Expand High Fidelity PCR Reactions

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Purpose: to generate PCR products with a minimum of mutations.
Note: while the frequency of errors is extremely small, the resulting PCR product must be sequenced in its entirely to ensure that there are no errors. This is essential for PCR products that derive from protein coding regions! WEAR GLOVES AND USE BARRIER TIPS FOR ALL STEPS BELOW.

1. Purify and determine concentration of oligonucleotides. If oligos are delivered in ammonium hydroxide, purify them by passing them over a Pharmacia MicroSpin S200 column (100ul oligo applied to a prespun column—see column protocol for details). For oligos that are supplied as lyophylates, resuspend at 1ug/ul in 1XTE. Read the concentration of this purified oligo.

2. Thaw two components of the Roche Expand High Fidelity PCR Kit: Tube 1 is the enzyme and must always be kept on ice or in a benchtop freezer. Tube 2 is the 10X reaction buffer. Thaw at room temperature of 37 degrees C and then place on ice.

3. Prepare template to be used in PCR reaction: Genomic DNA is used at full concentration (typically 0.1 – 1ug/ul), while pure plasmid DNAs should be diluted 1:10 in dH2O to a working concentration of 30-100 ng/ul .

4. Set up PCR reaction. Use individual tubes with lids, not strips of tubes and keep all components and tube on ice while setting up the reaction:

   Add in order:
   _____ul of dH20 for a final reaction volume of 50ul
   5 ul of Buffer in Tube 2 (supplied as a 10X solution)
   1 ul of DNA template (genomic DNA or diluted plasmid DNA)
   300ng of oligo 1
   300ng of oligo 2
   4 ul of dNTP (deoxyribonucleotides)(2.5mM concentration)
   0.75 ul of Enzyme in Tube 1
   Mix well by vortexing or flicking and then spin or flick hard to collect reaction volume at bottom of tube.

5. Program PCR cycles:

For products shorter than 3Kb:
1] 94C 2 min.
2] 94C 15 sec.
3](optimal annealing temperature) 30 sec.
4] 72C 1 minute/1Kb of product
5] go to 2] 9 times
6] 94C 15 sec
7](optimal annealing temperature) 30 sec.
8] 72C 1 minute/1Kb of product + 5 sec./cycle
9] go to 6] 9 times
10] 72C 7 min.
11] 4C 10 min.
12] end

For products longer than 3Kb:
1] 94C 2 min.
2] 94C 15 sec.
3](optimal annealing temperature) 30 sec.
4] 68C 1 minute/1Kb of product
5] go to 2] 9 times
6] 94C 15 sec
7](optimal annealing temperature) 30 sec.
8] 68C 1 minute/1Kb of product + 5 sec./cycle
9] go to 6] 9 times
10] 68C 7 min.
11] 4C 10 min.
12] end
(extension time should be programmed using the 1 minute/1 Kb of predicted PCR product size: a 3 Kb product should have 3 min. extension, an 8 Kb product should have 8 min. extension, etc.; minimum extension time is 30 sec.)
(optimal annealing temperature is determined empirically: oligos with restriction sites should have 18-24 nucleotides of homology to target and 10-15 nucleotides of restriction sites; these are usually annealed at 52C to 55C)

6. Cast an agarose gel of appropriate % according to expected PCR product size with ethidium bromide and wide sample well combs. Add 5 ul loading dye to PCR products and load into gel, leaving 1 empty well between each sample and between molecular weight marker/DNA ladders. Ladder should be loaded on each end of the gel.
7. Run gel until bands are resolved; photograph; cut out band with a clean razor blade (wiped down with an ethanol-treated Kimwipe). Measure the weight of the

gel slice and purify DNA according to the instructions included with the Qiagen QIAquick Gel Extraction Kit (Cat. # 28706). Elute DNA with 30 ul EB (elution buffer).

8. Ligate PCR product to pGEM-T vector: Mix together 4 ul of purified PCR product + 1 ul pGEMT-T + 5 ul Takara Rapida DNA Ligation Kit Component Tube 1. Incubate at 16C for 60 min.
9. Transform 5 ul of ligation to competent cells. Freeze rest of ligation for possible future use. Plate transformed bacteria on LB+ampicillin plates pretreated with 50 ul 40mg/ml X-GAL (dissolved in N,N-DMF or DMSO; stored at –20C) and 50 ul of 0.1M IPTG (dissolved in dH2O and filter sterilized; stored at 4C).
10. The next day, pick WHITE colonies and grow up minipreps. Disregard BLUE colonies.