Cassette hybridization for vector assembly application in antibody chain shuffling

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Gene assembly methods are an integral part of molecular cloning experiments. The majority of existing vector assembly methods stipulate a need for exonucleases, endonucleases and/or the use of single-stranded DNA as starting materials. Here, we introduced a vector assembly method that employs conventional PCR to amplify stable double-stranded DNA fragments and assembles them into functional vectors specifically for antibody chain shuffling. We successfully formed vectors using cassettes amplified from different templates and assembled an array of single chain fragment variable clones of fixed variable heavy chain, with different variable light chains – a chain shuffling process for antibody maturation. The method provides an easy alternative to the conventional cloning process.

Recombinant DNA technology is an important branch in synthetic biology that involves extensive manipulation of the microbial genetic information to design and bring DNA parts with distinct functions together into a novel system to facilitate programmed cellular functions as well as end product synthesis [1]. Therefore, construction of recombinant DNA molecules is the foundation for molecular research. Conventionally, a recombinant DNA vector is constructed through sequence-dependent cloning methods that rely mainly on restriction endonucleases and T4 DNA ligase [2]. However, this commonly used cloning strategy lacks flexibility because the choice of endonucleases needs to be reviewed carefully to avoid fragmentation within the insert and/or vector. Considerations such as availability and position of the endonuclease sites renders the application rigid while making simultaneous cloning of multiple gene fragments impossible. In addition, incomplete digestion and self-circularization of the vector also decreases the cloning efficiency [2,3].

To overcome these constraints, methods such as TA cloning, topoisomerase-based

cloning (TOPO) [4], BioBrick [5] and Golden Gate [6] have been introduced to improve the cloning performance. Besides this, sequence-independent cloning methods based on homologous recombination such as ligation-independent cloning (LIC) [7], sequence- and ligation-independent cloning (SLIC) [8], Gibson assembly [9] and LIC with uracil DNA glycosylase (USER) [10] have also been developed. These methods require exonucleases or DNA glycosylase to digest one of the DNA strands to create single-stranded sticky ends for annealing of the DNA [11]. However, the cleaving activity is difficult to control and might create a long single-stranded region, or in some instances it may digest the entire gene fragment. This leaves large gaps to repair and increases the risk of DNA loss, which will result in a reduced cloning efficiency [12–14]. Another sequence-independent approach that is entirely free from endonucleases and exonucleases is polymerase extension-based cloning. A DNA fragment flanked with sequences complementary to the site of insertion is PCR amplified and used as the megaprimer to amplify

the vector [15]. Circular polymerase extension cloning (CPEC) demonstrated the use of polymerase extension strategies to assemble and introduce multiple overlapping DNA fragments in a single step [16]. The process requires modification on the vector using restriction enzymes or a PCR method before inserts are introduced. Here, we proposed a vector construction method based on cassette hybridization that assembles not only the insert but the whole vector in any desired gene combination and arrangement simultaneously, using stable double-stranded DNA (dsDNA) as a template. The method provides flexibility in cloning any gene solely through PCR.

The vector assembly method introduced here can be applied in chain shuffling of monoclonal antibodies. Antibody chain shuffling is an *in vitro* antibody affinity maturation process that diversifies the antibody sequences to increase their binding affinity to a specific antigen [17]. The process is commonly achieved through restriction enzyme cloning [18,19] but the process is tedious and time-consuming. Another approach has also been shown

METHOD SUMMARY

To improve and reduce the cloning process time for antibody chain shuffling by adopting an assembly method based on a polymerase extension cloning method. Our method reduces the constraints in cloning by employing only two simple conventional PCR steps to amplify and assemble DNA cassettes that are designed with hybridization regions at both ends to direct assembly with specific adjacent cassettes.

with the use of lambda exonucleases to generate single-stranded DNA templates and using either Klenow Fragment [20] or *Bst* polymerase [21] to complete the single chain fragment variable (scFv) sequence. Here, we applied the cassette hybridization method to carry out antibody chain shuffling devoid of restriction enzymes and exonucleases to successfully generate an array of scFv clones with a diverse light chain repertoire rapidly.

