Reports

Directed evolution of nucleotide-based libraries using lambda exonuclease

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Directed evolution of nucleotide libraries using recombination or mutagenesis is an important technique for customizing catalytic or biophysical traits of proteins. Conventional directed evolution methods, however, suffer from cumbersome digestion and ligation steps. Here, we describe a simple method to increase nucleotide diversity using single-stranded DNA (ssDNA) as a starting template. An initial PCR amplification using phosphorylated primers with overlapping regions followed by treatment with lambda exonuclease generates ssDNA templates that can then be annealed via the overlap regions. Double-stranded DNA (dsDNA) is then generated through extension with Klenow fragment. To demonstrate the applicability of this methodology for directed evolution of nucleotide libraries, we generated both gene shuffled and regional mutagenesis synthetic antibody libraries with titers of 2×10^8 and 6×10^7, respectively. We conclude that our method is an efficient and convenient approach to generate diversity in nucleic acid based libraries, especially recombinant antibody libraries.

Directed evolution allows an in vitro mimicry of the natural in vivo evolution process to generate new or improved traits. The directed evolution process involves repetitive cycles of genotype diversification accompanied by a physical selection process to sieve out the best phenotype. The approach is synonymous with display techniques, such as phage, yeast, and ribosome display, that promote a physical linkage between genotype and phenotype. A basic requirement for any display technique is a diverse nucleotide library (1). Numerous methods have been developed to introduce diversity into these libraries, including random mutagenesis (2), in vitro or in vivo DNA shuffling (3–5) and site-specific recombination (6–8). Random mutagenesis can be carried out using several different methods, although most commonly through the use of a low fidelity DNA polymerase with $MnCl_2$ (9) and *Escherichia coli* mutator strains (10). Other methods include mutagenic polymerases (11), mixtures of triphosphate derivatives of nucleoside analogs (12), site directed mutagenesis (13), mutational hotspots (14), and parsimonious mutagenesis (15). Although random mutagenesis can generate randomization at any position in the sequence, the technique is mostly limited to short stretches of DNA. This prevents the alteration of entire binding sites, which are generally longer DNA regions. In addition, as some mutations may not be directed, errors such as frameshifts or stop codons could also occur during mutagenesis that may result in the disruption of the phenotype.

Antibody libraries are the mainstay in display approaches due to the nature of diversification present in antibody genes. Here, gene shuffling can be accomplished by numerous methods including chain shuffling (16), DNA shuffling (4) or staggered extension process (StEP) (17). Chain shuffling makes use of shuffling either the heavy or light chain variable regions of the antibody genes to generate new variants by conventional restriction digestion and ligation. In DNA shuffling, the antibody gene is digested with DNase I, randomly reassembled and amplified by PCR. StEP, which is also PCR-based, allows template switching by shortening extension times and, hence, will shuffle various portions of several parental antibody genes. DNA shuffling has been widely used for library generation, and a few recent modifications have been described that improve the efficiency of the technique including the use of single-stranded DNA (ssDNA) instead of double-stranded DNA (dsDNA) as template and use of restriction enzymes or endonuclease V instead of DNase I during DNAs fragmentation (18, 19). However, this method still presents limitations, including low frequency of chimeric genes due to preferred homoduplex formation, a limited distribution of restriction site, and a lack of high-resolution crossover (20, 21).

Lambda exonuclease is an enzyme that assists in the repair of dsDNA breaks in viral DNA (22). Lambda exonuclease is a highly processive 5′→3′ dsDNA exonuclease that selectively degrades a phosphorylated chain of the duplex to yield mononucleotides and ssDNA (23–25). ssDNA template used in directed evolution experiments is usually generated as the substrate for pairing enzymes that promote homologous recombination. The main characteristic of the enzyme is the requirement of a phosphate group at the 5′ dsDNA end. The use of lambda exonuclease to generate ssDNA has assisted in other method developments and technologies such as next-generation sequencing platforms (26), DNA-chips (27), SELEX (28), subtractive hybridization techniques (29), sample preparation for electrospray ionization mass spectrometry (30), and recombination methods (31).

DNA polymerase I is a single polypeptide chain with three separate functional domains: a polymerization domain, a 3′ exonuclease (or proofreading) domain, and a 5′ nuclease domain (32). Klenow fragment (KF) of DNA polymerase I is obtained by removing the 5′ nuclease domain from the protein by limited protease digestion. KF is a 68 kDa protein with the polymerase and 3′→5′ proof reading exonuclease activity, but lacking 5′→3′ exonuclease activity (33). Thus, KF is commonly used for fill-in reactions during molecular cloning as the enzyme has the ability to add nucleotides without degrading any overhangs. In addition, KF has been used to generate dsDNA from highly complexed templates, such as degenerate oligos for peptide-displaying phage library cloning (34). Another application of KFs is to conduct low temperature PCR cycling with the aid of proline (35).

A previous protocol described the use of lambda exonuclease to generate overhangs similar to overlapping regions created by restriction enzyme digestion (36). KF was used to repair the ssDNA by stabilizing the junction regions without strand displacement. This method focused on limited lambda exonuclease digestion to create short ssDNA 3′-overhangs for hybridization. The protocol was not as efficient for cloning smaller fragments, however, data showed cloning of an 89 bp fragment with reduced efficiency (36). Although this method may be efficient for antibody chain shuffling of larger fragments, it is not appropriate for short complementary determining region (CDR) mutagenesis. CDR regions vary from 3 to 12 amino acids in length (37, 38), depending on their location along the antibody sequence. Binding sites for antigens are formed by six CDRs, wherein three CDRs are found in the heavy chain and light chain, respectively. These CDRs loop out from the V region backbone to form interaction regions with target antigens. Hence, mutations on CDRs can directly increase antibody affinities for antigens and vice versa. However, only CDR3 in the heavy chain has a wide range of variation in terms of length and amino acid propensity, which contributes to antigen specificity (39, 40).

