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Original Article

Four-way ligation for construction of a mammalian cell-based full-length antibody display library

Ivan Zhou[1†], Zhe-Huan Zhang[2†], Chang-Zheng Li[3], Zhen-Rui Chen[2], Wei He[1], Ye Zhou[3], Shuwen Liu[1], Shuguang Wu[1], Yuanping Zhou[2], Wanlong Tan[2*], Shibo Jiang[1,4*], and Chen Zhou[1,5*]

[1]Antiviral Research Center, School of Pharmaceutical Science, Southern Medical University, Guangzhou 510515, China
[2]Nanfang Hospital, Southern Medical University, Guangzhou 510515, China
[3]Institute of Genetic Engineering, Southern Medical University, Guangzhou 510515, China
[4]Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY 10065, USA
[5]Dgen Biotech Limited, Hong Kong, China
[†]These authors contributed equally to this work.
*Correspondence address. Tel: +86-20-61648655 (C.Z.)/+1-212-570-3058 (S.J.)/+86-20-61647938 (W.T.); Fax: +86-20-61648655 (C.Z.)/+1-212-570-3099 (S.J.)/+86-20-61641763 (W.T.); E-mail: zyujun@yahoo.com (C.Z.)/sjiang@nybloodcenter.org (S.J.)/tanwanlong@gmail.com (W.T.)

A unique four-way ligation strategy was developed for rapid construction of a full-length antibody library. A mammalian expression vector was constructed that contained dual mammalian expression cassettes and sequences recognized by the unique restriction enzymes *Bsm*BI, *Bst*XI, and *Sfi*I. Both full-length light-chain and variable domain of heavy-chain genes were inserted into the vector in one step by four-way ligation, and full-length bivalent antibodies were displayed on mammalian cell surfaces. Using this strategy, only 2 weeks were required to successfully construct high-quality, full-length human antibody libraries.

Keywords four-way ligation; library construction; antibody library; antibody display; mammalian display

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Introduction

For >10 years, scientists have tried to develop mammalian display technologies [1–3]. By fusing a trans-membrane domain (TM) at the 3′-end of the heavy-chain (HC) or single-chain Fv antibody fragments (scFv), the full-length antibody [1,2] or scFv [3] can be displayed on mammalian cell surfaces. Unlikely to display scFv, both HC and light chain (LC) need to be expressed in a single cell to display a full-length antibody. This can be achieved by delivering both HC and LC expression vectors into cells by co-transfection or by delivering a vector with dual-expression cassette.

To construct the dual-expression cassette vector for expression of full-length antibody, the first step requires the insertion of both HC and LC into the vector. Currently, this can only be achieved by two separate insertions into the single dual-expression vector [1,2,4]. However, this approach is labor intensive and time consuming, and an ideal strategy would be with only one ligation to insert both HC and LC into one dual-expression vector.

We successfully constructed a universal single-expression cassette vector utilizing the restriction enzymes *Bst*XI, *Bsm*BI, and *Sfi*I, which could recognize and cleave unique non-palindromic sequences to prevent self-ligation, thus demonstrating the high insertion efficiency of either HC or LC [5,6]. Because the enzymes recognize non-palindromic sequences, they facilitate directional cloning and allow the ligation of multiple fragments in one reaction. By using a 1:1 molecular ratio of vector and insert without further optimization, the ligation and transformation efficiency of this single-expression cassette vector could easily reach 10^6 clones/μg of ligated DNA. However, only single HC or LC could be inserted in this vector. By chance, we also demonstrated that four fragments created by *Bst*XI digestion could be ligated together in the correct direction in a single reaction with high efficiency [5]. This observation prompted us to design a strategy utilizing two or more different enzymes in a vector that contains dual mammalian expression cassettes for the one-step insertion of both HC, or variable domain of HC (Vh), and LC genes through four-way ligation, which allowed for the efficient construction of full-length antibody expression libraries.

Therefore, here we report a strategy to design and construct a unique vector that contains dual-expression cassettes for simultaneous insertion of both Vh (or HC) and LC genes into the vector by a single four-way ligation with

an efficiency of 10^6 clones/μg of ligated DNA. Using this strategy, we constructed two high-quality, full-length antibody mammalian display libraries of 10^5 in size, and full-length antibody was expressed on mammalian cell surface with high efficiency.

