. In some cases, antigen-negative cells are used as absorber cells to bind Fab fragments: the absorber cells work to remove sticky clones (de Kruif et al., 1995). Cell sorting



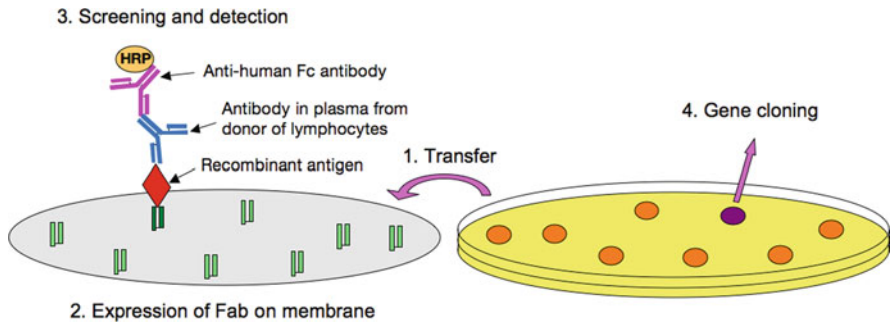
**Fig. 8.2** Panning for immobilized antigens. 1, Binding of recombinant phages with target antigen. 2, Washing off of free phages. 3, Elution of bound phages. 4, Propagation in *E. coli* and next round of panning

enables antigen-specific Fab enrichment (Siegel et al., 1997). Yeast *Saccharomyces cerevisiae* is also used as a cell display platform (van den Beucken et al., 2003), but the transformation efficiency is not as high and the Fab library size is limited. Ribosome display has been developed to expand the library size (Hanes et al., 2000; Hudson and Souriau, 2003), but the antibody is limited to a scFv, rather than a Fab fragment.

## 8.5 Bypassing Phage Display and Panning

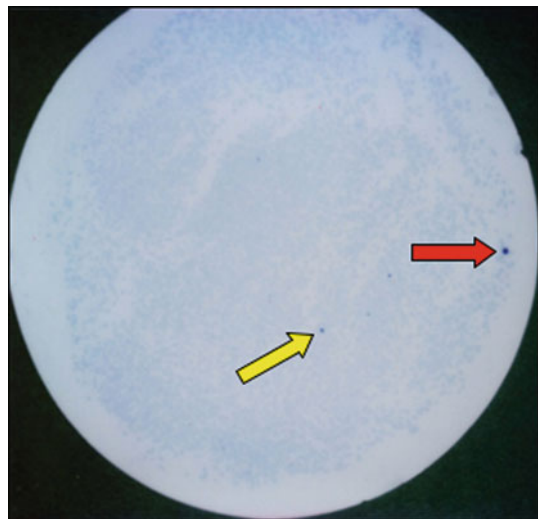
### 8.5.1 Colony Blot Screening Using Membrane Filters

Bypassing phage display and panning is possible if immunoglobulin gene libraries are constructed from PBLs of immune patients with positive serology. Colony blot screening is a relatively old approach for this purpose (Helfman et al., 1983). The principal of the method is shown in Fig. 8.3. Plasmid vector is used for preparation of combinatorial immunoglobulin gene libraries, instead of use of a phagemid vector. The size of the library is smaller than that of a phage display library, but is sufficient for isolation of Fab fragments to major antigens of pathogens. The number of bacterial colonies screened using filter membranes is also limited to less than 5,000 per 82-mm filter (Fig. 8.4). However, as shown in Table 8.1, positive rates in the first screening by colony blotting were 0.006 to 0.05% to antigens from various pathogens. Plasma recovered during the isolation process of lymphocytes from peripheral blood of the donor can be used for detection of positive clones,



**Fig. 8.3** Principle of colony blotting for selection of human Fab fragments. 1, Bacterial colonies transformed with expression vector containing light and Fd heavy chain genes are transferred to nitrocellulose membranes when the diameter of the colonies reaches 0.1–0.3 mm. 2, Filters are placed, colony side up, on the surface of fresh plates containing 1 mM IPTG and incubated for expression of Fab fragments. Then colonies are lysed. 3, Membranes are reacted serially with blocking solution, target antigen, plasma from donor, and horseradish peroxidase conjugated anti-human IgG Fc antibody and substrate. 4, Positive colonies are identified on original plates and plasmid DNA is isolated

