PCR Protocol

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Contents

1. Before performing the PCR

PCR is very sensitive to contamination from outside DNA. Steps should be taken to reduce the chance for contamination, such as wearing gloves and using filter tips (if necessary). It is important to assemble your reactions on ice.

-Wear a clean lab coat and clean gloves. Change gloves frequently. When you have gloves on,, avoid contact with materials that you don't necessarily have to touch (e.g. earphones, chairs & tables). Otherwise, change gloves!!

-Store *Taq* in the frozen blue box used for the PCR reagents. The original stock of primers should be kept in a box separate from your diluted working stocks. Store your DNA in a separate box from the PCR materials.

-Make a list of the premix (amounts of primers, buffer, dNTPs, enzyme, additions (BSA, DMSO)) that you are planning to make. Run always a negative control (ie. without any template DNA added). You may also want to run one positive control. This is a sample known to contain DNA that will be amplified by the primers that you are using. Calculate the amount of samples plus 1 extra to compensate for pipetting errors.

-Prepare all your premix on ice. Template DNA should not be left at room temperature!!! It degrades.

-Only take the *Taq* out of the freezer once you have all the premix ready and resuspended. Once the *Taq* is added, immediately place it back into the freezer. This is to keep the stability of the *Taq*.

-Gently vortex the mixture, or mix by pipetting up and down.

2. Recommendations for the PCR

DNA template:

50-250 ng/50 μl reaction.

If you are using cDNA, don't exceed 10% of the final PCR reaction volume.

Primers:

Generally 18-22 bp. This is long enough for adequate specificity.

Concentration 0.1-1.0 μM of each primer.

Taq:

Usually 1U/50 μl reaction volume (0.5-2.0 U/50 μl)

dNTPs:

Between 40 μM and 200 μM of each.

$MgCl_2$:

Total concentration must exceed the total dNTP concentration.

1.5 mM of $MgCl_2$ in presence of 0.8 mM total dNTPs. This leaves about 0.7 mM free for the DNA polymerase.

Note: It's recommended to place tubes in preheated cycler at 94ºC.

3. Performing the PCR

For a **25µL** PCR reaction:

Initial Concentration	**Final Conc.**	**Quantity for a 25 μl total volume**
10X Buffer	1 X	2.5 μl
10 mM forward primer	0.2 mM	0.5 μl
10 mM reverse primer	0.2 mM	0.5 μl
10 mM dNTPs	0.2 mM	0.5 μl
$MgCl_2$ (50mM)	5 mM	2.5 μl

4. General Thermocycler program

- Initial denaturation step: 4 min 94C
- Over 25 cycles:
    - Denaturation 92ºC – 94ºC for 30 sec – 1 min
    - Annealing –Dependent on primer (Average Tm of both primers minus 5)-
    - Extension 68ºC – 72ºC for 1-2 min, depending on length of PCR product
- Final elongation: 5 min 72ºC
- Storage at 4ºC

Note: After PCR, check **5 µl of the mix on a 1.5% agarose TBE gel**. Store remainder at -20ºC in a **labeled PCR box!!**

5. Stock solutions

dNTPs

2mM stock of dNTPs means that the final concentration of **each dNTP** (dATP, dCTP, dGTP, and dTTP) **is 2mM** -- NOT that all dNTPs together make 2mM. dNTPs come as 100mM stocks -- thaw and add 10µL of each dNTP to 460µL of MiliQ ddH_20 to make 2mM. Store at -20°C.

Template: You can get product using 50 ug DNA. It's not usually necessary to be incredibly fastidious about how much template you add to a reaction. You can get product with small amounts of starting DNA Generally, 1 µL of template is a convenient volume of template to add for each reaction.

$MgCl_2$: this is the greatest variable in PCR. The success of a PCR is very dependent on how much magnesium is present in the reaction. For this reason, it is usually advisable to do a magnesium optimization when performing new

PCRs. You can optimize the $MgCl_2$ concentration b doing reactions in sets of six, keeping all variables constant except for magnesium. I usually go from 1mM to 6mM $MgCl_2$. Since the stock is 25mM, usually, this means that 1µL of stock equals 1mM MgCl2 in a 25µL reaction -- it's convenient.

6. Primer dilution calculations

For a 100 uM Primer stock

1. Using nmole of oligo:

Example:

If the primer is at 28nmole, then add 280 µl of MiliQ water or TE

28 nmole x 1 µmole/1000 nmole = 0.028 µmole
0.028 µmole/ 100 µmole/liter = 0.00028 L
0.00028 L x 1000 ml/L = 0.28 ml or 280 µl