Benchmarks

Gene and library synthesis without amplification: polymerase step reaction (PSR)

Zhuo-Bin Lee, Christopher Firnhaber, Jesse Clarke, and Brian S. DeDecker

*Department of Molecular, Cellular and Developmental Biology,
University of Colorado, Boulder, CO*

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Current gene synthesis methods often incorporate a PCR amplification step in order to yield final material sufficient for resolution from multiple off-products. These amplification steps can cause stochastic sampling effects that propagate errors in gene synthesis or decrease variability when applied to the construction of randomized libraries. We have developed a simple DNA polymerase-based gene synthesis reaction, polymerase step reaction (PSR), that assembles DNA oligonucleotides in a unidirectional fashion without the need for amplification. We demonstrate that PSR is efficient, with little off-product production, no detectable error propagation, and maximized variability in the synthesis of a phage display library.

The prevalence of PCR amplification found in most gene synthesis methods is often due to the fact that these methods share similar means of assembly. As chemical synthesis is currently limited to synthesizing error-free oligonucleotides of lengths that are often shorter than the desired genes (1), many methods use DNA ligases or polymerases to assemble smaller purified oligonucleotide components into full-length gene products (2–4). However, successful assembly of the full-length sequence becomes dependent on the efficiency of a number of smaller successive reactions. The result of this approach is that the intended product may not

be produced in sufficient quantity—requiring PCR amplification to resolve the intended full-length sequence from the many incomplete and intermediate reaction products also present.

Of these methods, the polymerase cycling assembly (PCA) reaction and its variations are the most widely used of the polymerase-based methods of gene synthesis (5). As the intermediate steps and final product of PCA use PCR-type amplifications, stochastic sampling may amplify errors found in the initial oligonucleotide pools, thus requiring a linear amplification approach (6). PCR-type steps could also compromise the construction of libraries with targeted

randomization. Exponential amplification steps could reduce potential variability in the library by amplifying only a subset of the variation in the early rounds of thermal cycling. As such, improved gene synthesis methods without exponential amplification are desired to minimize error propagation and construct libraries with maximal diversity. For this reason, we developed a polymerase-based method to assemble genes in a linear fashion that resembles solid phase and non-polymerase based technologies (7–10).

Here we present an alternative method for the assembly of DNA oligonucleotides without amplification: polymerase step reaction (PSR). In PSR, a single forward primer is mixed with multiple template primers in the reverse direction. Importantly, the template primers are blocked with a propyl group on the 3' end, preventing their extension during thermal cycling (11). With the activity of the DNA polymerase effectively limited to the extension of the forward primer, any annealing and extension that does occur only serves to ratchet forward the construction of the primary gene product, all without amplification. This is in contrast with PCA, in which a pool of assembly primers extends in both directions to mostly generate off-products while the full-length sequence is undetectable and must be further amplified by PCR (12,13).

One strategy often employed during library synthesis is to incorporate targeted variation within the non-overlapping regions of the assembly primers (14). We employ a similar method using PSR and suggest that library diversity is likely maximized in the absence of amplification.

As a proof of principle, we assembled a library using the scaffold of the middle 288 bp of a single heavy chain variable (V_H) antibody domain (15). This monomeric domain is similar to camelid heavy chain antibodies but is designed to be completely humanized. Relatively small and efficient, the V_H domain is a promising scaffold for building next-

METHOD SUMMARY

Here we present a polymerase-based gene and library synthesis method, polymerase step reaction (PSR), that is unidirectional and does not require PCR-type amplifications. Error propagation due to stochastic sampling effects is eliminated, and full variability is obtained in the construction of a phage display library.

Figure 1. Polymerase step reaction (PSR). (A) Schematic diagram of PSR: (1) Forward primer in black is mixed with a collection of successive 3' blocked reverse direction templates. During thermal cycling, the product is ratcheted forward by DNA polymerase without off-product formation or exponential amplification. The positions of the three variable helices (H1–3) of the V_H domain are shown in red. (2) The single-stranded DNA (ssDNA) product can be filled in with an extendable reverse primer in a second thermal cycling reaction to produce a double-stranded DNA (dsDNA) product. (B) The ssDNA PSR product with some blocked primers associated (1) and the filled in dsDNA product (2) are shown on a 1.2% agarose gel stained with ethidium bromide. The markers are a 100 bp DNA ladder (New England Biolabs, Ipswich, MA).

generation diagnostic reagents and therapeutics. The V_H domain contains three separate variable loop regions, ideal for demonstrating the simultaneous incorporation of both dispersed and targeted variability by PSR (Figure 1A). Primers with an average length of 92 bases were assembled, with an average overlap of 27 bases. The 3 variable regions are separated by gaps of 58 and 115 bases, with a total of 20 variable nucleotide positions distributed between them (Figure 1A). With this construct we tested whether PSR could incorporate variability with minimal redundancy and minimal error propagation in the final pool. In addition, we used PSR

to synthesize a larger 758 bp product without any variability incorporated (Supplementary Material and Supplementary Figure S1).

The forward and template primers were synthesized and polyacrylamide gel electrophoresis (PAGE) purified by Gene Link (Hawthorne, NY). Forward primer (100 pmol) was mixed in a 2:1 molar ratio with each of the 3 blocked template primers. Reactions used KOD polymerase (EMD Millipore, Billerica, MA), and cycling was performed using a Bio-Rad T100 Thermal Cycler (Bio-Rad, Hercules, CA). Cycling conditions were as follows: 95°C for 2 min, then 30 cycles at 95°C for 20 s and 70°C for 2

min, followed by a 70°C hold for 5 min. Samples were then cooled and held at 4°C.

