Single-Primer Polymerase Chain Reactions

Abstract

The Polymerase Chain Reaction (PCR) [1] is an indispensable procedure in molecular biology. The success of the reaction depends crucially on the successful annealing of the two oligonucleotide primers to their targets, ideally at the same temperature. Extensive research has gone into developing primer design tools [2–8] to optimize their sequences to avoid laborious optimization conditions at the bench. We present here a simple method of modifying target sequences, whether short dsDNA constructs of circular plasmids, such that one oligonucleotide can act as both the forward and backward primers, thereby eliminating the need for optimization whether computationally or at the bench. Furthermore, the modification can be made such that such 2-way primer (2WP) can anneal to its two targets at the extension temperature, thereby eliminating the need for an extra annealing step.

Polymerase chain reaction (PCR) is an indispensable molecular biology technique used to selectively amplify a segment of DNA using a thermostable polymerase that extends two short oligonucleotides that complement the 3' ends of the target sequence. The annealing of the two primers to the target extremities at the same temperature is the most important factor in determining the success of the reaction, as misannealing of either or both to non-target regions (or to themselves, "primer-dimer") can lead to erroneous ampilicons. Having different annealing temperatures can also hinder the success of the reaction, requiring the setting of the reaction annealing temperature to the lowest of the two which can also lead to various problems. Primer design has therefore emerged as one of the most important steps to ensure successful reactions with high yields. There exist many bioinformatic tools to help achieve that, but in practice some primer pairs may still not work well together, often resulting in laborious reaction optimization at the bench. Furthermore, when the GC content of the two extremities are greatly different, it may become a difficult task to find a suitable primer pair, especially when the choice of primer locations is limited. For example, one primer at one extremity may be a T7 promoter while the other is part of a GC-rich coding sequence at the other extremity. Here we present a simple technique of inserting the complement of one primer into one extremity, thereby allowing the use of one primer to anneal to both extremeties of the target ampilicon, thereby eliminating the need to optimize the design or reaction conditions that suit two different primers. The involved procedure employ standard molecular biology procedure (phosphorylation, digestion, and ligation) and does not require any special sequence modification beside the insertion of one sequence. The method can simplify the engineering and mass production of plasmids and custom in-vitro transcription templates (for example in gene (protein) design for example). Designing the 2-way primer to have an annealing temperature equals to that of the extension also eliminates the need for an annealing step during the cycling program of a reaction, resulting in a significant reduction of total reaction time.