In order to overcome these limitations, we developed a new method to obtain and use ssDNA for chain shuffling and mutagenesis by degenerate oligonucleotides. Although conceptually similar in terms of ssDNA generation, rather than using a limiting digestion step, our approach produces the entire ssDNA for use. The ssDNA template generated will have an overlapping region of at least 7bp in length. This is important because the extension of KF is effective if the complementary region is longer than a heptamer (41). By hybridizing complementary ssDNA templates we are then able to generate a template strand of ssDNA which can be transcribed into dsDNA using KF (Figure 1).

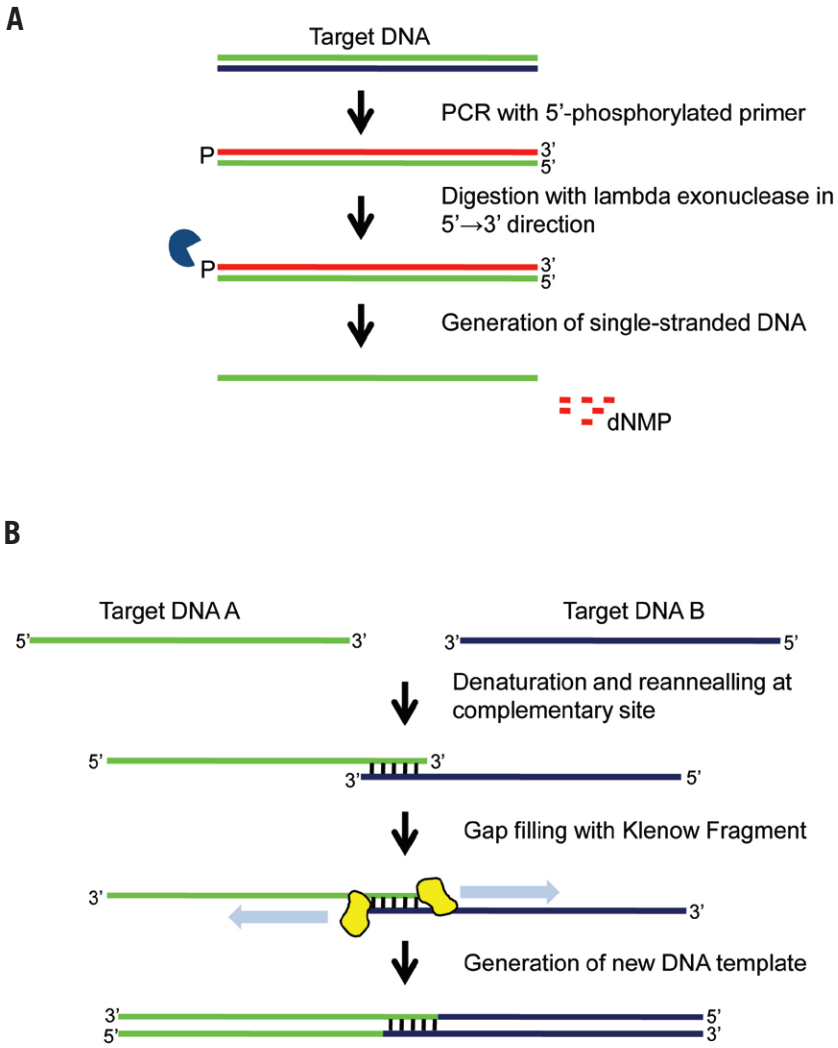

Figure 1. Schematic representation of the proposed protocol for generating new double stranded DNA templates. (A) Production of single-stranded DNA using lambda exonuclease digestion. (B) Generation of double-stranded DNA template using Klenow fragment filling with lambda exonuclease treated single-stranded DNA.

To demonstrate our new methodology, we use a semi-synthetic single chain Fragment variable (scFv) antibody molecule as a model. Two strategies to introduce directed sequence evolution were examined. First, we demonstrate chain shuffling between variable heavy chain (VH) against a specific variable light chain (VL) and secondly, we describe recombination of the VH framework with a synthetic CDR3 oligonucleotide. For chain shuffling, the protocol was used to introduce various light chains to a fixed heavy chain (Figure 2A) while for CDR mutagenesis, we introduced short randomized regions at the heavy chain CDR3 (Figure 2B).

Materials and methods

Ethics statement

Human peripheral blood mononuclear cells (PBMCs) were collected with written informed consent according to the guidelines approved by Universiti Sains Malaysia Human Ethics Committee.

