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Molecules and Cells

Successful Application of the Dual-Vector System II in Creating a Reliable Phage-Displayed Combinatorial Fab Library

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The dual-vector system-II (DVS-II), which allows efficient display of Fab antibodies on phage, has been reported previously, but its practical applicability in a phage-displayed antibody library has not been verified. To resolve this issue, we created two small combinatorial human Fab antibody libraries using the DVS-II, and isolation of target-specific antibodies was attempted. Biopanning of one antibody library, termed DVFAB-1L library, which has a 1.3×10^7 combinatorial antibody complexity, against fluorescein-BSA resulted in successful isolation of human Fab clones specific for the antigen despite the presence of only a single light chain in the library. By using the unique feature of the DVS-II, an antibody library of a larger size, named DVFAB-131L, which has a 1.5×10^9 combinatorial antibody complexity, was also generated in a rapid manner by combining 1.3×10^7 heavy chains and 131 light chains and more diverse anti-fluorescein-BSA Fab antibody clones were successfully obtained. Our results demonstrate that the DVS-II can be applied readily in creating phage-displayed antibody libraries with much less effort, and target-specific antibody clones can be isolated reliably *via* light chain promiscuity of antibody molecules.

INTRODUCTION

Phage display technology (Smith, 1988) enables us to isolate human antibodies specific to almost any antigen through the affinity-guided clonal selection of recombinant filamentous phage (Gavilondo and Larrick, 2000; Marks and Bradbury, 2004; Nissim et al., 1994). In a phage-displayed antibody library, antibody-binding domains are cloned into a phagemid vector and expressed as fusion proteins with the minor coat protein III (g3p), followed by super-infecting host *Escherichia coli* cells with helper phage to obtain recombinant phage progenies (McCafferty et al., 1996). Either single chain Fv (scFv) or Fab serves as a functionally binding antibody to be expressed as a phage-bound g3p fusion protein (Chapman et al., 1997), although it has been claimed that Fab-phage is superior for biopanning selection (Malone and Sullivan, 1996). To create a combinatorial Fab antibody library, light and heavy chain genes need to be cloned into a single phagemid vector one after another (Arakawa et al., 2007; Hoet et al., 2005). During this process, low transformation efficiency of ligated DNA into electrocompetent *E. coli* cells (Dower et al., 1988; Michelsen, 1995) frequently limits the size of an antibody library, making generation of an antibody library with more than 10^9 members challenging and painstaking (Ostermeier and Benkovic, 2000).

To circumvent this experimental difficulty, two-vector systems that allow introduction of heavy and/or light chains into a host cell through phage infection, rather than electroporation, were attempted using a phagemid-phagemid combination (Ostermeier and Benkovic, 2000) or a plasmid-phage vector combination system (Biard-Piechaczy et al., 1999; Cen et al., 2006; Geoffroy et al., 1994; Griffiths et al., 1994; Tsurushita et al., 1996; Sblattero and Bradbury, 2000; Waterhouse et al., 1993). However, library quality is difficult to control if phage progenies with non-functional antibody genes are present in the phage preparation, implying that a more straightforward and reliable methodology needs to be invented.

Previously, we developed the dual-vector system-II (DVS-II), composed of a set of pHf1g3A-2 phagemid and pLT-2 plasmid vectors, which are designed to encode heavy or light chain genes, respectively, and demonstrated that the functional Fab heterodimers are stably produced in both soluble and phage-displayed forms by the system (Joo et al., 2008). We also argued that the DVS-II system has a distinct advantage over a conventional one-phagemid vector system in building a large combinatorial Fab antibody library. In this study, we create combinatorial human Fab libraries using the DVS-II to support our argument, and we demonstrate the usefulness of our novel approach in a phage-displayed antibody library by isolating specific Fab antibodies from the libraries.

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Table 1. PCR primers used in this study

Name	Oligonucleotides
HuVH sense	5′-GCAACTGC*GGCCCAGCCGGCC*ATGGCCSAGGTGCAGCTGKTGCAGTCTGG-3′
HuJH anti-sense	5′-GGG*GGCCAATGTGGCC*GAT GAGGAGACGGTGACCAKGGTBCCTTGGCCCCA-3′
HuJH-syn anti-sense	5′-TGAGGAGACGGTGACCAKGGTBCCTTGGCCCCAAWMRDY $(SNN)_{4-8}$ GCGTGCACAG TACACGGCCGTGTC-3′
HuVLk1 sense	5′-GGG*GAGCTC*GACATCCAGWTGACCCAGTCTCC-3′
HuVLk2 sense	5′-GGG*GAGCTC*GAAATTGTGTTGACRCAGTCTCC-3′
HuVLk3 sense	5′-GGG*GAGCTC*GATATTGTGATGACYCAGTCTCC-3′
HuVLk4 sense	5′-GGG*GAGCTC*GTGTTGACGCAGTCTCCAGGCAC-3′
HuJk anti-sense	5′-CACAGT*TCTAGA*ACGTTTRATHTCCASYYKKGTCCC-3′

