Primer Design & PCR

Introduction

This protocol is designed to give you a basic understanding of how to design a PCR primer, make a thermocycler program, and troubleshoot your PCRs. Note that these are guidelines and not absolute requirements for these protocols to work.

Materials

- Ultrapure Water
- Template DNA
- Forward and Reverse Primers (At 10uM dilutions)
- 2X Q5 Master Mix (Alternatively Q5 Polymerase with Q5 Buffer can be used)
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Procedure

Primer Design (In order of importance)

1. Gibb's Free Energy of Homo/Heterodimerization is less than or equal to -7kCal/Mol
2. Hairpin structures should melt at least 5C below the ANNEALING temperature
3. **MELTING temperatures should be no more than 3C apart**
4. **MELTING temperature should be 60-64C ideally 62C**
5. **GC content between 30% and 70%**
6. **Last base in primer should be a G or a C**
7. Degenerate primers may be used to break up secondary structure or primer dimers if the mutations are silent (Subtract 4C from melting and annealing temperatures in this case).
8. Use the NEB Tm Calculator with 2X Q5 Master Mix to find annealing temp http://tmcalculator.neb.com/ - !/
9. Use the IDT Oligo Analyzer to find hairpin melting temperatures and free energy of homo/heterodimerization https://www.idtdna.com/calc/analyze

Thermocycler Program

10. Use NEB 2X Q5 Master Mix (Not Hot Start)

11. Prepare on Ice in this order

    9uL Ultrapure Water
    1uL Template DNA (Any Concentration less than 1 ug)
    1.25uL 10uM Forward Primer
    1.25uL 10uM Reverse Primer
    12.5uL 2X Q5 Master Mix

12. Follow the below thermocycler instructions

    Use the maximum number for each of the times below
    25 Cycles is usually sufficient but 35 can help with troubleshooting

13. Protocol copied from NEB's website

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25–35 Cycles	98°C	5–10 seconds
	*50–72°C	10–30 seconds
	72°C	20–30 seconds/kb
Final Extension	72°C	2 minutes
Hold	4–10°C	

Troubleshooting

14. If you are getting no bands you are probably using to high of an annealing temperature and primers are not binding. Lower your annealing temperature.

15. If you are getting smeary streaks or multiple bands you are probably using too low of an annealing temperature and have nonspecific priming. Raise your annealing temperature.

16. If you are getting smeary bands below 100bp you have primer dimers and should Gel purify if you are going to be using this in a reaction.

17. If you are having problems with a high GC content template, use NEB's Q5 high GC enhancer. DMSO and Betaine can also be used for this but they will lower the annealing temperature of your primers.