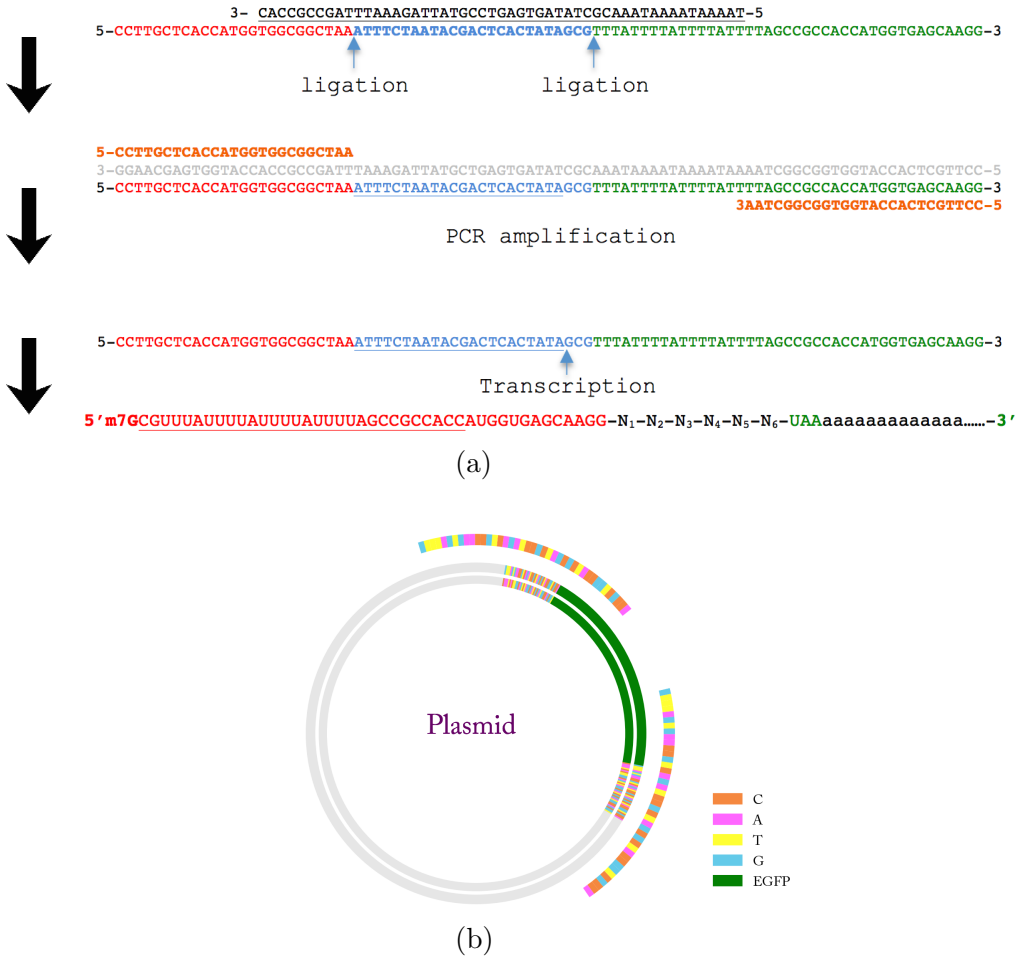

Figure 1: Construction of dsDNA templates ready for 2WP amplification

1 Results

Here we present ultraviolet images of gel electrophoresis showing DNA templates, RNA transcript, and RNA ligation results. Figure 2 (a) and (b) show double-stranded DNA (dsDNA) templates encoding nodes N0 to N6 and edges E0 →E1 to E5 →E6, respectively. The inset shows the reference molecular marker (ladder) used to verify that the observed length of each dsDNA template appears on the gel at the expected length. The PCR product of N6 dsDNA template (Figure 4a, well 7 from the left) shows erroneous bands, so the correct band is gel-excised under ultraviolet visualization and purified using the crush-and-soak method [28]. The transcription of N6 results in a clean band corresponding to the expected length of 98-nt (well 8 in Figure 4b). RNA transcripts of E0→E1, E1→E2, E2→E3, E3→E4, E4→E5, and E5→E6 in Figure 4b (wells 10-15) show smears as they are loaded immediately from in-vitro transcription reaction without purification, while purified transcripts of N0 to N6 are purified prior to loading (Zymo Research kit # R1019). The length of an RNA transcript is the length of its corresponding DNA template minus the T7 promoter region (25-bp) and leading sequence (25-32 bp). For example, the length of N6's RNA transcript is 98-nt = 152 - 29 – 25 (152-bp total dsDNA template length – 29-bp (leading) – 25-bp T7 (promoter)).