Restriction sites are written in italics, and degeneracy is denoted as S = G or C; K = G or T; B = G, T or C; W = A or T; M = A or C; R = G or A; D = G, A or T; Y = C or T; N = A, G, T or C; H = A, C or T.

MATERIALS AND METHODS

Molecular cloning procedure

All DNA cloning experiments were carried out according to standard procedures (Sambrook et al., 1989). Oligonucleotides of sequencing grade were synthesized by Bioneer Co., South Korea, and all PCR primers used in this study are listed in Table 1. For PCR reactions, *Pfu* or Ex-*Taq* DNA polymerase (Takara, Japan) was successfully used. All restriction endonucleases were also purchased from Takara.

Construction of a human heavy chain sublibrary

A two million human naïve Fd (V_H + C_{H1}) gene repertoire was prepared previously (IG Therapy, South Korea) and used as a PCR template to obtain V_H gene repertoires. To obtain a naïve heavy chain repertoire, HuVH sense and HuJH anti-sense primers were utilized in the PCR amplification using a thermal program of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min for 20 cycles. To obtain a semi-synthetic heavy chain repertoire, HuVH sense and HuJH-syn anti-sense primers and 157 naïve V_H backbones (IG Therapy) were utilized during the first PCR using the same thermal program as above, followed by purification of the 350 bp PCR product with a Wizard DNA Clean-Up system (Promega, USA). The second PCR was carried out with HuVH sense and HuJH antisense primers. The resultant human naïve and semi-synthetic V_H genes, and pHf1g3A-2 phagemid (Joo et al., 2008) were digested with a *Sfi*I restriction endonuclease and ligated using T4 DNA ligase (Takara) to create a heavy chain sublibrary. After extraction with phenol/chloroform and precipitation with ethanol, ligated DNA products were electroporated into *E. coli* ElectroTen Blue cells (Stratagene, USA) using a Gene Pulser (Biorad, USA) set to 2.5 kV, 25 μF and 200 Ω. The transformed cells were plated onto 2× YT plates containing 2% (w/v) glucose, 50 μg/ml ampicillin and 10 μg/ml carbenicillin (2× YT/ACG) and incubated overnight at 27°C. The *E. coli* colonies were harvested by scraping the plates with a sterile glass rod in the presence of fresh 2× YT medium. The pHf1g3A-2 phagemid DNA encoding the heavy chain antibody repertoire was purified from the cells using a Wizard plus SV minipreps kit (Promega).

Creation of combinatorial Fab antibody libraries

To construct the combinatorial Fab antibody libraries, termed DVFAB-1L and DVFAB-131L, electrocompetent TG1 cells with pLT-2 encoding a single or 131 independent human naïve light chains (IG Therapy) were transformed with 5 or 50 μg of supercoiled pHf1g3A-2 phagemid DNA from the heavy chain sublibrary using a Gene Pulser set to 2.5 kV, 25 μF and 200 Ω, as depicted in Fig. 1. Selection was performed by growing transformed *E. coli* TG1 cells in 2× YT/ACG medium containing 10 μg/ml tetracyclin (2× YT/ACGT) for 8 h at 37°C. Then, 100 ml of the culture was transferred to 500 ml of fresh 2× YT medium containing 50 μg/ml ampicillin, 10 μg/ml carbenicillin and 10 μg/ml tetracyclin (2× YT/ACT) and incubated at 37°C until an $OD_{600\ nm}$ reading reached approximately 0.5. The bacterial cell culture was then centrifuged at 3,300 × *g* for 10 min, and the cell pellet was resuspended with 500 ml of fresh 2× YT medium containing 2% glucose and Ex12 helper phage (IG Therapy), followed by a 1 h incubation at 37°C for phage rescue (Oh et al., 2007). Thereafter, the culture was centrifuged at 3,300 × *g* for 10 min, and the resulting cell pellets were resuspended in 5 L of fresh 2× YT/ACT medium supplemented with 50 μg/ml of kanamycin and 0.001% arabinose (w/v) (2× YT/ACTKA). After an overnight incubation at 27°C, the recombinant phage particles were harvested by centrifugation at 3,300 × *g* for 20 min. The phage supernatant was sterilized with 0.45 μm filters, and 40 ml aliquots were prepared for long-term storage at -80°C. Finally, phage in the 40 ml stock were precipitated using a PEG/NaCl solution and resuspended in 1 ml of sterile phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.3) before biopanning.