Materials and Methods

Reagents and cell lines

Restriction enzymes and T4 DNA ligase were purchased from Fermentas (Hanover, USA). Antibody reagents were purchased from BD Pharmingen (San Diego, USA). Primers were synthesized at Stanford University (Palo Alto, USA). Ready-to-use *Taq* DNA polymerase (2× Master Mix) was purchased from Promega (San Luis Obispo, USA). DH5α competent cells were purchased from TaKaRa (Dalian, China). Flp-in system, including vector pcDNA^{TM} 5/FRT, vector pOG44, Flp-in Chinese hamster ovary (FCHO) cell line, and related cell maintenance media, was purchased from Invitrogen (Carlsbad, USA). 293-T cells (ATCC, Manassas, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Miniprep, maxiprep, gel extraction, and polymerase chain reaction clean-up

All experiments were performed using the kits purchased from Axygen (Union City, USA), according to the manufacturer's guidelines.

DNA digestion and fragment purification

Vector DNA was isolated from overnight *Escherichia coli* bacterial cultures. After digestion with proper restriction enzymes, DNA fragments were separated through electrophoresis in 1% agarose gel. The target fragments were then isolated as described previously [7].

Polymerase chain reaction

Polymerase chain reaction (PCR) was carried out in a total volume of 50 μl containing 200 nM of forward primer and reverse primer, 50 ng of template vector containing HC, LC, or frame sequences [5], and 25 μl of 2× Master Mix. Amplification conditions were as follows: 94°C for 5 min to denature the template, then 30 cycles of 30 s at 94°C, 30 s at 55°C, and extension at 72°C for 1 min per 1 kb length of DNA to be amplified, ending with 7 min of extension at 72°C. The PCR products were electrophoresed on a 1% agarose gel and purified. The purified fragments were then digested by proper restriction enzymes according to experimental needs, purified by a PCR clean-up kit, and used for ligation.

Vector ligation and transformation

About 50–100 ng of total vector and insert fragments were mixed in a total volume of 10 μl with one unit of T4 DNA ligase. After ligation for at least 2 h, 1 μl of ligation mixture was used in transformation with 50 μl of competent cells following the manufacturer's guidelines. The proper amount of bacteria was plated on LB-ampicillin plate and cultured at 37°C overnight. The colonies were counted and the transformation efficiencies were calculated.

Transfection

Transfection was performed with either 293-T cells or FCHO cells according to experimental needs. Typically, transfection was performed in a 12-well plate, unless otherwise stated. The day before transfection, 4×10^5 cells were seeded in each well for transfection. DNA (2 μg) and the transfection reagent (5 μg; Dgen Biotech Ltd, Hong Kong, China) were separately diluted in 100 μl of DMEM each and then mixed. The mixture was incubated at room temperature for 30 min and directly added into each well without changing the culture medium. After 6 h of incubation at 37°C, the medium was changed with fresh culture medium. The antibody expression was then analyzed by fluorescence-activated cell sorting (FACS) at 48–72 h post-transfection.

FACS analysis of antibody expression on cell surface

Cells were dissociated by cell dissociation buffer (Invitrogen), followed by one wash with staining buffer (2% FBS in phosphate buffer saline). Cells (5×10^5) were stained by proper fluorescence-labeled anti-human IgG and/or anti-human Kappa chain antibodies in 50 μl of total volume at 4°C. Cells were washed and resuspended in 400 μl of staining buffer. The antibody expression was then analyzed by FACS.

Results

Design, construction, and analysis of a one-step dual-expression cassette vector

The universal single-expression cassette vector utilizes restriction enzymes *Bst*XI, *Bsm*BI, and *Sfi*I for the cloning of HC, LC, or Vh into the vector, respectively, with high efficiency [5,6]. Because the enzymes recognize non-palindromic sequences, the cleavage of these enzymes facilitates directional cloning and allows for multiple fragments to be ligated in one reaction, as demonstrated by *Bst*XI digestion [5]. Based on the structure of the universal single-expression cassette vector, we designed a new vector, pDGB4, for the simultaneous insertion of both HC (or Vh) and LC through four-way ligation as illustrated in **Fig. 1(A)**.