Materials & methods

Primers & template

A vector is divided into smaller cassettes based on gene functions, such as the origin of replication, f1 origin, T7 promoter, T7 terminator, affinity tag, antibiotic resistance and protein/antibody encoding gene. Each of these crucial genes were PCR-amplified separately from different in-house vectors into dsDNA gene cassettes. The primers for amplification were designed in such a way that the resulting cassettes have 17–24 bp of overlapping regions with the adjacent cassettes. The sequences of the primers used are listed in Supplementary Table S1.

Cassette amplification

Amplification of the cassettes was carried out using MyCycler (Bio-Rad, CA, USA) in a total volume of 20 μ l, containing 1x ThermoPol reaction buffer, 0.4U Vent[®] DNA polymerase (New England BioLabs, MA, USA), 200 μ M dNTPs (ABM Inc., Richmond, Canada), 0.5 μ M forward primer, 0.5 μ M reverse primer and 20 ng template. The amplification profile used includes an initial denaturation at 95°C for 1 min 30 s, 25 cycles of denaturation, annealing and extension (95°C for 30 s, 55°C for 1 min, 72°C for 1 min) followed by 10 min of final extension at 72°C. The amplified gene cassettes were extracted from 1% agarose gel by QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany).

Cassette hybridization

Equal amounts of the desired gene cassettes (50 ng each) were mixed in a single 25 μ l reaction, containing 1x OneTaq standard reaction buffer, 1.25U OneTaq[®] DNA polymerase (New England BioLabs) and 200- μ M dNTPs (ABM Inc., Canada). The hybridization was carried out using MyCycler (Bio-Rad) with the following thermo-profile: initial denaturation at

94°C for 30 s, ten cycles of denaturation, annealing and extension at 94°C for 30 s, 55°C for 1 min, 68°C for 5 min 30 s then a final extension at 68°C for 30 min. After hybridization, the assembled vector was ethanol precipitated, reconstituted in 5 μ l autoclaved distilled water, then transformed into *Escherichia coli* DH10 β (Invitrogen, CA, USA) by electroporation and plated on 2YT (Novagen, Darmstadt, Germany) agar plate supplemented with 100 μ g/ml ampicillin (Fisher Scientific, NH, USA) or 60 μ g/ml kanamycin (Amresco Inc., OH, USA) depending on the antibiotic resistance gene cassette used. The transformants were picked for colony PCR using specific primers listed in Supplementary Table S1. Successfully assembled vectors were extracted and sent for DNA sequencing.

Chain shuffling of monoclonal antibody

To achieve chain shuffling, the in-house monoclonal antibody clone C12 was PCR amplified and assembled based on the method described above. The variable heavy chain (V_H) was amplified from the clone C12, whereas the variable light chain (V_L) was PCR amplified from an in-house naive library [22]. 4 mM MgSO₄ (New England BioLabs) was supplemented when assembling the vector. In parallel, chain shuffling was done using the conventional ligation method as a comparison. The PCR amplified V_L cassettes and the C12 original phagemid were double digested in a final volume of 50 μ l with 20U XhoI and 10U NotI restriction enzymes (New England BioLabs) for 3 h at 37°C. The digestions were heat inactivated at 65°C for 20 min. The digested V_L cassette was PCR purified whereas the C12 phagemid was gel extracted from 1% agarose using QIAquick Gel Extraction Kit (QIAGEN). Then, 50 ng of digested V_L was ligated to 188 ng C12 phagemid at a 1:3 ratio using 400U T4 DNA ligase (New England Biolabs) in a total volume of 20 μ l. The reaction was carried out at room temperature for 1 h and heat inactivated at 65°C for 20 min. The ligation product was then ethanol precipitated with 2 μ l of sodium acetate (3M, pH 5.2) and 50 μ l of ice-cold ethanol at room temperature for 30 min, followed by 20 min centrifugation at 16000 $\times$ g. The pellet was washed with 300 μ l of ethanol (70% v/v) and air dried. The pellet was resuspended with 4 μ l of distilled water and transformed into DH10 β