Panning procedure

Enrichment of recombinant phage bound to target antigens was performed as described (Joo et al., 2008). Briefly, MaxiSorb ELISA plates (Nunc, Denmark) were coated with 10 μg/ml of fluorescein-conjugated with bovine serum albumin (fluorescein-BSA) (Sigma-Aldrich) in coating buffer (0.1 M $NaHCO_3$, pH 9.6), and 10^{10} phage from the DVFAB-1L library or 10^{11} phage from the DVFAB-131L library were applied to the plates for the first round of panning. Eluted phage were mixed with fresh TG1 cells, and antibiotic selection was performed by plating the cells onto 2× YT/AC plates with an overnight incubation at 27°C. Cells were harvested by scraping the plates with a sterile glass rod in the presence of fresh 2× YT medium, and phagemid DNA was isolated using a Wizard plasmid purification kit. Subsequently, 200 μl of electrocompetent TG1 cells containing the pLT-2 plasmid encoding a single light chain were transformed with 200 ng of phagemid DNA using a Gene Pulser. The transformed cells were plated onto the 2× YT/ACT plates and incubated overnight at 27°C. The cells were then harvested from the plates, and phage rescue was performed in 100 ml 2× YT/ACTKA in the presence of Ex12 helper phage as described above. Panning was repeated three times.

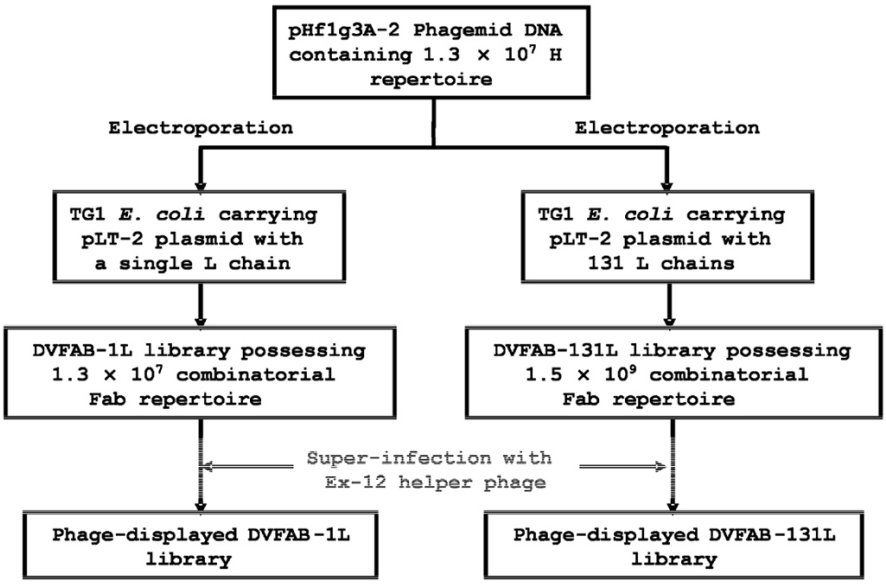

Fig. 1. Schematic diagram depicting construction of the DVFAB-1L and DVFAB-131L antibody libraries utilizing the DVS-II.

Polyclonal phage ELISA

Polyclonal phage ELISA was performed as described previously (Joo et al., 2008) by applying 5×10^7 PFU/well of recombinant phage into an ELISA plate coated with antigens such as fluorescein-BSA, biotin-BSA (Sigma-Aldrich), bovine superoxide dismutase (bSOD) (Sigma-Aldrich), recombinant glutathione-S-transferase (GST) (IG Therapy), L-glutamate dehydrogenase (L-Glu) (Sigma-Aldrich) or BSA (Sigma-Aldrich). Rat anti-M13 pAb (IG Therapy) followed by goat anti-rat IgG-HRPO (Sigma-Aldrich) was added into each well to detect phage particles bound to antigens. The binding signal was then visualized with a 3,3′,5,5′-tetramethyl benzidine (TMB) substrate (Sigma-Aldrich), and absorbance at 450 nm was measured using an ELISA reader (BioRad).

