Journal of Immunological Methods 450 (2017) 41–49

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

A universal phage display system for the seamless construction of Fab libraries

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ARTICLE INFO

Keywords:
Phage display
Type IIS restriction enzyme
Antibody library
Fab

ABSTRACT

The construction of Fab phage libraries requires the cloning of domains from both the light and the heavy chain of antibodies. Despite the advent of powerful strategies such as splicing-by-overlap extension PCR, obtaining high quality libraries with excellent coverage remains challenging. Here, we explored the use of type IIS restriction enzymes for the seamless cloning of Fab libraries. We analyzed human, murine and rabbit germline antibody repertoires and identified combinations of restriction enzymes that exhibit very few or no recognition sites in the antibody sequences. We describe three phagemid vectors, pUP-22Hb, pUP-22Mc and pUP-22Rc, which were employed for cloning the Fab repertoire of these hosts using *Bsm*BI and *Sap*I (human) or *Sap*I alone (mouse and rabbit). Using human serum albumin as a model immunization, we built a mouse/human chimeric Fab library and a mouse Fab library in a single step ligation and successfully panned multiple cognate antibodies. The overall process is highly scalable and faster than PCR-based techniques, with a Fab insertion success rate of around 80%. By using carefully chosen overhangs on each end of the antibody domains, this approach paves the way to the universal, sequence- and vector-independent cloning and reformatting of antibody libraries.

1. Introduction

In the early days of phage display, the construction of Fab libraries involved separate cloning of light chains and heavy chains, requiring twice the amount of work compared to production of a single chain display. A simple solution to this problem was to use two plasmids for the separate cloning of each chain. This technique has been shown to generate very large libraries (Griffiths et al., 1994; Ostermeier and Benkovic, 2000), but did not gain in popularity, likely because of the handling of multiple plasmids. Currently, most Fab libraries are still built on a single phagemid following well-tested restriction enzyme-based strategies such as those developed for pComb3H (Barbas et al., 1991) or pHEN2 (Hoogenboom et al., 1991), and many others (Qi et al., 2012).

An attractive and popular alternative to restriction enzyme cloning for building single-chain variable antibody fragment (scFv) libraries is the use of splicing by overlap extension PCR (SOE-PCR). Overlapping primers between the 3′ end of one antibody domain and the 5′ end of the other are coupled during the PCR to create the linker, and the assembled scFv fragments are then inserted into the phagemid using restriction enzymes. The advantages of SOE-PCR include single step assembly of the variable heavy chain (VH) and light chain (VL), absence of restriction site-induced mutations and one-step cloning of the final construct into the receiving vector. Consequently, similar approaches have been developed for the cloning of Fab libraries (Li and Yeh, 1997; Popkov et al., 2003; Andris-Widhopf et al., 2011). The SOE-PCR method is applied through multiple amplification steps to first assemble VL domains with a light chain constant domain and VH domains with a CH1 domain, followed by the assembly of the final construct containing both chains and an additional leader sequence and cloning into the recipient vector. Straightforward in appearance, these methods require multiple and complex PCR reactions which do not always produce large quantities of properly assembled double-stranded material (Sotelo et al., 2012). We decided to explore alternative strategies that would be more reliable in their procedure and outcome, and potentially more accessible to the novice molecular biologist. Because type IIS restriction enzymes have non-palindromic asymmetric recognition sites and cut outside of their recognition sequence, they allow unidirectional cloning with deletion of the recognition site during subsequent ligation. This unique characteristic enables the combination of multiple cloning reactions in a single tube, thus opening the path to seamless and simultaneous cloning of both VH and VL during library construction or antibody format conversion in a single tube. In this work, we have conducted a thorough analysis of the incidence of type IIS restriction sites in antibody genes and determined enzymes that were amenable to the cloning of entire repertoires with minimal losses. Overhangs were

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http://dx.doi.org/10.1016/j.jim.2017.07.011
Received 12 April 2017; Received in revised form 22 June 2017; Accepted 25 July 2017
Available online 26 July 2017

carefully chosen on key positions found in most antibody expression vectors, thus allowing a vector-independent strategy of cloning. As a proof of concept, we designed phagemid vectors for cloning of the human, mouse and rabbit Fab repertoire and were able to successfully display large Fab libraries on phage for these species.