PCR amplification of antibody genes

Phagemid DNA was prepared from overnight *E. coli* cultures of anti-eGFP

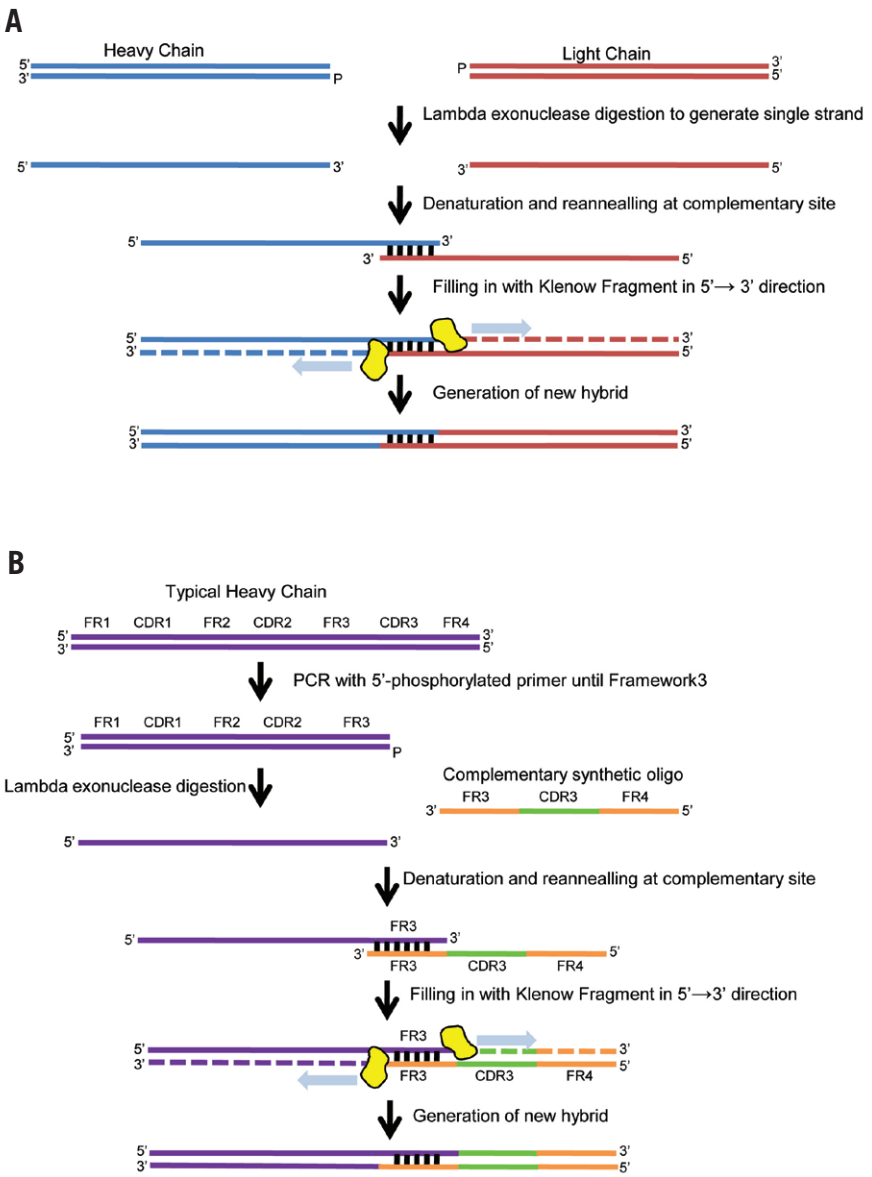

Figure 2. Application of the proposed protocol for directed evolution of antibodies. (A) Generation of new hybrids by applying the protocol to chain shuffling. (B) Generation of new hybrids by applying the protocol to randomize the CDR3 region of the heavy chain. The complementary synthetic oligo contains a stretch of degenerate nucleotides representing the CDR3 (marked in green).

scFv clones 44 and G1 using a MiniPrep Kit (Qiagen, Hilden, Germany). The final volume for all PCRs was either 20 or 50µL containing 40 or 100ng of phagemid DNA, respectively, 200 µM deoxynucleoside triphosphates (dNTPs), 0.2µM of each primer (1st Base, Kuala Lumpur, Malaysia), and 1× DreamTaq reaction buffer (Fermentas, Lithuania). The amount of polymerase used per reaction was 0.2 or 0.5 µL of 5 U/µL DreamTaq (Fermentas, Lithuania). All reactions were carried out using a MyCycler thermocycler (BioRad, Hercules, CA, USA). After heating at 95°C for 45 s, we performed 30 or 25 cycles, respectively (30 s at 95°C, 30 s at 55°C, and 30 s at 72°C) and ended after 5 min at 72°C.

PCR amplification of the variable heavy and light chain regions was carried out using the primers VH-NcoI-Fw (ACATGCCATGGCCGAGGTGCAGC) and VH-phos-tom-exo-Rv(5′-phos-CAGGACGGTGACCA-GGGTTCCCTG) as well as VL-phos-tom-exo-Fw (5′-phos-CAGGGAACCCTGGTCACCGTC) and VL-NotI-Rv (AAGGAAAAAAGCGGCCGCCCGTTTGATTTC) respectively. For CDR3 mutagenesis, primers used for VH framework were VH-NcoI-Fw (ACATGCCATGGCCGAGGTGCAGC), VH-Tom-Fr3-phos-Rv (5′-phos-ACAGTAATATACGGC) and synthetic CDR3 oligonucleotides (GACGGTGACCAGGGTTCCCTGGCCCCAGTAGTCAAA**MNNMNNMNNMNN**TTTCGCACAGTAATATACGGCCGT). Kappa light chain variable region gene sequence was amplified using cDNA template, synthesized from RNA that was extracted from human PBMCs according to Lim et al. (42); amplification of VK genes from family 2, 4, and 6, were conducted with the primers VK246-SalI-Fw (TGTGACAAAGTCGACGGATATTGTGMTGACBCAGWCTCC) and HscFv kappa-NotI-Rv (ATGATGATGTGCGGCCGCGAAGACAGATGGTGCAGCCACAGT). All PCR products were purified using the PCR Purification Kit (Qiagen) and eluted in 30 or 50 µL of distilled water. dsDNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