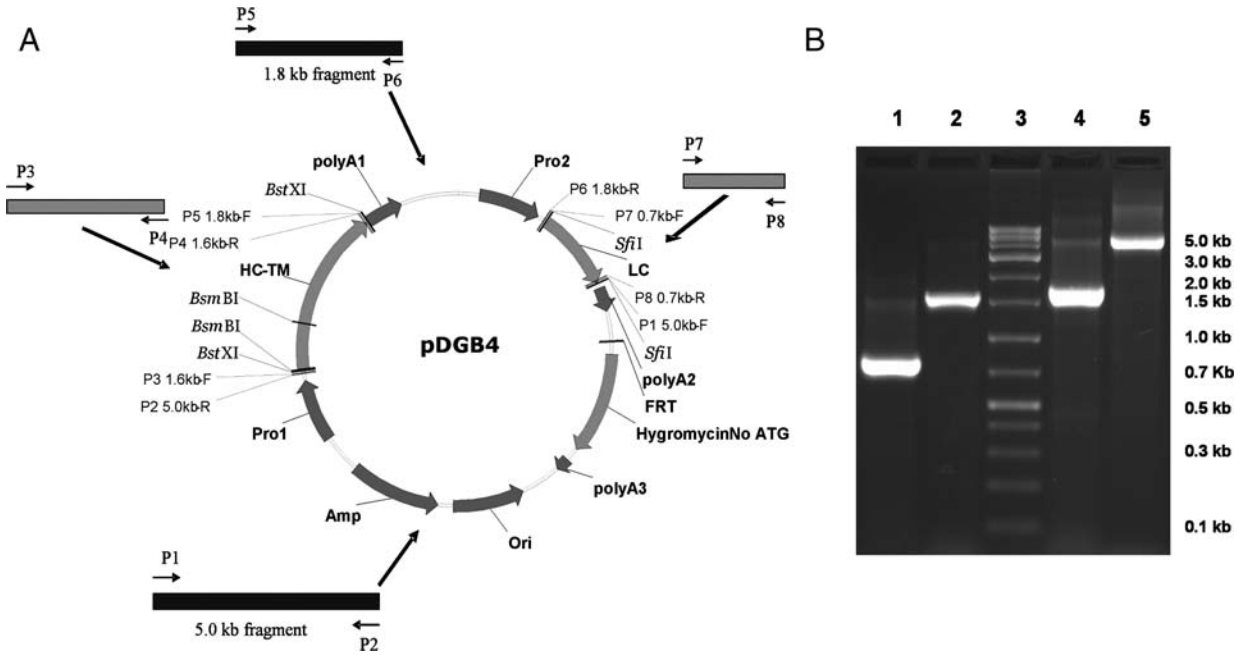

Figure 1 Schematic illustration of design and construction of dual-expression vector and analysis of PCR fragments (A) Vector design. Four fragments were PCR-amplified and ligated together by four-way ligation to form the vector pDGB4. (B) 1, 0.7 kb LC; 2, 1.6 kb HC- and TM-domain fusion fragment (HC–TM); 3, DNA marker; 4, 1.8 kb frame fragment; 5, 5.0 kb frame fragment. After PCR amplification, four fragments were digested by proper restriction enzymes, analyzed by electrophoresis, and purified for use in ligation.

Table 1 Primers used in the construction of pDGB4

Primer number	Primer name	Primer sequence (5′→3′)
P1	5.0 kb—F	AGTCTTGATAA***GGCCTGCACGGCC***CTCGAGTCTAGAGGGCCCG (*Sfi*I)
P2	5.0 kb–R	ACTGAGACG***CCAATCAGGTGG***CTAGCCAGCTTGGGTCTCC (*Bst*XI)
P3	1.6 kb–F	AAGCTGGCTAG***CCACCTGATTGGCGTCTCTAGT***CCACCATGGAC (*Bst*XI and *Bsm*BI)
P4	1.6 kb–R	TTCCCATGGT***CCAGAGTCATGG***TTATCAACGTGGCTTCTTCTGCC (*Bst*XI)
P5	1.8 kb–F	ACGTTGATAA***CCATGACTCTGG***ACCATGGGAAATGTCAGAGTGGAG (*Bst*XI)
P6	1.8 kb–R	TGGTGGTTCA***GGCCTATGTGGCC***TAGCCAGCTTGGGTCTCCC (*Sfi*I)
P7	0.7 kb–F	AAGCTGGCTA***GGCCACATAGGCC***TGAACCACCATGGTGTTGCAG (*Sfi*I)
P8	0.7 kb–R	TAGACTCGAG***GGCCGTGCAGGCC***TTATCAAGACTCTCCCCTGTTG (*Sfi*I)

The restriction enzyme sequence is shown as italic and bold.

