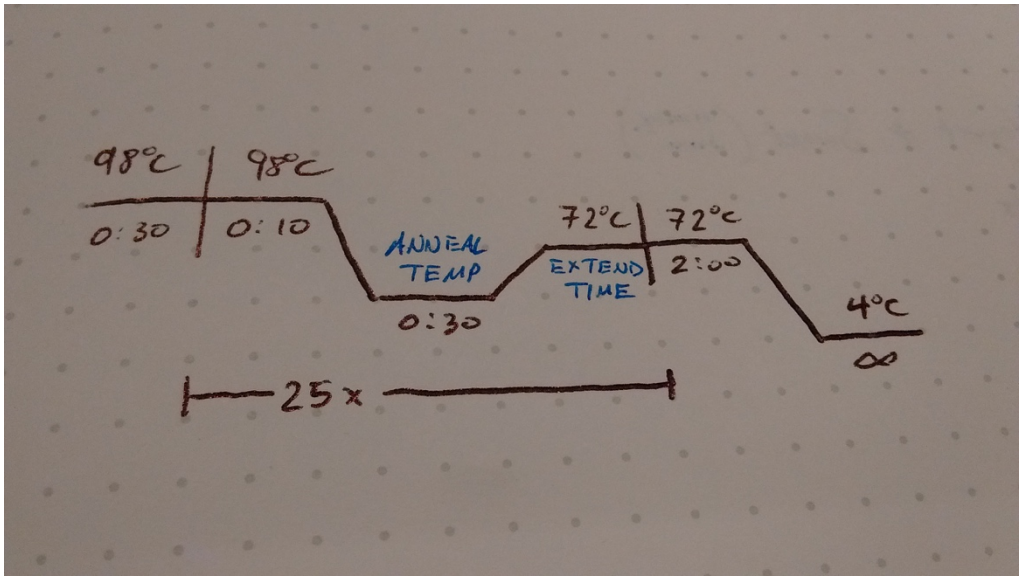
PCR

Overview

This protocol covers PCR with Q5 High Fidelity DNA Master Mix, using plasmid minipreps as the DNA template. PCR is used to amplify a specific region of DNA specified by the choice of primers. Generates a high concentration of linear double-stranded DNA fragments.

This protocol assumes a basic familiarity with the principles of PCR. We always start with a 30 second 98C denaturation, and end with a 2:00 minute extension at 72C. Each cycle starts with a 10 second denaturation, followed by a variable temperature anneal for 30 seconds, and then a variable time 72C extension. We usually run 25 cycles. For reference, here is a diagram of the typical cycling program.



This protocol is written from the perspective of performing a single 25µL PCR, but can obviously be extended to multiple samples. For PCRs with volumes greater than 25µL (up to 100µL), reaction can be scaled up appropriately.[i] It does not cover the creation of master mixes.

Materials

- .2mL Tubes
- Nuclease Free Water
- Plasmid DNA
  - Concentration should be ~1nM.[ii]
- 10µM Primers

[i] However, often DNA concentration can remain constant if desired.

[ii] Often PCR guides will give DNA in units of ng/µL (mass per volume). For most plasmids we are working with 1nM is ~1-4ng/µL.

- Q5 2x High Fidelity 2x Master Mix (MM)
    - Note that we have both hot start and non-hot start versions.
    - Do not confuse with 2x HiFi MM, which is several times more expensive.

Procedure

1. Determine and locate correct miniprep and primers. Retrieve from -20C and wait to thaw.
    - You may thaw in your hand if you like
2. While primers thaw, determine the correct annealing temperature and extension time for your reaction.
    - Annealing temperatures and Tms can be calculated from sequences using http://tmcalculator.neb.com/#!/main. Sequences and their calculated Tms and common annealing temperatures are listed on each primers specification page on Benchling.[iii]
    - Extension times are calculated based on the length of the intended product. Use 30 seconds per kb for amplicons smaller than 5kb in size. It is usually recommended to round up. Amplicons less than 1kb in size should use 30 seconds extension time. For amplicons larger than 5kb use 50 seconds per kb.
3. To a .2mL tube, add 9µL of NFW, 1µL of miniprep, and 1.25µL of each primer.
    - To minimize errors, it is recommended that you either check off boxes for each primer, or move each tube after you are done moving it.
4. Retrieve the Q5 2x MM from the freezer in a stratacooler.
5. If the Q5 is frozen, thaw it completely on the benchtop, and gently resuspend the precipitate using a P200.
    - Try to minimize both loss of Q5 and air bubbles introduced.
6. Add 12.5µL of Q5 to tube. Return Q5 to freezer.
    - If using non hot start, once Q5 has been added, minimize time on the bench.[iv] Hot start can sit for a little while longer if needed.
7. Flick tube to mix, and spin down briefly.
8. Turn on thermocycler by flipping switch in the back.
9. Program the thermocycler appropriately. To save time, hit edit, go to PCR and set annealing temperature and extension time to the desired values. Hit run. Make sure to put in 25µL for reaction volume.
10. Return primers and minipreps to appropriate locations in the freezer. After PCR is finished, visualize on gel to confirm amplification.

[iii] Annealing temperature for a given primer pair less than 5C apart is simply the lower Tm +1 C

[iv] This is to avoid non-specific amplification, as well as degradation of primers.