competent cell (Invitrogen). The colonies on plate were counted and randomly picked for colony PCR using LMB3_Fw (5' CAGGAAACAGCTATGAC 3') and pIII_Rv (5' GTTAGCGTAACGATCTAA 3') primers. Successfully assembled vector was sent for DNA sequencing. The gene allele of the V_L were analysed using IMG/V-QUEST database (www.imgt.org/IMG/V-quest/vquest) [23,24].

Phage ELISA

The positive clones with V_L chain shuffled were transformed into XL1-Blue electro-competent cells (Agilent Technologies). A single colony was picked for each clone and cultured overnight (o/n) at 37°C with agitation at 200 rpm. Then 50 μ l of the o/n culture was used to inoculate 5 ml of fresh 2YT (Novagen) medium supplemented with 2% D-glucose and 100 μ g/ml ampicillin. The culture was grown until OD_{600 nm} of 0.5 before co-infection with M13KO7 helper phage (New England BioLabs). The phagemid was packaged o/n at 30°C with agitation at 180 rpm in medium supplemented with 0.1% glucose, 100 μ g/ml ampicillin and 60 μ g/ml kanamycin. The culture was centrifuged down, phage-containing supernatant was collected and checked for titer. The binding affinity of the clones was compared through ELISA. A total of 100 μ l of antigen (10 μ g) in phosphate-buffered saline (PBS) was coated o/n at 4°C on Costar EIA/RIA microplate (Corning Inc., NY, USA). In parallel, the same amount of wells were coated with PBS without the antigens to act as background control. The plate was washed three times with PBS-T (0.1% Tween 20) and blocked with 300 μ l of 2% milk for 1 h. The phage (10¹² pfu/ml) was diluted in equal volume with 2% milk before 100 μ l was added to each sample and control wells. The plate was incubated for 2 h, then washed three times with PBS-T. Then 100 μ l of anti-M13 horseradish peroxidase diluted in 2% milk (1:5000) was added to each well and incubated for 1 h. Last, the plate was washed three times with PBS-T and 100 μ l of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt solution was added and left to develop in the dark for 30 min. All of the process above was carried out at room temperature with shaking at 500 rpm. The absorbance was measured at OD_{405 nm} with a microplate reader (Thermo Scientific Multiskan Spectrum, MA, USA).

Figure 1. The processes involved in the cassette hybridization vector assembly method. (A) Schematic illustration of hybridization of cassettes in proposed cassette hybridization vector assembly method. Gene cassettes were PCR-amplified from different vectors (step 1) and assembled into a functional recombinant vector through the site-specific hybridization of the complementary ends of each cassettes (step 2). The process is driven entirely by a conventional PCR reaction. **(B)** Vector constructs in different combinations. The gene cassettes amplified in step 1 form a repertoire to assemble vectors with desired gene combination and arrangement.

Results & discussion

The proposed cassette hybridization vector assembly method involves two major steps: cassette preparation and cassette hybridization (Figure 1A). During the design of the primers for cassette amplification, we divided a vector into smaller fragments based on their function. A vector is essentially built up of several important genes to form a functional unit. This includes the origin of replication that drives the vector replication, promoter and terminator for gene expression, affinity tag for downstream protein purification, antibiotic resistance gene for selection and the recombinant protein encoding gene [25]. Figure 1A shows the repertoire generation of the DNA cassettes containing all these fundamental elements of a functional vector. Each of the amplified cassettes contains a homologous overlapping region at the 5' and 3' end, which facilitates the assembly through hybridization. After the cassette repertoire is generated, these dsDNA cassettes are then assembled in different combinations (summarized in Figure 1B). A proof-of-principle experiment was done to successfully assemble several recombinant vectors (~3.6 kb in size) with cassettes of different sizes (130–1020 bp). The assembly process was driven entirely by high-fidelity polymerase and 50 ng of each cassette was found to be sufficient regardless of the size of the cassettes, unlike traditional ligation