**Fig. 8.4** Positive signals (strong, red arrow; weak, yellow arrow) on a nitrocellulose membrane in screening by colony blotting



instead of the use of monoclonal or polyclonal antibodies from immunized animals, if the donor's antibody titer for the target antigen is sufficient (Cheng et al., 2000). The antibody fraction purified from the plasma is labeled with an enzyme such as horseradish peroxidase and then used for detection. By using a secondary antibody specific for the Fc region of human IgG, direct binding of the second antibody to a human Fab fragment produced in *E. coli* is also avoided in the case of indirect detection (Liu et al., 2006). Although most of the positives in the first screening may become negative in the second and third screenings using ELISA or an indirect

**Table 8.1** Efficacy of colony blotting for screening of human Fab fragments from combinatorial immunoglobulin gene libraries derived from patients with infectious diseases

Disease in donors of lymphocytes	Target antigen	Positive rate (%)		
		First screening by colony blot	Final	References
Amebiasis (Liver abscess)	HGL of <i>Entamoeba histolytica</i>	0.054*	0.002	Cheng et al. (2000)
Amebiasis (Asymptomatic)	HGL of <i>E. histolytica</i>	0.0095*	0.0016	Tachibana et al. (2003)
SARS	Spike protein of SARS coronavirus	0.0063	0.0021	Liu et al. (2006)
Malaria	MSP1-19 of <i>Plasmodium falciparum</i>	0.0078	0.00038	Cheng et al. (2007)
Toxoplasmosis	SAG1 of <i>Toxoplasma gondii</i>	>0.005	0.00017	Fu et al. (2011)

\*Positive rate to crude antigen.

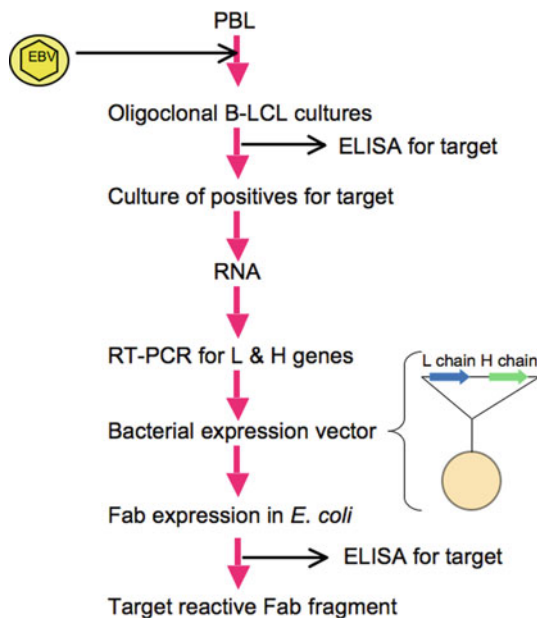
HGL, heavy subunit of galactose- and *N*-acetyl-D-galactosamine-inhibitable lectin; MSP1-19, C-terminal 19 kDa fragment of merozoite surface protein 1; SAG1, surface antigen 1.

immunofluorescein antibody test, colony blotting is a simple and effective procedure for screening for human Fabs with neutralizing activity to pathogens. Screening by colony blotting is especially suitable for relatively small libraries such as chain-shuffled libraries.

Screening of bacterial colonies using two filter membranes is also used (Skerra et al., 1991). Bacteria secreting Fab fragments into the periplasm are grown on a membrane. The secreted Fab fragments are allowed to diffuse to a second membrane coated with anti-globulin, and are probed with antigen. The binding of antigen is detected on the second membrane using enzyme or colloidal gold conjugates. Positive colonies can be grown on the first membrane. The use of a filter membrane for screening is also applicable to phage-expressed antibody libraries (Wu et al., 1998).

### 8.5.2 Oligoclonal B Lymphoblastoid Cell Culture

In this method, PBLs are collected from healthy human adult volunteers with high antibody titers to the target antigens. The PBLs are infected with Epstein-Barr virus (EBV) strain B95-8 at a dose of  $10^5$  transformation dose 50 (TD<sub>50</sub>)/mL, and plated on 96-well plates at  $10^4$  cells per well (Fig. 8.5). Half of the medium is changed every 4 days and the culture is continued for 4 weeks without any treatment, including single isolation. During the process, EBV-transformed B lymphoblastoid cell lines (B-LCL) consisting of several clones are established (Takekoshi et al., 2001). Antibody titers in the culture medium are then checked and the antibody-producing cells are expanded in a 6-cm dish. Cells and medium are harvested and total RNA is