To construct the vector, eight primers (**Table 1**) were designed and synthesized to introduce the proper restriction enzyme-recognizing sequences into the vector at the desired location and to generate four fragments after PCR amplification: P1 and P2 for the 5.0 kb frame fragment; P3 and P4 for the 1.6 kb HC–TM (HC fused to TM domain) fragment; P5 and P6 for the 1.8 kb frame fragment; and P7 and P8 for the 0.7 kb LC fragment. Electrophoresis analysis confirmed their molecular sizes [**Fig. 1(B)**].

After digestion of the fragments with proper enzymes and purification, four fragments were mixed in a molar ratio of 1:1:1:1 with a total amount of 91 ng in 10 μl of total ligation mixture. One microliter of ligation mixture was then used in the transformation. Four colonies (clones #65–68) were selected for analysis using the enzymes *Bsm*BI and *Sfi*I, and all four clones showed fragments of the correct size [**Fig. 2(A)**]. To confirm that the constructed vector contained all of the desired restriction enzyme-cutting sites and could express both HC and LC, clone #65 was chosen for further analysis.

Maxiprep DNA of clone #65 was digested in five different ways [**Fig. 2(B)**]. Results showed that all five different digestions gave the expected fragments with correct sizes, suggesting that the vector contained all the designed enzyme-cutting sites at the correct locations. However, since the fragments were from PCR amplification, and the expression was not guaranteed, to confirm that the HC and LC contained in the vector could be expressed appropriately, DNA was transiently transfected into 293-T cells, and the expression of both HC and LC was analyzed by