Enzyme-linked immunosorbent assay (ELISA)

Monoclonal ELISA was carried out using *E. coli* culture supernatants containing soluble Fab molecules. To prepare the culture supernatant, *E. coli* TG1 colonies obtained after the 3rd round of panning were grown in 10 ml of 2× YT/ACTG medium at 37°C until an $OD_{600\ nm}$ reading reached approximately 0.5. The bacterial cell culture was then centrifuged at 3300 × *g* for 10 min, and the cell pellet was resuspended with 5 ml of fresh 2× YT/ACT medium supplemented with 0.02% arabinose and 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). After overnight incubation at 27°C, the culture supernatant was harvested by centrifugation. MaxiSorb ELISA plates were coated with 10 μg/ml of fluorescein-BSA or five different antigens (fluorescein-BSA, biotin-BSA, bSOD, GST and BSA) diluted in coating buffer, and 50 μl of *E. coli* culture supernatant was added into each well. Goat anti-human kappa L chain antibody-HRPO (Sigma-Aldrich) was added into each well to determine the specific binding of the induced Fab molecules to the antigens.

Competitive ELISA

To measure the binding affinity of Fab clones, a competitive ELISA was performed (Lee et al., 2004). *E. coli* culture supernatants containing soluble Fab molecules were mixed with or without 10^{-5} M-10^{-12} M fluorescein diluted in 0.5% BSA (w/v) in PBS and incubated for 2 h at room temperature. The Fab and antigen mixtures were then transferred to MaxiSorb ELISA plates coated with 10 μg/ml of fluorescein-BSA, and an ELISA was performed as above. IC_{50} was calculated as the concentration of solution-phase fluorescein-BSA that inhibited 50% of the Fab molecules from binding to immobilized antigen without competitive antigens.

DNA sequencing analysis

The pHf1g3A-2 phagemid and the pLT-2 plasmid were isolated from *E. coli* cells producing Fab molecules specific for fluorescein-BSA by using a Wizard plus SV minipreps kit (Promega). Two different sequencing primers (5′-GTGCCGTTCTATAGCCATAGCAC-3′ and 5′-GGCACTGGCTGGTTTCGCTA CCGTG-3′) complementary to pHf1g3A-2 and pLT-2 were utilized to read V_H and V_L genes, respectively, and automatic DNA sequencing was performed by Solgent Co., South Korea.

RESULTS

Construction of the DVFAB-1L library and isolation of target-specific Fab antibodies

The DVFAB-1L combinatorial Fab antibody library was constructed by electroporating TG1 cells expressing a single human kappa light chain with pHg3f1A-2 phagemid DNA encoding 1.3×10^7 human heavy chains, as illustrated in Fig. 1. The light chain was arbitrarily chosen from 131 naïve kappa light chains that expressed well in *E. coli*. Since there were only 2×10^6 members of the naïve V_H repertoire available in our laboratory, degenerative CDR3H were included in naïve heavy chain backbones to build a heavy chain sublibrary with larger diversity. After constructing the DVFAB-1L library, 24 *E. coli* transformants were randomly chosen, and expression of soluble Fd-g3p fusion and kappa L chain molecules by the *E. coli* clones were determined by ELISA using culture supernatants. The result revealed that more than 80% of the *E. coli* clones (21 out of 24) produced soluble Fd-g3p fusion molecules, and all 24 *E. coli* clones produced soluble molecules (data not shown). Therefore, actual antibody diversity of the DVFAB-1L library was about 10^7.

Recombinant phage was prepared from the library by phage rescue using Ex12 helper phage, and panning against fluorescein-BSA was carried out as a model system as described

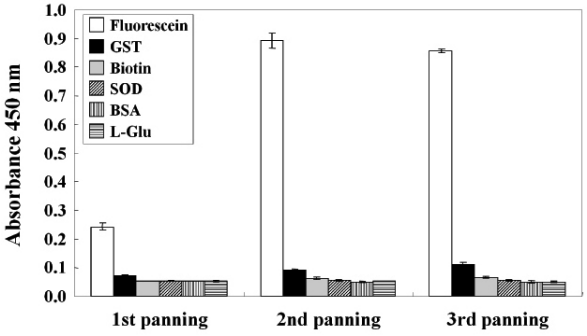

Fig. 2. Phage ELISA showing antigen-specific binding reactivity of the phage preparations obtained after each round of panning by using fluorescein-BSA as a target antigen. An identical concentration of the recombinant phages (5×10^7 PFU) was applied to each well of the microtiter plates coated with fluorescein-BSA (Fluorescein), glutathione S-transferase (GST), biotin-BSA (Biotin), bovine superoxide dismutase (bSOD), bovine serum albumin (BSA) or L-glutamate dehydrogenase (L-Glu). Anti-M13 Ab-conjugated with HRPO was used as a secondary antibody to detect phage particles bound to antigens. Binding signals were visualized with TMB substrate and analyzed at $OD_{450\ nm}$. Data represent the average of three experiments ± standard deviation.