**Fig. 8.5** Oligocloning procedure. PBLs are infected with EBV and cultured for 4 weeks without isolation. The EBV-transformed B-LCL is propagated in oligoclonal pools. RNA is extracted from positive clones. Antibody genes are amplified with RT-PCR and cloned into a bacterial expression vector. The vector is transformed into *E. coli* and antibody titers are checked by ELISA. Positive clones are selected

extracted from the cells. The immunoglobulin genes encoding the Fab fragments are amplified by RT-PCR and cloned into the bacterial expression vector. The vector is transformed into *E. coli* and each colony is picked up and incubated for expression of Fab fragments. Screening of 100 clones is generally sufficient to obtain positives in a subsequent assay of the Fab fragments by ELISA.

EBV-transformed B-LCL culture supernatants from a donor were found to be reactive with 71 target antigens (Table 8.2) (Hamatake et al., 2010). There was a tendency to produce autoantibodies, but autoantibody titers in the donor were normal. Using this method, Fab fragments against human cytomegalovirus (Takekoshi et al., 1998), hepatitis B virus s-antigen (Maeda et al., 2005), human tumor necrosis factor- $\alpha$  (Takekoshi et al., 2001), ganglioside GM1 (Nagatsuka et al., 2003), and human CD4 (Hamatake et al., 2010) have been obtained. Single isolations of EBV-transformed LCLs frequently cause loss of many clones, which may make it difficult to isolate monoclonal antibodies. However, with oligoclonal B-LCLs, immunoglobulin genes for positive clones are obtained at the semi-cloned level, and there is a low risk of loss of clones. Oligoclonal-LCLs can be stocked in a deep freezer for a long period, and genes for Fab fragments can be rescued by RT-PCR even if there is a loss of cell viability.



**Table 8.2** List of antigens recognized by EBV-transformed B-LCL culture supernatants derived from a donor

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1. Nuclear antigens
Nuclear staining, and SSB and RNP antigens
2. Viral antigens
Hepatitis B virus and human cytomegalovirus
3. Bacterial antigens
<i>Pseudomonas aeruginosa</i> (13 serotypes), <i>P. maltophilia</i> , <i>P. cepacia</i> , <i>Staphylococcus aureus</i> , <i>S. epidermidis</i> , <i>S. faecalis</i> , <i>Escherichia coli</i> , <i>Salmonella enteritidis</i> , <i>Enterobacter aerogenes</i> , <i>Proteus morgani</i> , <i>P. mirabilis</i> , <i>Klebsiella pneumoniae</i> , <i>K. ozaenae</i> , <i>Serratia marcescens</i> , <i>Shigella dysenteriae</i> , <i>Bacillus subtilis</i> , <i>B. cereus</i> , <i>Listeria monocytogenes</i> , <i>Corynebacterium diphtheriae</i> , LPS-Rc, and LPS-Re
4. Cytokines and cell surface molecules
TNF-alpha, IL-8, monocyte chemotactic protein (MCP)-1, and CD4
5. Glycolipids
CMH (GlcCer), CDH (LacCer), CTH, Globoside, Forssman, paragloboside, CPH, Gal-CMH (Galactocerebroside), CSE (Sulfatide), NAGM3, NGGM3 (including N-glycosialic acid or HD antigen), blood group i-type antigen, glycosphingomyelin 1 (GM1), gangliotetraosylceramide 1 (GA1 or asialo-GM1), and GA2
6. Human blood cells
B cells, T cells, NK cells, granulocytes, red blood cells, and 5 leukemic cell lines
7. Animal red blood cells
Sheep, rabbit, chicken, and guinea pig

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## 8.6 Expression and Purification of Fab Fragments

Fab fragments are produced in the periplasm of *E. coli*, and the expression of recombinant Fab in *E. coli* can vary significantly from antibody to antibody. Severe limitations in yield, folding and functionality are sometimes encountered in bacterial production of Fab fragments. The expression efficacy is affected by vector design and culture conditions; that is, the temperature and concentration of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) (Corisdeo and Wang, 2004; Shibui and Nagahari, 1992). Many cases of successful expression have been shown at relatively low temperature and with gentle induction at a low IPTG concentration. The amount of antibody expressed also depends on its composition. Poor Fab expression is linked to poor intrinsic stability, whereas increases in Fab stability are correlated with higher Fab yields and higher levels of properly folded and functional protein (Demarest et al., 2006). This is also affected by the growth rate of *E. coli* and secretion of Fab fragments into the medium. Limitations on protein expression may be overcome by single amino acid substitutions (Knappik and Pluckthun, 1995). Storage of fragments in periplasmic spaces requires breaking of the outer membrane of the bacteria to release the fragments (Selisko et al., 2004; Takekoshi et al., 1998).