Generation of ssDNA through lambda exonuclease treatment

1.0 µg of purified targeted dsDNA (for chain shuffling: anti-eGFP scFv clone 44 for VH and anti-eGFP scFv clone G1 for VL; CDR3 mutagenesis: heavy chain framework from anti-eGFP scFv clone 44) was incubated at 37°C for 30 min respectively with 1× lambda exonuclease reaction buffer and 10 U of lambda exonuclease (New England Biolabs, Ipswich, MA, USA) in a total of 50 µL reaction volume. The enzyme was then heat inactivated at

75°C for 10 min. To visualize ssDNA after lambda exonuclease treatment, samples were loaded on a 1% agarose gel with ethidium bromide. ssDNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

Evaluation of ssDNA generation by 6 M urea PAGE

6 M urea polyacrylamide was prepared using 1.875 mL 40% acrylamide/bis-acrylamide (29:1), 0.6 mL 10XTBE, 2.88 g urea, 19.8 µL 30%APS, 2.4 µL TEMED and 3.525 mL dH_2O. 15 µL of DNA sample was mixed with 3 µL of 6× loading dye. The mixture was heated at 95°C for 5 min and left to cool before loading. For DNA separation, electrophoresis was carried out at a current of 25mA using mini protean tetra cell (BioRad) for 50 min in 0.5×TBE buffer. Gel was stained with EtBr solution and visualized under UV.

Evaluation of ssDNA generation by fluorescence intensity reading

100 ng of DNA in a 10 µL aliquot with 5 µL of iTaq universal SYBR green supermix (BioRad) was used to measure fluorescence using Rotor-Gene 3000 (Corbett Research, Cambridgeshire, UK) at 55°C. Synthetic oligo20 ssDNA (CCGGCCATGGCC$(NNK)_{20}$GCGGCCGCATAGACTGTT) from Integrated DNA Technologies, USA was used as the ssDNA control, while oligo20 dsDNA was generated by KF filling in of the oligo20 ssDNA as the dsDNA control.

Annealing two ssDNA templates

0.4 µg of each digested target ssDNA (for chain shuffling: anti-eGFP scFv clone 44 for VH and anti-eGFP scFv clone G1 for VL; CDR3 mutagenesis: heavy chain framework from anti-eGFP scFv clone 44 and synthetic CDR3 oligonucleotides) in 1×NEB buffer 2 (New England Biolabs) were annealed to create a substrate for KF. Under non-cyclic conditions, the reaction mixture was heated at 95°C for 5 min and left to cool to 30°C with a ramp of 0.1°C/s. The reaction was then left to incubate at 30°C for 5 min. For cycling conditions, the reaction mixture was heated at 95°C for 5 min, and then slowly cooled to 70°C, 60°C, 50°C, 40°C, 30°C, and 25°C with a ramp of 0.1°C/s and every temperature kept constant for 5 min. All temperature cycling was carried out using the MyCycler thermocycler (BioRad).

Filling in reaction by Klenow fragment

For filling in ssDNA, 20U KF (New England Biolabs) and 1.5mM dNTPs (Fermentas, Lithuania) were added to a final reaction volume of 50 µL with the annealed ssDNA. The mixture was incubated at 37°C for 4 h and then heat inactivated at 75°C for 20 min. To determine which DNA strand was extended from the annealing site, same amount of reaction sample was loaded on 1% agarose gel with ethidium bromide for visualization and then gel purified with Purification Kit (Qiagen).

Table 1. Amino acid sequence analysis of hybrids generated via directed chain shuffling.

Chain	Heavy Chain		Light Chain		GenBank Accession no.
	CDR 2	CDR 3	CDR 2	CDR 3	
Anti-eGFP 44	S**Y**I**S**G**T**G**N**T**T**A**Y**	K**D**T**N**Y**F**	Y**Y**A**S**Y**L**	Q**T**A**A**G**P**S**T**	JX028841
Anti-eGFP G1	S**T**I**S**S**N**G**G**Y**T**Y**Y**	K**A**Y**S**A**F**	Y**T**A**S**T**L**	Q**T**S**A**N**P**G**T**	JX028842
Shuffle Hybrid 1	S**Y**I**S**G**T**G**N**T**T**A**Y**	K**D**T**N**Y**F**	Y**T**A**S**T**L**	Q**T**S**A**N**P**G**T**	JX028843
Shuffle Hybrid 2	S**T**I**S**S**N**G**G**Y**T**Y**Y**	K**A**Y**S**A**F**	Y**Y**A**S**Y**L**	Q**T**A**A**G**P**S**T**	JX028844

Bold letters represents amino acids in randomized positions.