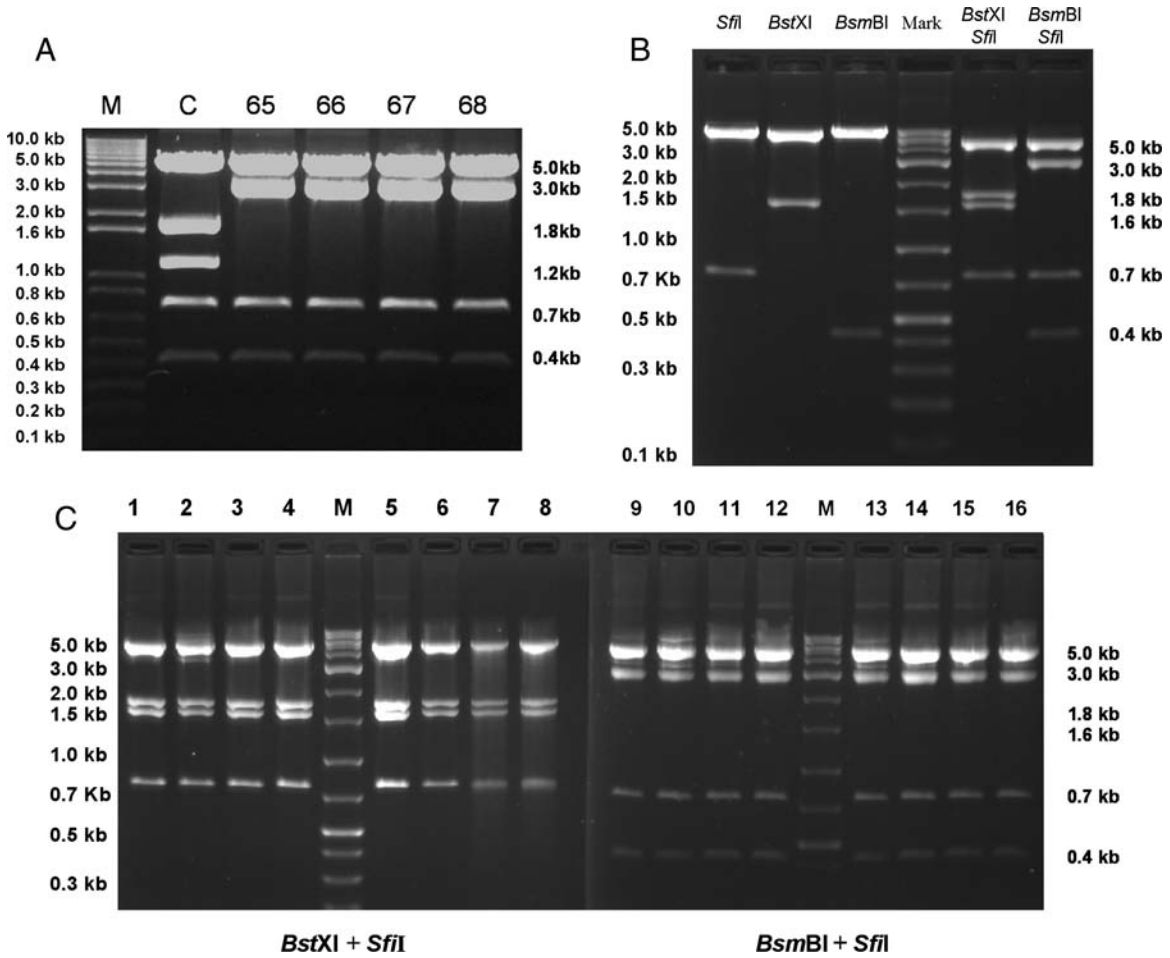

Figure 2 Restriction enzyme digestion analysis of vector pDGB4 and library clones The sizes of DNA markers are labeled on the left side of each panel and the sizes of fragments are labeled on the right side of each panel. (A) Four clones (#65–68) from four-way ligation were digested by *Bsm*BI plus *Sfi*I and electrophoresis was analyzed in 1% agarose gel. M, ladder; C, control vector with fragments of known size. (B) Purified plasmid DNA of clone #65 was digested by proper restriction enzymes as marked on the top of each lane and electrophoresis analyzed in 1% agarose gel. (C) Plasmid DNAs of 16 clones were isolated from overnight bacteria cultures. About 0.5 μg of each clone was digested by proper restriction enzymes and analyzed by electrophoresis. M, marker; clones #1–8 from the *Bst*XI + *Sfi*I group; clones #9–16 from the *Bsm*BI + *Sfi*I group.

FACS (**Fig. 3**). Compared with the control [**Fig. 3(A–C)**], both HC and LC could be separately [**Fig. 3(D,E)**] and simultaneously [**Fig. 3(F)**] detected. These results demonstrate that our vector pDGB4 can successfully express full-length antibodies on the cell surface.

Four-way ligation efficiency analysis

The purpose of this vector construction is to facilitate the simultaneous and efficient cloning of HCs and LCs. To analyze the efficiency of the ligation and transformation, the vector DNA of clone #65 was digested by *Bst*XI + *Sfi*I [**Fig. 2(B)**, lane 5] and *Bsm*BI + *Sfi*I [**Fig. 2(B)**, lane 6], and the fragments were individually purified after electrophoresis separation. Two four-way ligations and transformations (*Bsm*BI + *Sfi*I and *Bst*XI + *Sfi*I) were carried out, and ligation and transformation efficiency was calculated as described in Materials and Methods. The results are shown in **Table 2**.

To confirm that the clones used to calculate the efficiency actually contained the right vectors, 16 colonies were selected (1–8 from the *Bst*XI + *Sfi*I group and 9–16 from the *Bsm*BI + *Sfi*I group) for analysis. Plasmid DNAs of the 16 clones were digested by proper enzymes. Results showed that all 16 clones contained correct fragments [**Fig. 2(C)**], further supporting the significantly high efficiency of our four-way ligation. Results also showed that the vector-only backgrounds were in the range of 0.9–2.7%, demonstrating that the four-way ligation results in both high quantity and high quality (**Table 2**).