Fab fragments can easily be purified from culture medium or an extracted fraction of bacteria if a tag such as a His-tag is added during the cloning process (Skerra, 1994; Tachibana et al., 2003). The expression level of the light chain is sometimes

higher than that of the Fd chain, and light chains also have a natural tendency to form homodimers, whereas Fd fragments do not usually dimerize. Therefore, attachment of a His-tag to the C-terminus of the Fd region is useful to avoid contamination of the purified fraction with light chain dimers. With this system, only functional heterodimers are purified. Affinity chromatography using anti-human Fab or anti-human F(ab')<sub>2</sub> antibody is also used for purification of Fab fragments (Cheng et al., 2000; Takekoshi et al., 1998).

## 8.7 Maturation of Fab Fragments

In the human immune system, affinity of antibodies is matured in a stepwise fashion by incorporation of somatic hypermutation and selection of variants under increasing selective pressures. Various *in vitro* strategies have been used to mature the affinity of recombinant Fab fragments (Hoogenboom, 2005). These include chain shuffling, CDR shuffling, and site-directed or random mutagenesis. Site-specific mutagenesis uses modifications based on structural information, whereas random mutagenesis includes use of error-prone PCR (Gram et al., 1992). Shuffling of light or heavy chain genes is effective for finding a better combination of heavy and light chains from the combinatorial library (Hur et al., 2010; Lou et al., 2010; Zhu et al., 2008). When light chain genes are shuffled, the positive rates in colony blot screening are 10- to 20-fold higher than those in shuffling analysis of heavy chain genes (Tachibana et al., 2003). If there is a loss or a partial deletion in the cloned gene, chain shuffling is also useful to find complete genes (Fu et al., 2011; Jia et al., 2008).

Residues in the CDR, and especially in the CDR3s of the heavy and light chains of the antibody, are thought to be responsible for high-affinity interactions with antigen. Therefore, an increased affinity may occur by mutation if the native residue exhibits a negative effect on the interaction. However, the effect of a mutation is not restricted to contact residues (Winkler et al., 2000). For instance, the CDR3 of the light chain comprises amino acids 89–97 in the Kabat numbering system (Johnson and Wu, 2004; Wu and Kabat, 1970). Although the residue at position 91 may not interact directly with antigenic molecules, it can affect the binding of residue 93 (Hall et al., 1992). Thus, amino acid substitution at position 91 may result in a conformational change that allows redistribution of the neighboring amino acids involved in the antigen-antibody interaction. For site-directed mutagenesis, recombination PCR with high fidelity DNA polymerase is feasible to introduce amino acid substitution and improve the affinity of the original human Fab (Jones and Winistorfer, 1997; Tachibana et al., 2004). The framework in the variable region is also important for establishing the correct conformation of the CDR loops, and mutations in the framework may affect the flexibility of the CDR loops.

It is possible to increase the affinity of antibody Fab fragments using various procedures, and the increased affinity may correlate with biological efficacy. However, if the original antibody already demonstrates high affinity above a threshold, the effect of affinity maturation on biological function may be limited (Hoogenboom, 2005; Tachibana et al., 2003).

## 8.8 Concluding Remarks

Phage display followed by panning is widely used for preparation of recombinant antibody Fab fragments from large combinatorial immunoglobulin gene libraries from naïve and immune donors. Here, we have shown that bypassing the phage display and panning processes is practical when immunoglobulin genes are derived from seropositive donors. Identification of bacterial colonies using membrane filters and donor plasma is useful for the initial screening of human Fab fragments to major antigenic molecules of pathogens. Preparation of immunoglobulin gene libraries from EB virus-transformed oligoclonal B-LCL can also be used for preparation of human Fab fragments to pathogens and self-antigens. Recently, a new technique bypassing the phage display platform has also been developed. B cells are cultured at near clonal density, culture supernatants are screened, and positives are used to clone authentic antibody genes (Walker et al., 2009). Thus, improvement of technologies for antibody engineering is still in progress to generate high-affinity human antibodies.

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