methods, which need the optimization of the molar ratio of vector and insert. The existing gaps generated by the process are then repaired within the *E.coli* using the host bacteria repair mechanism. The correct construct was confirmed through sequencing. The functionality of antibiotic resistance genes was verified by plating out on different antibiotic plates whereas the gene expression functionality was tested through expression of the green fluorescent protein (EGFP) and red fluorescent protein (DsRed), where the phenotypic features were observed easily under UV illumination. In addition, we also successfully assembled a vector with two genes of interest – scFv and IgG1 hinge-Fc originating from two different vectors. The design included the removal of the Avi-His tag and swapping of the His-tag from the N terminus to C terminus (Figure 1B). This highlights the robustness of the cassette hybridization vector assembly method to introduce multiple inserts simultaneously and allows user-defined vector construction for

specific experiment requirements without the concern of availability of desired or undesired features in commercial vectors.

Besides creating a vector of interest, the proposed vector assembly method was applied to carry out the antibody

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Table 1. Summary of positive chain shuffling clones with variation in V-gene and J-gene using cassettes hybridization vector assembly method.

Number	Clone name	V-gene and allele	J-gene and allele
0	Original (C12)	<i>IGLV1-47*01F</i>	<i>IGLJ3*02F</i>
1	C1	<i>IGLV2-11*01F</i>	<i>IGLJ2*01F</i>
2	C2	<i>IGKV3-15*01F</i>	<i>IGKJ1*01F</i>
3	C3	<i>IGLV3-19*01F</i>	<i>IGLJ3*02F</i>
4	C4	<i>IGLV1-44*01F</i>	<i>IGLJ3*02F</i>
5	C5	<i>IGKV3-20*01F</i>	<i>IGKJ1*01F</i>
6	C6	<i>IGLV1-44*01F</i>	<i>IGLJ2*01F</i>
7	C7	<i>IGKV3-20*01F</i>	<i>IGKJ2*01F</i>
8	C8, C9	<i>IGKV1-39*01F</i>	<i>IGKJ3*01F</i>
9	C10	<i>IGKV3-20*01F</i>	<i>IGKJ5*01F</i>

Table 2. Summary of positive chain shuffling clones with variation in V-gene and J-gene using conventional restriction digestion and ligation method.

Number	Clone name	V-gene and allele	J-gene and allele
0	Original (C12)	<i>IGLV1-47*01F</i>	<i>IGLJ3*02F</i>
1	C1	<i>IGKV3-15*01F</i>	<i>IGKJ3*01F</i>
2	C2, C3, C4, C9	<i>IGLV3-19*01F</i>	<i>IGLJ2*01F</i>
3	C5	<i>IGLV7-46*01F</i>	<i>IGLJ1*01F</i>
4	C6	<i>IGKV2-30*02[F]</i>	<i>IGKJ1*01F</i>
5	C7	<i>IGKV3-20*01F</i>	<i>IGKJ4*01F</i>
6	C8	<i>IGLV3-10*01F</i>	<i>IGLJ1*01F</i>
7	C10	<i>IGLV2-11*01F</i>	<i>IGLJ3*02F</i>
8	C11, C16	<i>IGKV4-1*01F</i>	<i>IGKJ1*01F</i>
9	C12, C15	<i>IGKV3-20*01F</i>	<i>IGKJ1*01F</i>
10	C13	<i>IGKV4-1*01F</i>	<i>IGKJ2*01F</i>
11	C14	<i>IGLV6-57*02F</i>	<i>IGLJ3*02F</i>
12	C17	<i>IGLV3-1*01F</i>	<i>IGLJ2*01F</i>
13	C18	<i>IGLV3-21*03F</i>	<i>IGLJ3*02F</i>