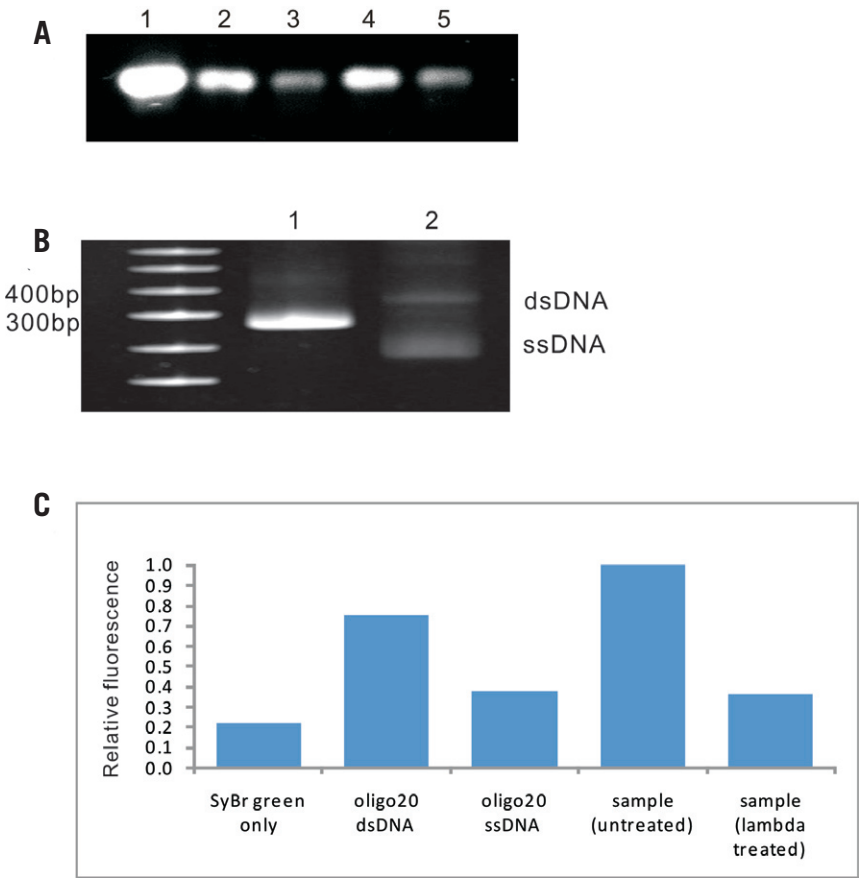

Figure 3. Digestion with lambda exonuclease generated single stranded DNA. Production of single stranded DNA using lambda exonuclease at 37°C with 1µg DNA. (A) Optimization of lambda exonuclease concentration and incubation time analyzed on a 1% agarose gel; Lane 1: negative control (without lambda exonuclease), Lane 2: 5U enzyme and 30mins, Lane 3: 10U enzyme and 30mins, Lane 4: 5U enzyme and 1hr, Lane5: 10U enzyme and 1hr (B) 6M Urea-PAGE showing generation of ssDNA from dsDNA; Lane 1: negative control (without lambda exonuclease), Lane 2: 10U enzyme and 30mins incubation. (C) Fluorescence measurements of SYBR green in the presence of ssDNA or dsDNA samples.

Cloning for sequencing

The final purified product was digested with appropriate restriction enzymes (New England Biolabs) and ligated to the pIT2 plasmid using T4 DNA ligase and 1× T4 DNA ligase buffer (New England Biolabs) with a final volume of 20 µL according to the manufacturer's protocol. The DNA was prepared using MiniPrep DNA kit from Qiagen according to the manufacturer's protocol. The DNA was sent for sequencing using BigDye Terminator v3.1 cycle sequencing chemistry at 1st Base (Malaysia) using LMB3 Forward primer (CAGGAAACAGCTATGAC) and pIII Reverse primer (GTTAGCGTAACGATCTAA). Sequencing results were analyzed using VBASE2 (43). The

Table 2. Amino acid sequence analysis of hybrids generated via shuffling of a natural repertoire of human VK family 2, 4, and 6 genes.

Clone	V gene	CDR1	CDR2	CDR3	GenBank Accession no.
1	humIGKV101	QNISSNF	GSS	QQYGNSLPFT	JX028845
2	humIGKV109	HSLVHFDGNTY	EVS	MQTTDWPYT	JX028846
3	humIGKV125	QNLLHTSGDNY	LSS	MQSLQTPPT	JX028847
4	humIGKV083	QSISSY	WAS	QQYYTTPRT	JX028848
5	humIGKV034	QSLLHSDGKTY	EVS	QQSSTLPIT	JX028849
6	humIGKV083	QSVLYSSNNKNY	WAS	QQYYSTPWT	JX028850
7	humIGKV109	QSLVHSDGNTY	KVS	MQATHWPPVT	JX028851
8	humIGKV034	QSLLHSDGKTY	EVS	MQALQSPLT	JX028852
9	humIGKV109	QSLVHSDGNTY	RVS	MQATHWPA	JX028853
10	humIGKV109	QGLGYSDGSTY	KVS	MQATHWPA	JX028854

sequences of all clones have been deposited by GenBank under the accession no. JX 028841 to JX028874.

Determination of library diversity by DiStRO

500 ng dsDNA sample with 10 µL iTaq universal SYBR green supermix (BioRad) in a total volume of 40 µL was prepared and the assay was performed using Rotor-Gene 3000 (Corbett Research) instrument. Reannealing was initiated by denaturation for 2 min at 95°C and subsequent measurements were taked during annealing at 50°C for 180 min taking one measurement per minute. Re-melting was performed starting at 25°C with incremental steps of 0.5°C for 7 s each until the final temperature of 98°C was reached.

Table 3. Amino acid sequence analysis of hybrids generated via directed mutagenesis of CDR3.