Construction of mammalian display antibody libraries by four-way ligation

To mimic the real conditions in pharmaceutical library construction, two pairs of PCR-amplified human kappa chain library (*Sfi*I digested) and human HC variable domain library (*Bsm*BI digested) were used in the four-way ligation

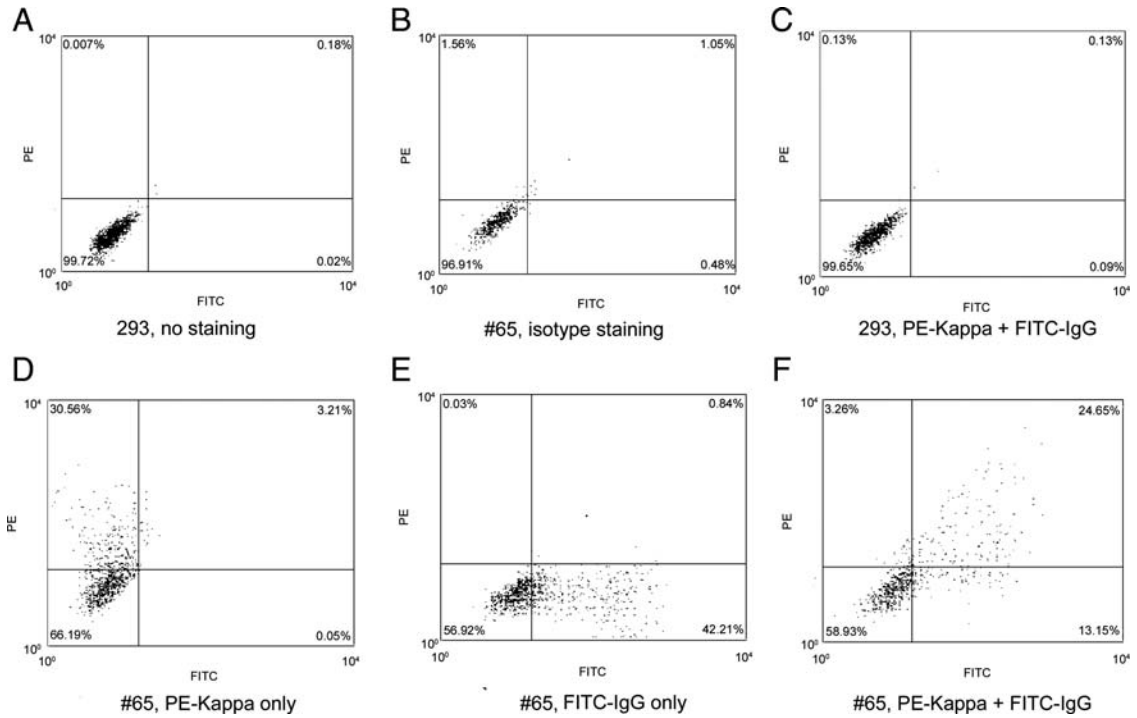

Figure 3 Antibody expression on cell surface 293-T cells transiently transfected by plasmid DNA of clone #65 were stained by fluorescein isothiocyanate (FITC)-conjugated mouse anti-human IgG (FITC-IgG) or/and phycoerythrin (PE)-conjugated mouse anti-human kappa chain antibodies (PE-kappa) and then FACS analyzed. *Y*-axis indicates the signal of PE fluorescence and *X*-axis indicates the signal of FITC fluorescence.

Table 2 Cloning efficiency of four-way ligation

Enzymes used in digestion	Number of colonies		Vector only (%)	Colonies/μg of DNA
	Vector + insert	Vector only		
*Bsm*BI + *Sfi*I	233	16	2.70	259,000
*Bst*XI + *Sfi*I	754	17	0.90	838,000

The final volume of transformation mixture is 500 μl, and 50 μl was plated for later colony analysis. The transformation efficiency of positive control pUC19 is 107,000/ng DNA. The negative control is DH5α competent cells without vector transformation, and the colony number is 0. One microliter of ligation mixture (9.1 ng of total DNA) was used for each transformation.

to replace the LC gene (0.7 kb *Sfi*I-fragment) between two *Sfi*I sites and the HC variable domain (0.4 kb *Bsm*BI-fragment) between two *Bsm*BI sites in the vector clone #65. Two libraries were constructed with sizes in the range of 10^5. The ligation and transformation efficiency is in the range of 10^6 clones/μg of ligated DNA with a background of $<0.5\%$ (**Table 3**). Typically, a background of $<10\%$ is acceptable [8]. Our results showed a significant improvement in antibody library construction when compared with the quality and efficiency of past ligation methods.