2. Material and methods

2.1. Antibody sequence repertoires and restriction analysis

Antibody gene sequence reference sets were assembled for IGHV, IGHJ, IGVK, IGJK, IGLV and IGLJ loci using the following databases and sources: (a), the IMGT database for all sets except murine IGHV, counting only genes marked as functional (Lefranc et al., 2015); (b), the UNSWIg germline repertoire for human IGHV and IGHJ sets (Wang et al., 2011); (c), 11 human IGHV genes recently identified by TIgGER (Gadala-Maria et al., 2015); and (d), mouse BALB/c and C57BL/6 IGHV sets, kindly provided by Dr. Andrew Collins (Collins et al., 2015). All IGHD functional repertoires were taken from the IMGT databases. Restriction site analysis was conducted using GenBeans software (www.genbeans.org).

2.2. Library construction

2.2.1. Reagents

All enzymes were from New England Biolabs (Ipswich, MA), except *Lgu*I (Thermo Fisher Scientific). All DNA purifications were performed using NucleoSpin Plasmid and NucleoSpin Gel and PCR Clean-up kits from Macherey-Nagel (Bethlehem, PA). The vectors pUP-22Hb, pUP-22Mc and pUP-22Rc were derived from the pADL-22c phagemid (Antibody Design Labs, San Diego, CA), using synthetic DNA fragments (Integrated DNA Technologies, Coralville, IA) inserted into the double *Sfi*I/*Bgl*I cloning site.

2.2.2. Mouse-human chimeric Fab phage library

Five female BALB/c mice were immunized with human serum albumin (HSA, A8763, Sigma-Aldrich) in PBS in emulsion with Freund complete adjuvant for the first immunization and incomplete adjuvant for the boosts. Five days after the last boost, the spleen of the best responder was harvested, total RNA fraction was prepared with Trizol (Thermo Fisher Scientific) and cDNA was prepared by reverse-transcription using ProtoScript II (New England Biolabs). This cDNA was also used to prepare a mouse Fab phage library, described further below. Variable domains were amplified using Taq polymerase and a degenerated murine primer set (Antibody Design Labs). The immunoglobulin heavy joining (IGHJ)-directed primers were designed to mutate the valine codon GTC into GTT, which removes the *Bsm*BI site located in the TVSS motif. The J primers were mixed and one PCR was performed for each V primer with the corresponding J primer mix. All light chain PCR reactions were mixed and gel purified; 480 ng of the mix were digested by *Sap*I for 2 h at 37 °C followed by heat-mediated inactivation for 20 min at 65 °C. All heavy chain PCR reactions were mixed and gel purified; 480 ng of the mix were digested by *Bsm*BI for 2 h at 55 °C. Vector pUP-22Hb (7.5 μg) was digested by *Bsm*BI (2 h at 55 °C) and, after clean-up, 5 μg were further digested by *Sap*I (2 h at 37 °C) followed by heat-mediated inactivation for 20 min at 65 °C. All three reactions (light chain, heavy chain and vector) were combined in a molar ratio 1:1.2:1.2, purified and eluted in 200 μl of water. The ligation reaction was conducted in 400 μl with 30 units of T4 DNA ligase for 2 h at room temperature. After heat-mediated inactivation (15 min at 65 °C) and purification, the ligation product was digested by 100 units of *Mlu*I-HF in a 100 μl reaction overnight at 37 °C. After heat-mediated inactivation (20 min at 80 °C), any digested DNA was dephosphorylated by 5 units of shrimp alkaline phosphatase for 60 min at 37 °C followed by heat-mediated inactivation for 5 min at 65 °C. The ligation was tested by heat shock-mediated bacterial transformation. Analysis of the transformants for the presence of an insert was done by colony-PCR using the primers phiS4 (5′-ATGAAATACCTAT TGCCTACGG) and psiR3 (5′-GCGTAACGATCTAAAGTTTTGTCG). After purification, the ligated products were eluted in 30 μl of water. A total of 50 μl of electro-competent TG1 cells (Lucigen, Madison, WI) were electroporated with 10 μl of the ligation using a MicroPulser (Biorad, Hercules, CA) following the manufacturer's recommendations, re-suspended into SOC medium, incubated for 1 h at 37 °C and diluted into 100 ml of 2xYT medium supplemented with 1% (w/v) glucose and 100 μg/ml ampicillin. A small culture aliquot was immediately submitted to serial 10-fold dilutions and plated. The bulk of the cells was further incubated overnight at 37 °C. The next day, bacteria were spun and an aliquot re-suspended in 25 ml of 2xYT medium at a density of 0.5 $OD_{600\ nm}$ and incubated for 45 min at 37 °C with 25 μl of M13KO7d3 helper phage (Antibody Design Labs). Kanamycin (50 μg/ml) and ampicillin (100 μg/ml) were added and the culture was incubated overnight at 30 °C. Subsequently, virions were purified by PEG precipitation and re-suspended in 2.5 ml (1/10th of the initial culture volume) of TBS. Virions were quantified by UV spectrophotometry using the George Smith formula (Day and Wiseman, 1978): virions/ml = $[OD_{269\ nm} - OD_{320\ nm}] \times 6 \times 10^{16}$ / plasmid length. In parallel, plated transformants were counted and analyzed for the presence of insert by colony PCR as described above. Inserts were sequenced using primer phiS4 for the light chain and psiR2 (5′-CGTTAGTAAATGAAT-TTTCTGTATGAGG) for the heavy chain.