Clone	CDR3 Sequence	GenBank Accession no.
CDR Hybrid 1	CAK**PVAE**FDY	JX028855
CDR Hybrid 2	CAK**CIIL**FDY	JX028856
CDR Hybrid 3	CAK**SDMA**FDY	JX028857
CDR Hybrid 4	CAK**HGYQ**FDY	JX028858
CDR Hybrid 5	CAK**EAIS**FDY	JX028859
CDR Hybrid 6	CAK**DMTP**FDY	JX028860
CDR Hybrid 7	CAK**MVLA**FDY	JX028861
CDR Hybrid 8	CAK**GMSH**FDY	JX028862
CDR Hybrid 9	CAK**STFI**FDY	JX028863
CDR Hybrid 10	CAK**PRPI**FDY	JX028864
CDR Hybrid 11	CAK**RKTT**FDY	JX028865
CDR Hybrid 12	CAK**VADS**FDY	JX028866
CDR Hybrid 13	CAK**TLPP**FDY	JX028867
CDR Hybrid 14	CAK**TLLT**FDY	JX028868
CDR Hybrid 15	CAK**TQTP**FDY	JX028869
CDR Hybrid 16	CAK**CATA**FDY	JX028870
CDR Hybrid 17	CAK**KSTR**FDY	JX028871
CDR Hybrid 18	CAK**LPPH**FDY	JX028872
CDR Hybrid 19	CAK**SGGC**FDY	JX028873
CDR Hybrid 20	CAK**HRQG**FDY	JX028874

Bold letters represent amino acids in randomized positions.

Results and discussion

Generation of ssDNA by lambda exonuclease digestion

To obtain ssDNA, we chose lambda exonuclease as it has processive 5′→3′ exoDNase activity selectively digesting the 5′-phosphorylated strand of dsDNA with 20 times more affinity for a 5′-phosphorylated terminus than a 5′-hydroxylated terminus (44). Although lambda exonuclease selectively digest its preferred substrate, the 5′-phosphate group, it also possesses very low background activity on single-stranded and non-phosphorylated DNA templates. Thus, it is crucial to optimize enzyme concentration and incubation time to generate sufficient single-stranded material.

For enzyme optimization, 1 µg of a dsDNA template phosphorylated at only one end was incubated with 5 U or 10 U of lambda exonuclease at 37°C for either 30 min or 1 h. Analysis on a 1% agarose gel showed that 5 U enzyme was insufficient to produce an adequate amount of single-stranded material as a strong residual band of dsDNA was observed. In contrast, the amount of ssDNA template generated using 10 U of enzyme was greater with a lower band intensity of dsDNA template observed. Variations in incubation time (30 min and 1 hr) did not show any difference in the amount of ssDNA product generated (Figure 3A). In the end, we determined the best conditions for ssDNA generation were 1 µg of dsDNA template incubated with 10 U of lambda exonuclease at 37°C for 30min. After digestion with lambda exonuclease, all single-stranded templates have free 3′-OH overhangs for filling in reaction.

To further confirm the successful generation of ssDNA using our optimized lambda exonuclease digest method, PAGE analyses and fluorescence measurements in the presence of SYBR green were performed. Difference between double-strand and single-strand DNA could be clearly observed using 6 M urea PAGE (Figure 3B) as ssDNA shows faster migration (45). Hence, lambda exonuclease successfully generates sufficient

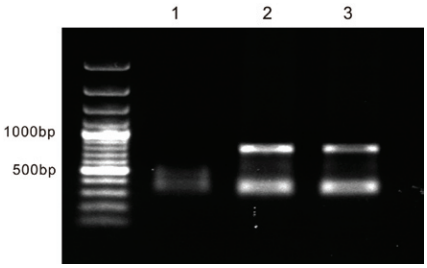

Figure 4. Optimization of incubation time for the KF filling reaction. 20U KF was tested at 37°C with different incubation times on 400 ng complementary overlapping ssDNA VH and VL molecules without temperature cycling ; Lane 1: negative control (sample without Klenow Fragment), Lane 2: 4 h, Lane 3: 8 h.

ssDNA using the optimized conditions. As additional confirmation, we generated fluorescence measurements in the presence of SYBR green. SYBR green strongly binds dsDNA molecules with weaker binding to ssDNA, emitting a strong fluorescence when bound to dsDNA (46). We determined the relative fluorescence reading of SYBR green alone to be 0.2241 rfu. Control samples of oligo20 dsDNA and oligo20 ssDNA gave intensity values of 0.7540 rfu and 0.3821 rfu, respectively. The dsDNA sample treated with lambda exonuclease produced a fluorescence reading of 0.3600 rfu (Figure 3C). This drop in fluorescence signal upon lambda exonuclease treatment to levels comparable to the control ssDNA oligo further confirms the generation of ssDNA template in agreement with the urea PAGE gel results.

Extension of ssDNA using Klenow fragment

To anneal ssDNA fragments prior to Klenow treatment, a mixture of 400 ng of lambda exonuclease digested ssDNA molecules of VH and VL with complementary overlapping ends was heated at 90°C for 5 min and cooled to 30°C for reannealing. This mixture was then treated with KF, which is able to synthesize new complementary DNA strands in the presence of dNTPs (47). KF works by adding bases to the 3′ hydroxyl groups of a blunt-ended DNA duplex, working in a 5′ to 3′ direction (48). To determine the optimal conditions for synthesis of dsDNA, the double-stranded products of different KF titrations were evaluated on a 1% agarose gel.

10 U and 20 U of KF were evaluated for either 4 h or 8 h at 37°C with 400 ng of ssDNA templates. The amount of dsDNA generated with 10 U was insufficient to complete the synthesis of full dsDNA (data not shown).