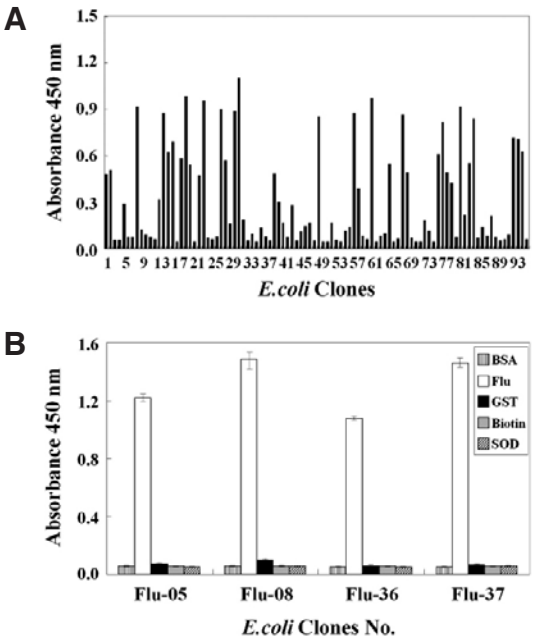

Fig. 3. Determination of antigen-binding specificity of Fab molecules in the *E. coli* culture supernatant. Culture supernatant containing soluble Fab molecules was harvested by growing TG1 cells obtained after the 3rd round of panning, and ELISA was performed to identify *E. coli* clones producing target-specific Fab molecules at the clonal level (A) and to determine cross-reactivity of anti-fluorescein-BSA Fab clones (B). The microtiter plates coated with fluorescein-BSA for (A), or five different antigens including fluorescein, GST, Biotin, bSOD, or BSA for (B). Goat anti-human kappa L Ab-conjugated with HRPO was used as a secondary antibody. Binding signals were visualized with TMB substrate, and analyzed at $OD_{450\ nm}$. Data represent the average of three experiments ± standard deviation.

in "Materials and Methods". The Ex12 helper phage was used because it provides a much higher display level of antibody fragments on phage than M13KO7 helper phage (Joo et al., 2008). Three rounds of panning were carried out. Polyclonal phage ELISA performed using phage progenies after each round of panning indicated that antigen-specific enrichment of phage was clearly undertaken even from the 2nd round of panning (Fig. 2). Monoclonal ELISA was then performed using the culture supernatants of ninety six *E. coli* colonies obtained after the last round of selection, and the results showed that approximately 60% of the *E. coli* clones produced anti-fluorescein-BSA Fab molecules at the clonal level (Fig. 3A). Among those positive clones, four Fab clones (named Flu-05, Flu-08, Flu-36 and Flu-37), which produce a high binding signal to fluorescein-BSA, were further selected. A subsequent ELISA using four additional irrelevant antigens, biotin-BSA, GST, bSOD and BSA, showed that the human Fab antibody clones did not cross-react to those antigens at all (Fig. 3B).

In addition to fluorescein-BSA, the library was also screened with four different antigens, biotin-BSA, GST, bSOD and L-Glu, and target-specific Fab antibodies were successfully isolated from the library, except for L-Glu (data not included).

Characterization of the Fab clones specific for fluorescein-BSA

Binding affinities of those four Fab clones were measured by competitive inhibitory ELISA (Fig. 4). The results showed that three clones (Flu-05, Flu-36 and Flu-37) had an IC_{50} of 5×10^{-6} M and Flu-08 had an IC_{50} of 10^{-7} M. Interestingly, DNA sequencing analysis of V_H and V_L genes revealed that Flu-05, Flu-36 and Flu-37, which had the same IC_{50} in the competitive inhibitory ELISA, were indeed identical. Therefore, V_H amino acid sequences of only two Fab clones, Flu-36 (EMBL accession no. FM160409) and Flu-08 (EMBL accession no. FM160410), are presented in Fig. 5. Both of them belong to V_H subgroup I. To find out if there was more diverse V_H gene usage, six additional anti-fluorescein-BSA Fab clones were further identified, but DNA sequencing analysis of those clones showed that they also utilized one of