2.2.3. Mouse Fab phage library

The cDNA obtained above was amplified using Taq polymerase and a different degenerated murine primer set (Antibody Design Labs). Primers were designed as above with only a *Sap*I restriction site for both VL and VH. The PCR reactions were done as described above. Vector pUP-22Mc (5 μg) was mixed with 480 ng of each of the light and heavy chain PCR products in a molar ratio of 1:1.2:1.2 and digested with 5 μl of the restriction enzyme in a reaction volume of 50 μl for 2 h. The reaction was done 3 times, each time with a different restriction enzyme, either *Sap*I in CutSmart buffer at 37 °C, *Lgu*I in Anza buffer at 37 °C, or *Bsp*QI in 3.1 buffer at 50 °C. The procedure then continued as described above for the mouse-human chimeric Fab library.

2.2.4. Rabbit Fab phage library

The cDNA prepared from the spleen of an immunized New Zealand rabbit was amplified using Taq polymerase and a degenerated rabbit primer set (Antibody Design Labs). Primers were designed as above with only a *Sap*I restriction site for both VL and VH. The PCR reactions were done as described above. Both ligation and digestion were conducted in the same reaction containing vector pUP-22Rc (5 μg), 630 ng of each of the light and heavy chain PCR products in a molar ratio of 1:1.5:1.5, 5 μl of T4 Ligase, 5 μl of *Sap*I and an equal mix of the two buffers in a final volume of 100 μl for 4 h at room temperature. The procedure then continued as described above for the mouse-human chimeric Fab library.

2.3. Library screening

2.3.1. First round of selection

Costar EIA/RIA Easy Wash™ plates (Corning, NY) were coated with 50 μl/well of 4 μg/ml HSA in TBS overnight at 4 °C and blocked after one TBS wash with 100 μl TBS containing 0.05% (v/v) Tween20 (TBST) and 5% (w/v) non-fat dry milk power (blocking buffer) for 1 h at 37 °C. After 3 washes with TBST, 50 μl of each HSA library diluted to 0.2 $OD_{269\ -\ 320\ nm}$ in blocking buffer were added to 4 wells and incubated for 4 h at 37 °C. After 5 washes with TBST and 5 washes with TBS, bound phages were eluted for 10 min at room temperature with 50 μl/well of glycine/HCl buffer 0.1 M, pH 2.2. Phages were collected in a single tube and neutralized with 64 μl of 1 M Tris/HCl, pH 8.0. The entire eluate was mixed with 264 μl of TG1 Phage Competent cells