For 20 U enzyme, a comparison between 4 h and 8 h incubation at 37°C was also tested (Figure 4). Treatment with 20 U enzyme

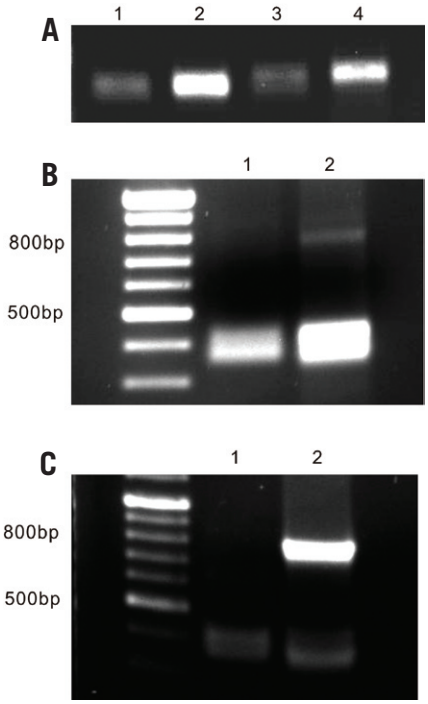

Figure 5. Klenow fragment reaction on ssDNA templates under different conditions analyzed on a 1% agarose gel. (A) Filling in reaction with KF on a single ssDNA template; Lane 1: Negative VH control (sample without KF), Lane 2: VH with KF, Lane 3: Negative VL control (without KF), Lane 4: VL with KF. (B) Annealing efficiency of VH and VL ssDNA without temperature cycling; Lane 1: negative control (without KF), Lane 2: VH and VL with KF. (C) Annealing efficiency of VH and VL ssDNA with temperature cycling; Lane 1: negative control (without KF), Lane 2: VH and VL with KF.

for 4 h and 8 h did not yield a significant difference in the amount of dsDNA template generated.

When applying non-cycling conditions, we did note a significant generation of dsDNA from the individual 350 bp ssDNA templates (Figure 4). We rationalized that this increase in band intensity was likely caused by residual dsDNA from the exonuclease reaction. We examined this effect by incubating VH and VL ssDNA templates separately with 20 U of KF for 4 h. Similar amounts of ssDNA template (400 ng) were used, and band intensity of KF treated samples visualized on a 1% agarose gel increased (Figure 5A). This demonstrates the presence of residual dsDNA template after exonuclease treament. However, we hypothesized that even with a small amount of leftover dsDNA template, an increase in annealing efficiency would reduce background dsDNA without the need for sample clean up.

To test this, we compared the influence of annealing efficiency under temperature cycling and non-cycling conditions. 400ng of VH and VL ssDNA template were mixed

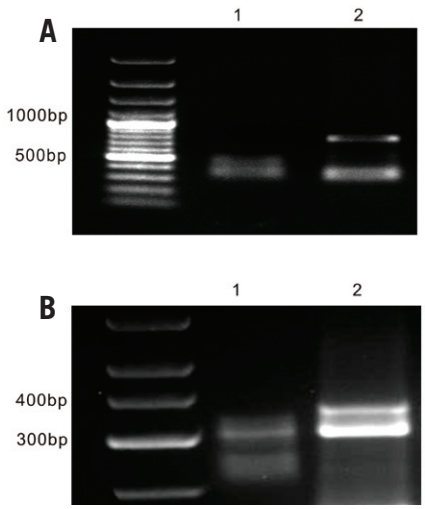

Figure 6. Klenow fragment filling reaction for individual hybrid and library generation. Generation of new hybrids with KF after successful annealing. (A) Chain shuffling between heavy and light chains from two different anti-eGFP scFv clones; DNA marker (100 bp plus ladder); Lane 1: negative control (without KF), Lane 2: new hybrid formation. (B) CDR3 mutagenesis of a scFv; DNA marker (Low range marker); Lane 1: negative control (without KF), Lane 2: new hybrid formation.

in a reaction tube and incubated. Agarose gel visualization of dsDNA formation under non-cyclic conditions showed a weak intensity band for the targeted 700 bp fragment with a stronger intensity band for the individual VH and VL ssDNA templates at 350 bp (Figure 5B). On the other hand, the band intensity after cycling steps showed a strong increase in the properly formed VH and VL hybrid templates (700 bp) with a coordinated reduction in the individual ssDNA templates (350 bp), indicating that cycling conditions greatly improve ssDNA annealing efficiency to form proper dsDNA (Figure 5C).

We concluded that for 400 ng of ssDNA template, temperature cycling is best for ssDNA template annealing followed by incubation at 37°C with 20 U of KF for 4 h to generate sufficient dsDNA template for cloning.

Diversity generation through chain shuffling

The ability to shuffle between heavy and light chains of an antibody is vital. Heavy chains play a crucial role in affinity, while light chains are more involved in antibody specificity (49). To demonstrate chain shuffling using our new ssDNA protocol, heavy and light chain primers were designed to give products with a complementary stretch of nucleotides and only a single 5′-phosphorylated end. As lambda exonuclease digests along the 5′-phosphorylated strands of both chains, this will lead to the sense strand of the