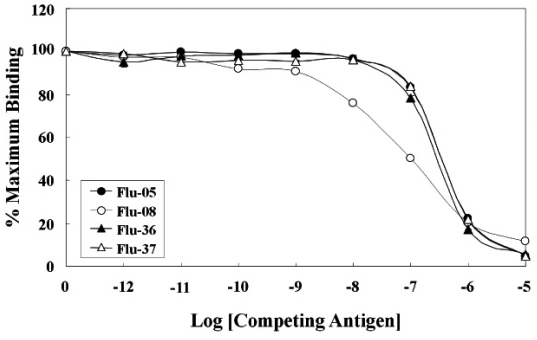

Fig. 4. Competitive inhibitory ELISA for estimating affinities of anti-fluorescein Fabs. Culture supernatants containing soluble Fabs were harvested by growing four *E. coli* clones expressing anti-fluorescein Fab, and pre-incubated with 10^{-5}-10^{-12} M fluorescein-BSA. A standard ELISA was then performed using ELISA plates coated with fluorescein-BSA. The y axis shows the ratio of the ELISA signal (A_{450}) in the presence of 10^{-5}-0 M antigens to that in the absence of solution-phase antigens. Data represent the average of three experiments ± standard deviation.

those two V_H genes (data not shown). The amino acid sequence of a single V_L kappa (EMBL accession no. FM160412) used in

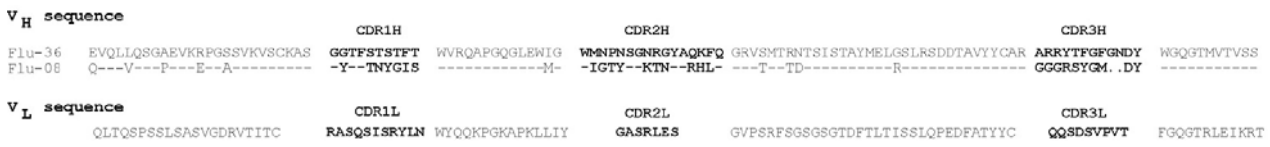

Fig. 5. Deduced amino acid sequence of anti-fluorescein-BSA Fab clones isolated from the DVFAB-1L library.

V_H CDR

Name	CDR1H	CDR2H	CDR3H	Subgroup
Flu-A	GFTFSSYSMN	SISSSSSYIYYADSVK	NLAVG..RRAP.....ANY	V_H3
Flu-B	GFTFINAWMS	SISSYSVNKYYADSVK	VWGSN..WNDRR.....LDY	V_H3
Flu-C	GYTFSRYYMH	IINPSGGSTRYAQKFQ	ARKGKIVVVTGRHLKYYYMDV	V_H1
Flu-D	GGTLSNYAVS	RIIPIVSSTKIAPKFQ	ARR....YTFG.....FSNDY	V_H1

V_L CDR

Name	CDR1L	CDR2L	CDR3L	Subgroup
Flu-A-V_L1	RASETVSSRQLA	GASSRAT	QQYG.SSP.RT	V_K4
Flu-A-V_L2	RASQSVSSNYVA	GASSRAT	QQYA.YSP.YT	V_K3
Flu-A-V_L3	RASQSISNSYLG	GASSRAT	QQYGSSPP.YT	V_K3
Flu-A-V_L4	RASQSVSS.NLA	GASTRAT	QQYNNWPP.YT	V_K3
Flu-A-V_L5	RASQNIGS.WLA	RASNLRS	QQA.TIFP.LT	V_K2
Flu-B-V_L	RASQSVRSDYLA	NAYARAT	QQYG.SSP.RT	V_K3
Flu-C-V_L	RASQSIFN.YVA	DASNRAT	QQRSKWPPTWT	V_K3
Flu-D-V_L	RASQSISS.YLN	AASSLQS	QQSYSTPP.YT	V_K2

Fig. 6. Deduced amino acid sequence of V_H and V_L genes of the anti-fluorescein-BSA Fab clones isolated from the DVFAB-131L library

the DVFAB-1L library is also included in Fig. 5.

Construction and affinity-guided selection of the DVFAB-131L library

Construction of a large antibody library was attempted by combining 131 human kappa light chains with a 1.3×10^7 complexity of heavy chains, as depicted in Fig. 1, to demonstrate a systemic advantage of the DVS-II in building a large antibody library. The resulting library, named DVFAB-131L, has a total combinatorial Fab diversity of 1.5×10^9, which is 131-fold larger than that of the DVFAB-1L library. The transformation efficiency of TG1 cells housing pLT-2 with the pHf1g3A-2 phagemid DNA was approximately $10^8/\mu g$ DNA, which was at least 100-fold higher than that of the ligated DNA (~$10^6/\mu g$ DNA) in our experimental setup (data not shown). By using 50 μg of the pHf1g3A-2 phagemid DNA, we were able to obtain 5×10^9 independent *E. coli* colonies exhibiting both amp^R and tet^R phenotypes within two days.