chain shuffling process. In this experiment, our in-house monoclonal antibody clone C12 phagemid was divided into several fragments based on their functions, which includes the lac operator, V_H , V_L , pIII, f1 origin, ampicillin resistance gene and origin of replication. Each gene fragment was amplified

into dsDNA cassettes as described above. The variable heavy chain (V_H) was fixed whereas the variable light chain (V_L) was PCR-amplified from an in-house naive human scFv phage library [22]. The primers were designed to anneal at the linker and pIII region, which is universal for the phage library. Thus, the resulting V_L cassettes are made up of the diverse V_L gene repertoire originating from the library. Addition of a supplement such as Mg^{2+} , a cofactor for polymerase, during assembly of larger sized vectors can greatly improve the yield. This was evident in this case where the phagemid has a size of approximately 5300 bp. A total of 117 colonies was observed after transformation and incubated o/n. After colony PCR with LMB3_Fw and pIII_Rv primers, 18 positive clones

(18/30) were sent for sequencing, nine clones were reported to be in the correct frame with distinct V_L germline gene family, V-gene and J-gene (summarized in Table 1).

In parallel, the conventional method of chain shuffling through restriction digestion and ligation was carried out. To accommodate the experimental time frame limit to a single day reaction, the digestion and ligation reaction was carried out at 3 h and 1 h respectively instead of o/n incubation, which would otherwise lengthen the cloning process to 3 days. A total of 93 colonies were obtained on plate after transformation and incubated o/n. Out of 24 positive clones (24/30) sent for sequencing, 13 clones showed distinct V_L germline gene family usage (summarized in Table 2). The percentage of successfully chain-shuffled clones was 54% (13/24) using the conventional restriction digestion and ligation method while our proposed vector assembly method was around 50% (9/18). Therefore, the yield of the proposed vector assembly method is comparable with the conventional ligation method. This suggests

Figure 2. Result of phage ELISA after background deduction. Standard deviation was performed in triplicate. Clone C12 was the original parent clone whereas the remaining clones are chain-shuffled clones using the cassette hybridization vector assembly method.

the potential of the proposed method as an alternative to carry out monoclonal antibody chain shuffling using only DNA polymerase, without the need of exonucleases and ligase for a reduced cost.

To test the functionality of the chain-shuffled clone, all ten clones obtained from the cassette hybridization vector assembly were transformed into XL1-Blue electro-competent cells, packaged into phage and used to conduct phage ELISA. The absorbance reading at 405 nm after removing the background reading is demonstrated in Figure 2. To ensure a consistent phage input, which is 10^{12} pfu/ml, clone 2 and 3 with a lower titer were omitted from the analysis. All the remaining eight chain-shuffled clones showed positive binding to the antigen, with readouts ranging from 0.064 to 0.215. The variations in readings suggest that the V_L from different families does affect the binding of the scFv towards the antigen. As compared with the original clone C12, some of the chain-shuffled clones showed binding functionality close to the original clone. Although we did not obtain a clone with a higher binding characteristic, the results show the applicability of the cassette hybridization vector assembly method as an effective and robust affinity maturation method. The isolation of clones with an improved binding characteristic can be improved by increasing the sample size to generate a chain-shuffled mini-library for a new round of panning.

In conclusion, we have successfully assembled several functional vectors with the gene of interest in a desired order through the cassette hybridization vector assembly method. The method is highly flexible, and allows the desired gene to be put together from different sources to achieve full customization of vectors by two simple conventional PCR steps. We also demonstrated the feasibility of the cassette hybridization vector assembly method to shuffle the V_L (or V_H) of scFv in order to achieve affinity maturation of a monoclonal antibody, simplifying the conventional way achieved through restriction cloning.

Author contributions

JYL and QL performed the experiments. JYL, YSC and TSL analyzed the data. All authors were involved in the design of the experiments and preparation of the manuscript.

Competing & financial interests disclosure

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Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.2144/btn-2018-0031

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