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PCR – High Fidelity

Materials:
Sterile dH_2O
2 U /µl Phusion DNA Polymerase (NEB, F530S)
5X PCR Buffer (comes with Phusion)
10 mM each dNTP solution (Invitrogen 18038-042)
10 - 20 µM Forward and Reverse primers
Agarose
TAE solution (1X and 10X)
Gel casting mold, combs, and electrophoresis apparatus
EZ-vision Loading buffer / DNA dye (Amresco, N472-Q-0.5ml)
1 Kb ladder (Amresco, K181-500µl)

Method:
Do the following methods on ice
1. Prepare a "Master Mix" in a 0.5 ml microfuge tube for your PCR reactions according to the following table
** Make sure to add dNTP's before primers otherwise the Phusion may "chew up" the primers

Reagent (conc in PCR tube)	**1X (µl)**
Sterile dH_2O	12.4
5X PCR Buffer (1X)	4
$MgCl_2$	0
10 mM dNTPs (150 µM)	0.4
5 U/µl Taq (1 U)	0.25
Sub Total	**17**
10 µM Forward Primer (1 µM)	1
10 µM Reverse Primer (1 µM)	1
DNA	1
Total Volume	**20**

2. Add 0.5 - 4 µl of DNA sample to each PCR tube
   0.5 µl for purified plasmid to 4 µl for colony PCR from liquid culture
3. Add Master Mix to each PCR tube
4. If you did not add your primers to the master mix add them now
5. Place your PCR tubes in the thermocycler and run your program[1]
6. While you are waiting for the PCR you can make a gel to check your results
7. In a 250 - 500 ml bottle make up a 1 % agarose solution by adding 0.5 g agarose to 50 ml 1X TAE
8. Microwave agarose and TAE buffer for 1 minute or until boiling
9. Set up gel mold in empty try by placing combs in mold and clamp mold tight
10. Slowly pour agarose TAE solution into gel mold, allow to cool and solidify for 30 min
11. When the agarose gel has solidified carefully and slowly remove comb and place in electrophoresis apparatus, don't worry if some of the wells are stuck closed they will open when you add TAE buffer in the next step. You can also store your gel in the refrigerator overnight. If your using a mold or comb hat is in "high demand" remove the gel from the mold too. Place the gel in a plastic bag so that it does not dry out.

13. Place your gel in the electrophoresis system and fill with 1X TAE to the fill line, you can use the TAE olution that is already in the system, no need to drain and refill.
14. When your PCR is finished, add 5 µl of loading buffer to 20 µl DNA in separate PCR tube
15 Mix 10 µl of 1 Kb ladder with 2.5 µl EZ-vision loading dye
16. Place 5 – 20 µl of PCR product with loading buffer into each well.
17. After loading place cover on electrophoresis system and run gel at approximately 85 V for 30 – 45 minutes
18. Turn power supply off and remove cover. Carefully place gel in an empty tray to carry to the imager.

[1] The program you will run in the thermocycler will depend on the size of your primers and the size of the expected product, and the Taq polymerase you are using, below is an example program

Typical Program
1. 98C – 30 sec
2. 98C – 15ec
3. 60C[*2] – 30ec
4. 72°C – 1.5 min[*3]
5. Cycle to step 2 35 more times
6. 72°C – 4.5 min (3 * the extension time length)
6. Incubate at 16°C forever

[*2] The annealing temperature is the most critical, a rule of thumb is to subtract 5°C from the Tm given to you by the primer 3 web program or you can check this temp by entering your primer sequence in promega's website program getting the "base stacking Tm" subtracting 2 - 3°C and using that as your annealing temperature

http://fokker.wi.mit.edu/primer3/input.htm
http://www.promega.com/biomath/calc11.htm

[*3] The extension time should be ≈ 1.5 – 2 kb per minute (It IS faster than Taq)

[2] The % of agarose in your gel will be determined by the size of the PCR product you are trying to isolate. The smaller the product size (in base pairs) the higher the % of agarose you will need to use. In general 0.5% = 20 kbp – 2 kpb, 1% = 10kpb – 500bp, 1.5% = 5kbp – 250bp, 2% 2.5kbp – 100bp

Phusion DNA polymerase is very thermo stable and can be left out at room temp for days with no effect on activity. Best practice however is to keep Phusion frozen when not in use

PCR Reagents

10 X TAE Buffer 1L
+ 48.4 g Tris
+ 7.44g $Na_2EDTA * 2H_2O$
+ 11.42 ml glacial acetic acid
+ 100 µl 0.5 M EDTA
Add H_2O up to 1 L
pH to 8.5
Note – Do not make up more then 1 L it will go bad before you can use it all

1 X TAE Buffer 1 L
In 900 ml dH_2O
+ 100 ml 10 X TAE buffer (40 mM Tris)

100 µM Primers ≈ 1 ml
Multiply 10 * the nmol amount of primer printed on the side of the tube
This is the amount of sterile dH_2O you should add to the tube
Mix by up and down pipetting try not to vortex
store in -20°C

20 µM Primers 500 µl

In 400 µl sterile dH_2O
+ 100 µl of 100 µM primer
store in -20°C