To determine whether anti-fluorescein-BSA Fab clones can be isolated from the DVFAB-131L library, three rounds of panning were performed against fluorescein-BSA. After a final round of panning, 96 *E. coli* clones were randomly chosen, and monoclonal ELISA was performed as described previously. The ELISA data showed that the frequency of *E. coli* clones producing soluble Fab molecules specific for fluorescein-BSA was about 4% (data not shown). Nine Fab clones that showed specific and strong binding reactivity to the antigen, termed Anti-Flu-1-9, were further selected, and DNA sequencing analysis of the V_H and V_L genes was carried out. A total of four different V_H genes [Flu-A (EMBL accession no. FM160413), Flu-B (FM160414), Flu-C (FM160415) and Flu-D (FM160416)] and eight different V_L genes [Flu-A-V_L1 (FM160417), Flu-A-V_L2 (FM160418), Flu-A-V_L3 (FM160419), Flu-A-V_L4 (FM160420), Flu-A-V_L5 (FM160421), Flu-B-V_L (*FM160422*), Flu-C-V_L (FM160423) and Flu-D-V_L (FM160424)] were identified among the clones (Fig. 6). It was found that the Anti-Flu-4 Fab clone appeared twice, and pairing of the heavy and the light chains in each anti-fluorescein-BSA Fab clone is summarized in Table 2. All eight Fab clones had a similar K_D value of approximately 10^{-6} M as determined by IC_{50} (data not shown).

Table 2. Pairing of V_H and V_L genes by anti-fluorescein-BSA Fab clones isolated from the DVFAB-131L library

Name	V_H gene	V_L gene	Frequency
Anti-Flu-1	Flu-A	Flu-A-V_L1	1/9
Anti-Flu-2	Flu-A	Flu-A-V_L2	1/9
Anti-Flu-3	Flu-A	Flu-A-V_L3	1/9
Anti-Flu-4	Flu-A	Flu-A-V_L4	2/9
Anti-Flu-5	Flu-A	Flu-A-V_L5	1/9
Anti-Flu-6	Flu-B	Flu-B-V_L	1/9
Anti-Flu-7	Flu-C	Flu-C-V_L	1/9
Anti-Flu-8	Flu-D	Flu-D-V_L	1/9

DISCUSSION

Low transformation efficiency of *E. coli* cells with ligated DNA is one technological bottleneck in creating a large antibody library, and various two-vector systems have been reported to circumvent the difficulty by utilizing highly efficient phage transduction for vector delivery into *E. coli* cells (Ostermeier and Benkovic, 2000; Sblattero and Bradbury, 2000). However, a major disadvantage of this approach was that the antibody clones having deletions of light chains, heavy chains or both are frequently observed, and the presence of these non-functional clones severely undermines the quality of a library (Hong et al., 2004; Kwon et al., 2003; Zahra et al., 1999). Our unpublished observations using the antibody clones having both functional heavy and light chains, light chain deletions, heavy chain deletions, or deletions of both, revealed that *E. coli* host cells that fail to express functional antibody fragments grow about 2-fold faster in the liquid culture and produce at least 3-5 times more non-

functional phage progenies upon helper phage super-infection compared to the host cells producing functional antibodies. Therefore, it seems critical to reduce the antibody clones that fail to express functional antibody fragments in the library repertoire as much as possible to maintain the quality of an antibody library.

Our DVS-II is different from previous two-vector systems. First, our DVS-II uses conventional electroporation, instead of infectious phage transduction, to deliver vectors encoding antibody genes into *E. coli* host cells, so that deterioration of library quality caused by phage progenies possessing non-functional antibody genes can be alleviated. Second, our DVS-II does not adapt *in vivo* recombination to combine heavy and light chains in *E. coli* host cells. Admittedly, *in vivo* recombination is a convenient way to randomly combine heavy and light chains into a single vector. However, it is impossible to recombine every heavy or light chain gene located in the donor vector into the recipient phagemid vector containing counterpart light or heavy chain genes. Thus, a large proportion of antibody clones in the antibody repertoire may fail to encode functional antibody fragments, which is not problematic in our DVS-II.

