PCR Protocols

General considerations:

(1) **Reagents.** These are stored in the PCR box in the -20 ºC freezer. Make sure to keep the enzymes and dNTP stocks on ice when taken outside the freezer. Everything else can be thawed to room temperature.
(2) **dNTPs.** Our lab dNTP stocks contain 10 mM **each** of dATP, dTTP, dCTP, and dGTP. Some companies sell "10 mM" stocks that actually only contain 2.5 mM of each, which effectively makes them about "10x." Apparently, the reason for this is historical.
(3) **Templates.** When amplifying from a plasmid DNA clone, make a working stock at 10 ng/µL (you will need 1 µL of this). When amplifying from a cDNA library, see the tube label for the DNA concentration (you will generally need 100-500 ng, depending on the relative abundance of your gene and the complexity of the library).
(4) **Primers.** These are generally at 100 µM in water, unless otherwise indicated on the tube. Primers in the lab should be generally designed with T_m ! 65 ºC (~80 ºC for Quikchange primers).
(5) **Take care not to cross-contaminate the reagents, especially the templates and primers!**
(6) Use low-retention pipette tips, especially for volumes " 1 µL. Barrier tips are usually not required for standard amplifications, provided the pipettors are clean.
(7) 8-strip 0.2-µL PCR tubes and lids are stored by the thermal cycler. These tubes hold a maximum of 50 µL of reaction mix.
(8) We generally use Vent (made by NEB) and Phusion (made by Finnzymes, available through NEB). **Learn about these enzymes first before using them.**
(9) **Pipetting order.** In general, add the **water first** and the **enzyme last**. For a large number of reactions, it is good practice to first set-up a master mix of the common reagents and then aliquot them, rather than to pipet the reagents separately for each individual tube.

General insert amplification with Vent:

This works well for amplification of DNA fragments up to 3 kb or so.

(1) Reaction mix:

Reagent	Stock concentration	Volume
Thermopol buffer	10x	5 µL
dNTP mix	10 mM of each dNTP	1 µL
Forward primer	100 µM	0.5 µL
Reverse primer	100 µM	0.5 µL
DNA template	10 ng/µL	1 µL
$MgSO_4$ (generally not required)	10 mM	2 µL increments
Vent polymerase	2 U/µL	0.5 µL
ddH_2O	X µL	to 50 µL total volume

(2) Cycling parameters:

95 ºC, 3 min
95 ºC, 30 sec
60 ºC, 30 sec } 30x
72 ºC, 30 sec/kb

72 ºC, 10 min
4 ºC, hold

(3) Optimization:

- Smeared bands are generally due to non-specific priming. Use a higher annealing temperature.
- Using too high of an annealing temperature can lead to low product yield.
- Yields can also be improved by optimizing [Mg^{2+}].
- Primer dimers are generally caused by sub-optimal primer design (unavoidable sometimes).

Insert verification with Vent:

(1) Make the master mix. Generally, for x samples make enough for x+1.

Reagent	Stock concentration	Volume
Thermopol buffer	10x	2 μL
dNTP mix	10 mM of each dNTP	0.4 μL
Forward primer	100 μM	0.4 μL
Reverse primer	100 μM	0.4 μL
Vent polymerase	2 U/μL	0.2 μL
ddH_2O		16.6 μL

(2) Aliquot 20 μL of the master mix into separate tubes, then add the plasmid template. Use 0.1 μL for high-copy plasmids (e.g., pFastBac). Use 0.2-0.3 μL for ColE1-based plasmids, e.g., pET vectors. You can also use *E. coli* colonies, but this only works with high-copy plasmids. When amplifying from colonies, it helps to start with a couple of cycles of freeze-thaw to lyse the cells.

(3) Cycling parameters:

95 ºC, 3 min
95 ºC, 30 sec
60 ºC, 30 sec } 20x
72 ºC, 30 sec/kb
72 ºC, 5 min
4 ºC, hold

Whole plasmid amplification with Phusion:

This method has been tested with plasmids generally used in the lab (i.e., pFastBac, pET). It can also be used as a Quikchange-type mutagenesis protocol. Primers should be at least 27 (preferably 30) bases long with T_m ! 65 ºC. Otherwise, you will have problems with proper annealing. Phusion has very high fidelity and processivity because it contains an ssDNA binding domain — it will rapidly chew up your primers in the absence of dNTPs! The Phusion HF and GC buffers contain a high salt concentration, which elevates T_m. The HF buffer should generally work well — use DMSO to optimize difficult templates. Note that DMSO decreases primer T_m, and (probably) lowers enzyme fidelity. For this reason, try not to go above 3% DMSO (and avoid using the GC buffer). Read the Phusion manual for details.

(1) Reaction mix:

Reagent	Stock concentration	Volume
HF buffer	5x	10 µL
dNTP mix	10 mM of each dNTP	1 µL
Forward primer	100 µM	0.5 µL
Reverse primer	100 µM	0.5 µL
DNA template	10 ng/µL	1 µL
DMSO (generally not required)	100%	1 µL increments
Phusion polymerase	2 U/µL	0.5 µL
ddH_2O	X µL	to 50 µL total volume

(2) Cycling parameters:

98 ºC, 2 min
98 ºC, 15 sec
72 ºC, 30 sec + 15 sec/kb } 35x
72 ºC, 10 min
4 ºC, hold

(3) DpnI digest (if required):

- Add 1 µL DpnI (NEB).
- Incubate at 37 ºC for at least 4 hr (preferably overnight).

Mutagenesis with the Quikchange method:

Read the Quikchange manual and follow the protocol.

Things to look up / think about:

Learn about how DNA is amplified using PCR. Is it practically possible to amplify DNA from a single molecule of template?
What is the relationship between the annealing temperature and the primer T_m? How do you calculate the T_m? Why do you need a higher annealing temperature for amplifications with Phusion vs. Vent, even if you use the same primers?
Why do we use thermostable polymerases?
What do the terms "fidelity" and "processivity" mean in reference to polymerases?
What would happen if you added the enzyme to primers in the absence of dNTPs? When can you get away with doing this?
dNTPs are generally used at 0.2-0.4 mM. How was this working range determined? What would happen if you used a higher or lower dNTP concentration? What would happen if you used different concentrations for each dNTP?
What is the purpose of Mg^{2+}? How does the concentration of Mg^{2+} affect enzyme fidelity and processivity? Can you use Mn^{2+}?
Why is it good practice to use a master mix for common reagents rather than to pipet them separately?