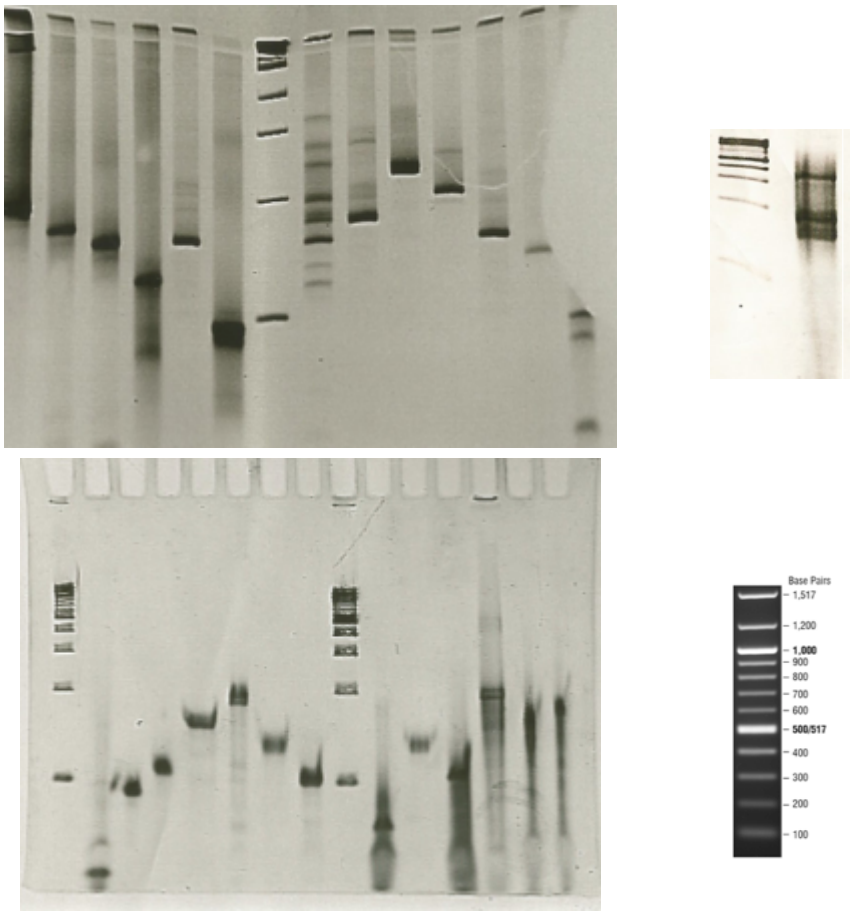

Figure 2: Gel electrophoresis results of DNA templates, RNA transcripts, and RNA ligation products. Inset: standard 100-bp molecular marker (NEB). (a) Double-stranded DNA (dsDNA) template strands encoding for nodes & edges, run on 4% non-denaturing polyacrylamide gel. Wells left to right, w1-w7: templates N0 to N6 of lengths 91, 134, 151, 194, 224, 170, and 152 base pairs (bp), respectively; w8: marker (inset); w9-14: partial set of dsDNA templates encoding for edges E0→E1, E1→E2, E2→E3, E3→E4, E4→E5, and E5→E6 of lengths 70, 123, 98, 182, 133, 149 bp, respectively. N6 template (well 7) shows erroneous PCR byproducts subsequently excluded by excising the correct band & crush-and-soak purifying it [28]. Each sequence is primed upstream with a T7 promoter. (b) Partial set of purified in-vitro transcribed RNA nodes & edges run on 4% TBE-Urea denaturing polyacrylamide gel; left to right, w1/w9: marker (inset), w2-w8: RNA transcripts of N0 to N6 with lengths 44, 87, 102, 147, 177, 120, 98-nt, respectively; w10-w15: RNA transcripts of edges E0→E1, E1→E2, E2→E3, E3→E4, E4→E5, and E5→E6 with lengths 70, 123, 98, 182, 133, 149-nt, respectively. (c) Example RNA ligation; w1: marker (inset); w2: ligation of N3 RNA transcript (147-nt) to N4 (177-nt) by splint ligation with edge 3→E4 transcript (182-nt) to form a 324-nt RNA strand (ligation product).

2 Method

DNA templates were constructed using ligation from smaller synthesized oligonucleotides (Biocorp). Segments of each node/edge sequence were concatenated using splint ligation with T4 DNA ligase. The following example illustrates the assembly and ligation of N0's DNA template:

The splint oligonucleotide (black, underlined) and the constituent oligonucleotides of a node (red, blue, and green) were mixed at 10 uM concentration each in a 30-ul ligation reaction (50 mM Tris-HCl, 10 mM MgCl2, 1 mM ATP, 10 mM DTT. 13u/ul T4 DNA ligase (NEB)) for 1 hour at room temperature. Donor oligonucleotides (blue and green) are phosphorylated with T4 Polynucleotide Kinase (PNK) (NEB) prior to ligation, and in the same ligation reaction conditions.