To determine whether our DVS-II is practical in a phage-displayed antibody library, we constructed a small antibody library, the DVFAB-1L, in the beginning. Only a single kappa L chain was included in the DVFAB-1L library to prevent any possible experimental complications, since phage progenies produced in the DVS-II carry only the heavy chain genotype. In this regard, previous reports showed that antigen-specific Fab clones are successfully isolated from combinatorial libraries constructed by paring a single light chain with diverse heavy chain repertoires from an immunized (Czerwinski et al., 1998; Rojas et al., 2002) or synthetic (Sidhu et al., 2004) source. In accordance with those reports, we were able to isolate anti-fluorescein-BSA Fab clones from the DVFAB-1L library. In addition, we were also able to isolate human Fab clones specific for three different target antigens including biotin-BSA, GST or bSOD (data not included in this manuscript). These results clearly demonstrate the feasibility of the DVS-II in a phage-displayed antibody library, although the antibody diversity of the DVFAB-1L library would not be large enough to be a 'single pot'. Relatively low binding affinities of the anti-fluorescein-BSA Fab antibodies were not surprising because antibodies obtained from an antibody library having a similar antibody complexity (10^7-10^8) also had comparable K_d values (Rojas et al., 2002). Nevertheless, the results that we obtained from the DVFAB-1L library were not able to demonstrate the distinctive usefulness of the DVS-II in a phage-displayed antibody library.

The experimental evidence demonstrating advantages of the DVS-II over a conventional single phagemid vector system was provided by the DVFAB-131L library. It is known that transformation efficiency is influenced by *E. coli* strains (Song et al., 1993). Among the *E. coli* strains that we have tested, the *E. coli* ElectroTen Blue strain showed the best transformation efficiency (~10^7/μg ligated DNA), though it is not suitable for phage display technology because its kan^R genotype prevents the antibiotic selection of cells that are super-infected with M13-derived helper phage. On the other hand, *E. coli* strains that are usually used in a phage-displayed antibody library, such as *E. coli* XL-1 Blue or TG1, exhibited approximately 100-fold less transformation efficiency than *E. coli* ElectroTen Blue under the same experimental conditions (our unpublished data). To take advantage of the high transformation efficiency of the *E. coli* ElectroTen Blue strain, we used this strain to construct the heavy chain sublibrary in the pHf1g3A-2 phagemid vector. Thereafter, the super-coiled pHf1g3A-2 phagemid DNA purified from the *E. coli* ElectroTen Blue cells was electrotransfected into *E. coli* TG1 cells, which already accommodated the pLT-2 plasmid encoding light chain genes. In this way, we were able to create the DVFAB-131L library with a 1.5×10^9 combinatorial antibody complexity with approximately 100-fold less effort compared to a conventional method using a single phagemid vector.

However, inclusion of more light chains in the antibody library seemed to complicate the panning procedure because the selected heavy chains are randomly reshuffled with 131 different light chains after each round of panning. We assumed that our DVS-II was still workable considering that antibody molecules exhibit a great deal of light chain promiscuity while retaining binding capacity to the antigen (Collet et al., 1992). Indeed, the frequency of anti-fluorescein-BSA Fab clones after three rounds of panning of the library was ~4%, which is only 18-fold lower than that of the DVFAB-1L library. Therefore, this result demonstrates that target-specific antibody clones can be isolated reliably from the DVFAB-131L. Since there are more diverse light chains in the combinatorial antibody repertoire of the library, we were able to obtain anti-fluorescein-BSA Fab clones utilizing more diverse heavy chains than those from the DVFAB-1L library, although not as diverse as we expected. This may be due to the fact that Fab clones having stronger light chain promiscuity are enriched more preferentially during panning, as supported by Fig. 6 and Table 2. Interestingly, none of the anti-fluorescein-BSA Fab clones from the DVFAB-131L library possessed higher binding affinities than those from the DVFAB-1L library. We might fail in isolating high affinity antibodies because too few positive antibody clones were studied, since Roche et al. reported that only 6% of the target-specific antibody clones isolated from the antibody library having 1.6×10^{10} diversity have affinities in the subnanomolar range (Rothe et al., 2008). It is also possible that antibody diversity in the DVFAB-131L library is still not large enough to contain high affinity antibody clones specific for fluorescein-BSA, although this is not within the scope of this study.

In conclusion, we demonstrate herein that the DVS-II can be applied in creating a reliable phage-displayed antibody library with much less effort than using a conventional one-phagemid system, and target-specific antibodies can be isolated stably by taking advantage of the light chain promiscuity of antibody molecules. It is also worthy to mention that the DVS-II has a potential for humanizing non-human antibodies through guided-selection or chain shuffling, since once a human heavy chain sublibrary of high quality is constructed in the pHg3f1A-2 phagemid, this sublibrary can be shuffled with any specific non-human light chains in the pLT-2 plasmid to obtain specific human heavy chain counterparts without building chimeric antibody libraries for every occasion.

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