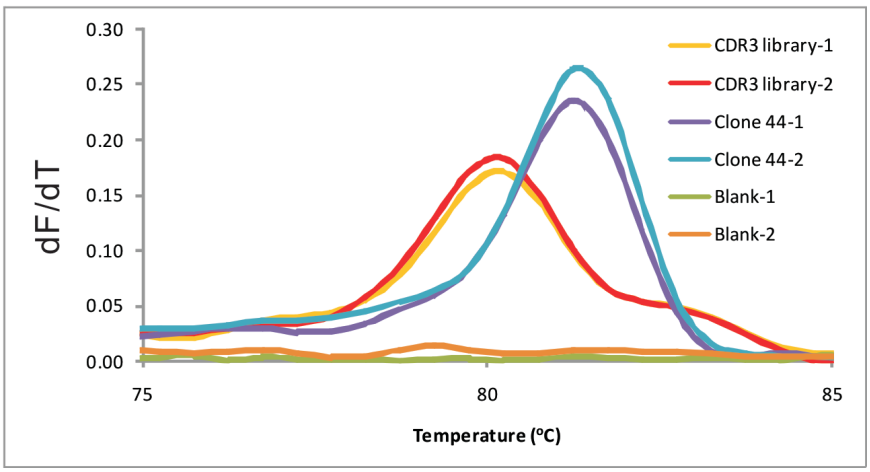

Figure 7. Re-melting curves for library diversity using the DiStRO assay. CDR library 1 and 2 are duplicates of the generated CDR3 library. Clones 44–1 and 44–2 are duplicates of a single scFv clone, 44. Blank 1 and 2 are duplicates of a SYBR green run without DNA samples.

heavy chain and antisense strand of the light chain remaining following enzyme treatment. Subsequently, the VH sense and VL antisense strands can anneal at the complementary region resulting in ssDNA templates of the scFv construct.

We performed our chain shuffling experiments as illustrated in Figure 2A. The VH and VL regions from anti-eGFP scFv clones 44 and G1 were amplified. Lengths of both fragments are approximately 400bp, and a negative control without the addition of the KF showed no band formation at 800 bp, while the sample with KF resulted in a band at the expected size (Figure 6A). The resulting 800 bp constructs then were ligated into the phagemid vector pIT2 and 10 clones were randomly selected and sequenced. All 10 clones sequenced showed successful exchange of the VL chains (Table 1).

By using our ssDNA and KF approach, we eliminated the primerless PCR step that is widely used in chain shuffling to get a full-length sequence. The primerless PCR step is cumbersome, as DNA smearing often occurs due to amplification of non-full-length sequences. The use of KF to generate full length sequences is easier, as it involves only one incubation step at 37°C (50).

Next we applied this protocol in the construction of a mini-library consisting of the heavy chain from anti-eGFP clone 44 and a natural pool of human kappa variable light chains (VK family 2, 4, and 6 genes) from human donors obtaining a clonal diversity of 2×10^8. The library size was estimated by a standard titration method (51). As the scFv heavy chain originates from a single clone, the only variability can be found in the light chain. Therefore, the method aims at producing new variants of a scFv with a given characteristic, such as binding to the same epitope on the target molecule with another affinity. A total of 12 clones were randomly picked and analyzed by DNA sequencing of both heavy and light chains. Ten clones (83%) showed successful introduction of new light chains from a human VK246 pool (Table 2).

Diversity generation with synthetic CDR3 oligonucleotides

To demonstrate another possible application of our directed evolution approach, we performed CDR3 mutagenesis where the 5′-phosphorylation was placed along the antisense primer binding to VH framework 3. The VH region was amplified by PCR and single strand templates were generated using lambda exonuclease according to the protocol described above. The synthetic CDR3 region is a 72-base antisense oligonucletide with a 9bp complementary region to VH framework 3 for annealing. Mutagenesis of CDR3 was accomplished through the incorporation of degenerate bases, as illustrated in Figure 2B. The resulting NNK degeneracy (where N represents a 25% use of adenine, thymine, guanine and cytosine nucleotides; and K represents a 50% mix of thymine and guanine) reduces the standard genetic code from 64 to 32 codons. It allows the coding of all 20 amino acids and eliminates 2 of 3 possible known stop codons (50). Next, the ssDNA of the VH and the synthetic CDR3 were mixed and extended using KF as described before. Agarose gel analysis demonstrated successful ligation as well as dsDNA formation of the CDR3 region with the VH backbone (Figure 6B).

The method was successfully used to generate a mini scFv library with a randomized CDR3 containing 6×10^7 clones. Out of the 27 clones selected for sequencing, 20 clones (75%) showed amino acid sequence variability in the CDR3 region (Table 3). All 4 degenerate positions along the CDR3 region were randomized without any repetition in the sequences of new hybrids. The measure of success for this newly developed protocol is the ability to generate a highly diverse collection of genes. Several assays such as Amplicot (52), DiVE (53) and DiStRO (54) have been developed to assay combinatorial nucleotide library diversity. The diversity of the generated CDR3 library was evaluated using DiStRO assay, which is a method designed to monitor nucleotide diversities based on DNA re-annealing kinetics (52). The re-melting curve from the assay is correlated to the diversity of a library. For a highly diverse library, more imperfect heteroduplexes are formed and the re-melting profile will be shifted. As shown in Figure 7, the re-melting curve of the CDR3 randomized library shows a left sided shift when compare with the control sample, anti-eGFP 44. The re-melting point of the library is around 80°C, which is within the range of the standard 12N library from DiStRO (54).

In summary, we demonstrate successful randomization at CDR3 using our new simplified directed evolution protocol, while framework sequences were unaffected and chain shuffling. This protocol allows regionally pre-determined randomization of genes. The success rate for the method is 75% to 83%, which falls well within range of other protein evolution methods, such as DNA shuffling (84%) (3), synthetic shuffling (72%) (16) and StEP (84%) (17).

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Competing interests

The authors declare no competing interests.

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