PNK adds a phosphorous residue at the 5' end of donor strands, a prerequisite for ligation.

The ligation product is used as template in a PCR reaction. 0.5 ul of ligation reaction (5 picomole total concentration) is used as template in a 25-ul PCR reaction using Phusion DNA polymerase (NEB) and carried out for 40 cycles. By design, one oligonucleotide serves as both the forward and backward primer (since the primer sequence, red in this example, is purposefully ligated 5' of the template). This eliminates the need to optimize melting temperature condition to satisfy two primers. The following illustration shows the resulting dsDNA template from PCR for N0, with primer sequence shown in bold orange, and the T7 promoter region underlined:

PCR products are used as template in in-vitro transcription (IVT) reactions at a concentration of 20 ng/ul in total reaction volume of 50ul (40 mM Tris-HCl, 6 mM MgCl2, 1.5 mM DTT, 2 mM spermidine, 1U/ul T7 (NEB)). The reaction is carried out for 2-4 hours at 37 degrees Celsius and subsequently treated with 5 units of DNAse I (NEB). Transcription reactions contained 2mM concentration of each NTP, except for N0 where GTP was added to 0.5mM concentration while m7G analog (NEB) was added to 4 mM concentration (to facilitate ribosomal translation of transcripts beginning with N0). In IVT reactions of N1 to N6, guanosine monophosphate (GMP) (Sigma) was added to a 2mM concentration while guanosine triphosphate (GTP) was added to a 0.5mM concentration (to facilitate RNA ligation, since ligase requires monophosphate at the 5' donor RNA). The example below shows N0's IVT, with the arrow indicating the transcription start site of T7 polymerase. In all templates, the 1st transcribed base is G (preferred by T7) and the 2nd/3rd are CG when possible, as this has been shown to further improve transcription yield [26]:

(4) N6 RNA sequence is polyadenylated using E. coli. poly(A) polymerase (NEB) in a total reaction volume of 10ul at concentration of 5 ng/ul (50 mM Tris-HCl 250 mM NaCl 10 mM MgCl2, 0.5U/ul poly(A)), in order to facilitate ribosomal translation of sequences ending with N6 since the ribosomal translation mix to be used is from eukaryotes (Promega's Human In Vitro Translation system) and polyadenation is a prerequisite for mRNA stability and successful translation [27].

The RNA transcripts are ligated using T4 RNA Ligase 2 (NEB) at a concentration of 10uM each transcript (nodes and edges) in a total reaction volume of 30ul (50 mM Tris-HCl 10 mM MgCl2 2 mM DTT, 1U/ul T4 Ligase).

The resulting sequences, if ligated, represent the mRNA sequence encoding for the enhanced fluorescent green protein (EGFP). Underlined sequence = ribosome binding site (RBS); AUG = start codon, which is part of N0, UAA=stop codon, which is part of N6; lower-case sequence at the 3' = polyadenylation of N6):

References

1. Saiki, R. K. *et al.* Primer-Directed Enzymatic Amplification of DNA. *Science* **239,** 487–491 (1988).
2. Apte, A. & Daniel, S. PCR Primer Design. *Cold Spring Harbor Protocols* **2009,** pdb–ip65 (2009).
3. Hyndman, D. L. & Mitsuhashi, M. PCR Primer Design. *PCR protocols,* 81–88 (2003).
4. Neff, M. M., Turk, E. & Kalishman, M. Web-Based Primer Design for Single Nucleotide Polymorphism Analysis. *TRENDS in Genetics* **18,** 613–615 (2002).
5. Singh, V. K. & Kumar, A. PCR Primer Design. *Mol. Biol. Today* **2,** 27–32 (2001).
6. Vandenbroucke, I. *et al.* PCR Primer Design. *Biotechniques* **51,** 322–327 (2011).
7. Ye, J. *et al.* Primer-BLAST: A Tool to Design Target-Specific Primers for Polymerase Chain Reaction. *BMC Bioinformatics* **13,** 134. ISSN: 1471-2105 (2012).

8. Yuryev, A. *PCR Primer Design* (Springer Science & Business Media, 2007).