To analyze the expressibility of the libraries constructed, the plasmid DNA was isolated from 20 colonies randomly picked from the libraries (10 colonies from each library) and transiently transfected into FCHO cells. The expression of antibodies was analyzed by FACS. Compared with negative control, 8 out of 10 clones from library 1 (clones #2–5, 7–10) and 6 out of 10 clones from library 2 (clones #11, 12, 15, 16, 19, and 20) showed detectable antibody expression on the cell surface (**Fig. 4**). The results demonstrated that the strategy reported here was very powerful not only in regular cloning but also in construction of full-length antibody display libraries with very low background.

Discussion

The future of antibody library construction and pharmaceutical applications of antibodies depends largely on screening and selecting platforms. Although past display technologies have been effectively used in a wide variety of applications [9–13], the quantity and quality of the libraries

Table 3 Efficiency of library construction

Library and enzymes #	Number of colonies per μl of ligation mixture		Vector only (%)	Colonies/μg of DNA	Library size
	Vector and insert	Vector only			
Library 1 (*Bsm*BI + *Sfi*I)	13,500	54	0.40	1,483,516	2.20×10^5
Library 2 (*Bsm*BI + *Sfi*I)	11,040	54	0.49	1,213,187	1.45×10^5

One microliter of ligation mixture (9.1 ng of total DNA) was used for each transformation into top-10 competent cells.