The single-stranded DNA (ssDNA) synthesis product was isolated from free primer on a PureLink PCR purification column (Life Technologies, Carlsbad, CA). The yields were measured by UV absorbance on a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE). Approximately 51 pmol of ssDNA product (with some blocked primers still associated) was recovered from the reaction, giving a yield of 51% compared with forward primer input.

Next, approximately 24% of the ssDNA from the PSR reaction was annealed to a complementary fill-in primer and made double-stranded with KOD polymerase. Thermal cycling is required as some blocked primer is expected to carry through to this stage and must be competed off. Thermal cycling conditions were as follows: 95°C for 2 min, then 25 2-step cycles at 95°C for 20 s and 70°C for 3 min. Samples were then cooled and held at 4°C.

The double-stranded DNA (dsDNA) product was isolated by agarose gel (1.5%) electrophoresis and purified using

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A megaprimer strategy similar to QuikChange (Agilent Technologies, Santa Clara, CA) was used to clone the full-length filled-in PSR product into a phage display vector containing the V_H domain fused to the P3 coat protein (16). After *DpnI* digestion of the product, 1 μ l was transformed into Top10 Competent Cells (Life Technologies, Carlsbad, CA). Of ~800 colonies per plate, 93 were prepared with QIAprep columns (Qiagen, Hilden, Germany) and sequenced. All sequences contained the PSR synthesis product with variability incorporated into all 20 nucleotide sites. Of the sequences, 67% were considered viable without frame shifting insertions or deletions, of which 10% had a substitution mutation outside of the designed variable sites. Of the frame-shifted sequences, 61%

Overall, the uniqueness of the errors sequenced and the full variability of engineered sites demonstrates that PSR assembles DNA oligomers without stochastic sampling effects. The simple, one-day PSR synthesis reaction will allow for primary and secondary maturation libraries to be made quickly and efficiently. In principle, any scaffold could have variability incorporated by

Primer 1								Primer 2								Primer 4							
CDR H1				CDR H2								CDR H3											
Kabat numbering																							
K	30	31	32	33	51	52	52a	53	54	55	56	57		94	95	96	97	98	99				
AAA	NDT	NDT	TAT	...	ATT	NDT	CCG	NDT	NDT	GGC	NDT	ACT	...	TGC	NDT	NDT	NDT	NDT	TCG				
AAA	AAT	CGT	TAT	...	ATT	AGT	CCG	ATT	AAT	GGC	GAT	ACT	...	TGC	CAT	CGT	AAT	TGT	TCG				
AAA	AGT	TAT	TAT	...	ATT	GAT	CCG	AAT	AAT	GGC	TAT	ACT	...	TGC	GTT	GGT	TTT	CAT	TCG				
AAA	TAT	AGT	TAT	...	ATT	ATT	CCG	GTT	AAT	GGC	AGT	ACT	...	TGC	GGT	AAT	CGT	CAT	TCG				
AAA	TTT	AAT	TAT	...	ATT	AGT	CCG	AGT	CGT	GGC	GAT	ACT	...	TGC	GTT	GGT	AGT	TGT	TCG				
AAA	TTT	GAT	TAT	...	ATT	CTT	CCG	GGT	ATT	GGC	TTT	ACT	...	TGC	AAT	GGT	AGT	CAT	TCG				
AAA	GAT	TGT	TAT	...	ATT	TTT	CCG	TAT	CAT	GGC	TTT	ACT	...	TGC	AAT	TAT	AGT	GGT	TCG				
AAA	GAT	CGT	TAT	...	ATT	TTT	CCG	CAT	AGT	GGC	TTT	ACT	...	TGC	GAT	GGT	AAT	GAT	TCG				
AAA	GAT	CGT	TAT	...	ATT	GGT	CCG	AGT	GTT	GGC	AAT	ACT	...	TGC	AGT	AAT	ATT	TGT	TCG				
AAA	GGT	CTT	TAT	...	ATT	TAT	CCG	AGT	TGT	GGC	AAT	ACT	...	TGC	GTT	TGT	ATT	AGT	TCG				
AAA	CAT	TAT	TAT	...	ATT	CAT	CCG	CTT	CGT	GGC	AAT	ACT	...	TGC	CGT	AAT	GTT	AGT	TCG				
AAA	CTT	AAT	TAT	...	ATT	AGT	CCG	GAG	AGT	GGC	TAT	ACT	...	TGC	CGT	CGT	TGT	AGT	TCG				
AAA	CGT	GGT	TAT	...	ATT	GTT	CCG	GGT	CGT	GGC	CTT	ACT	...	TGC	TAT	TAT	CTT	CGT	TCG				

Figure 2. A sample of the sequenced polymerase step reaction (PSR) product clones without frame shifting insertions or deletions (67% of total). The variable region of the forward primer (H1) contained 9 duplications in the sample, as expected from a fully randomized population (example duplication in cyan). The variable regions of primers 2 and 4 (H2 & H3) showed no duplications in the sequenced sample, as expected. Errant substitution mutations (example in green) were found in 10% of the viable sequences, but were derived from unique original primers.

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PSR in the non-overlapping areas of the oligonucleotide assembly. Other applications for PSR may be found in the synthesis of single genes without errors. Current methods of error removal require unique errors that are revealed by mismatches and endonuclease cleavage (3,17). PSR synthesis could be ideal for this and other applications where off-products and amplification of initial DNA synthesis errors during gene assembly is undesirable.

Author contributions

Z.-B.L., C.F., J.C., and B.S.D. designed and performed the experiments. B.S.D. conceived the method. Z.-B.L., C.F., J.C., and B.S.D. analyzed the data and wrote the manuscript.

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Competing interests

The authors declare no competing interests.

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Address correspondence to Brian S. DeDecker, University of Colorado at Boulder, MCD Biology, 347 UCB, Boulder, CO 80309. E-mail: dedecker@colorado.edu

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