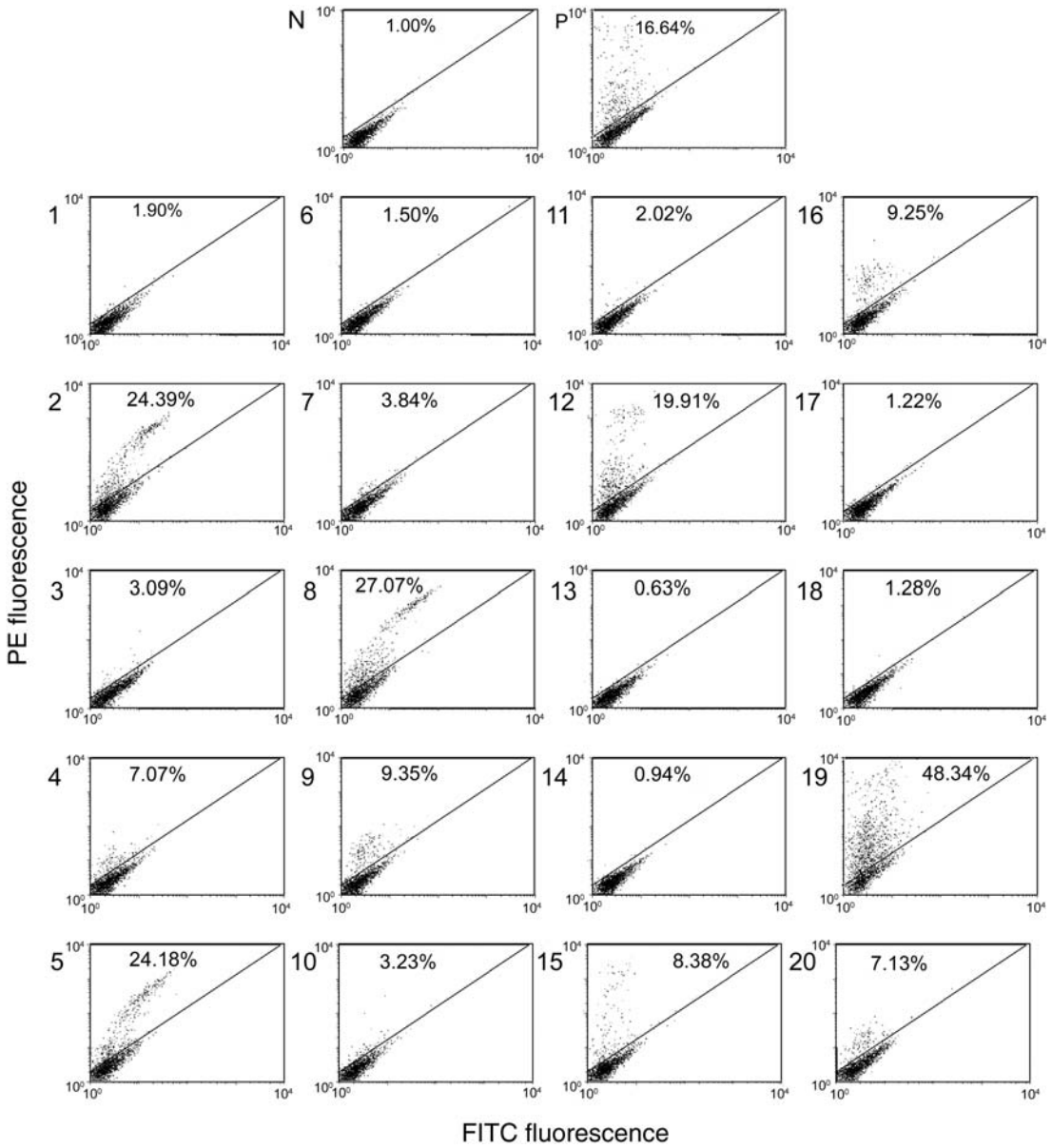

Figure 4 FACS analysis of antibody expression of clones from four-way ligation Plasmid DNAs of 20 library clones were isolated and transfected into FCHO cells. Cells were stained by PE-conjugated mouse anti-human kappa chain antibodies (PE-kappa) only and then FACS analyzed. *Y*-axis indicates the signal of PE fluorescence. N, negative control; P, positive control; 1–10, clones from library 1; 11–20, clones from library 2.

constructed have not yet met current needs for more efficient development of antibody drugs [14]. With the increasing need for therapeutic antibodies to treat human diseases, the strategy will transit more toward mammalian cell display, and advancement in all aspects of library construction will be required. Our vector provides a step toward this transition by combining the major benefits of past display techniques and overcoming their limitations.

Instead of using classic restriction enzymes, such as *Hin*dIII or *Bam*HI, we have used three different restriction enzymes in our vector: *Bst*XI, *Bsm*BI, and *Sfi*I. Utilization of these enzymes is the key to increasing the ligation efficiency of our vector and inserting both HCs and LCs in only one step. Because these enzymes are designed to cleave non-palindromic sequences, self-ligation is impossible. Moreover, the individual border of every single vector fragment is unique and can only bind to its complementary border during ligation. However, since we can control which borders will bind, the hindering variable of self-ligation, as demonstrated previously, is virtually eliminated [5]. Therefore, we can ligate all four fragments in the vector at once. To the best of our knowledge, this use of a one-step four-way ligation for library construction has not been reported so far. The use of our one-step vector system and the removal of self-ligation not only reduce the time expended for library construction, but also increase the overall quality of the antibody library, as demonstrated by the high efficiency and low background achieved by our strategy (**Table 3**). The two libraries mentioned above were constructed by a single scientist within 2 weeks, and each of them showed a background of $<0.5\%$, much lower than the usual threshold background of 10% [9]. In this report, competent cells with a transformation efficiency of 3×10^7 clones/μg of pUC19 were used and the transformation efficiency of our ligation mixture was $>10^6$ clones/μg of ligated DNA (**Table 3**). Therefore, it should be possible with reasonable effort to construct a full-length antibody display library of 10^8 or larger using high-transformation efficiency competent cells and multiple transformations, which are common strategies used in the construction of large libraries [9].

Libraries that we previously constructed have shown 80% in right reading frame for the HC library and 90% for the LC library [6]. In this report, we paired both HC and LC libraries in a dual-expression cassette vector using four-way ligation. FACS analysis of individual clones demonstrated that the detectable expression rate of the libraries was in the range of 60–80%, which was consistent with the calculated expression rate (72%), even though the expression levels of analyzed clones varied, and the percentage of positive cells ranged from 2.02% to 48.34%.

Using this strategy, any two genes of interest can be inserted into a single mammalian expression vector within a separate expression cassette. This prospect will also be very useful for a pair of genes, such as receptor and ligand, or any two genes that need to be expressed in a single cell at the same time for analysis of their interactions.

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