

Some PCR-related Protocols

By Alisa Ramakrishnan, updated May 2005

Here's a webpage that gives some good background: http://www.promega.com/guides/pcr_guide/default.htm

Resuspending primers

To resuspend stock primers to 100uM (100umol/L): on the tube, it tells you how many nmoles there are. You can calculate it yourself or go to a webpage <http://biotools.idtdna.com/resuspension/> (The extinction coefficient is usually written on the page that comes with the oligos (primers)).

But there's a shortcut: if you look under how many nmoles you have, just multiply that number by 10 and add that many uL of water.

Example: 90.31nmoles primer = add 903.1uL water, vortex for 20sec, put in freezer.

OR – to do it on your own:

1nmol=1000pmol

1uM=1pmol/uL

$$\frac{90310 \text{ pmol}}{x \text{ uL}} = \frac{100 \text{ pmol}}{1 \text{ uL}}$$

Diluting primers to a working concentration

Stock primers are all at 100uM, or 100pmol/uL

I like to use 2.5uM primers in my reactions. You can use any amount you want, just so long as your final PCR reaction has about 0.25uM primers in it. (Some people troubleshoot primer amount, but this should work for our stuff.)

$$C_1V_1=C_2V_2$$

Example: make 200uL of 2.5uM primer from 100uM stock
(200uL working primers)(2.5uM)=(x uL)(100uM stock primers)
Solve for X and make your final volume 200uL.

Running a PCR reaction

PCR recipe (non-fluorescent primers)

If just running fragments, you can use a 7.5 uL reaction just fine. Here's a slightly different version:

10uL rxn:

5uL Qiagen HotStarTaq mix

1uL genomic DNA – DNA from plants that I'll help you find in the freezer

1uL Forward Primer (2.5uM)

1uL Reverse Primer (2.5uM)

2uL ddH₂O

Pretty easy, huh? It's because of the Qiagen mix. This is a fancy, expensive PCR mix that has several nice features:

- It contains almost all the components you need for a PCR reaction: dNTPs, MgCl₂, buffer, and Taq Polymerase. (It doesn't have the primers or DNA, of course, as those are unique to each reaction.)
- The Taq Polymerase is fancy, in that it requires a 15min incubation at 95C before it will be able to synthesize new strands of DNA. This is nice 'cause often, even while setting up PCR reactions, the Taq will start polymerizing on its own and give false products, or smears. It's also nice 'cause the hot start really separates those DNA strands and that seems to help the Taq get in there and do its job well.

Even though the HotStarTaq is fancy and nice, I still like to set up my reactions on ice and keep the Taq cold. It loses activity, like any enzyme, when stored at room temperature for any length of time.

PCR thermal cycling

For everyday use, we have a simple program we use:

1. 95°C for 15min (initial denaturing step ONLY for HotStarTaq or similar hot-start taqs)
2. 95°C for 30sec (denature, or melt DNA strands apart)
3. 55-60°C for 30sec (anneal, or primers stick to DNA)
4. 72°C for 30sec (extend, or let Taq do its job)
5. cycle between 2-4 for about 35 times
6. 72°C for 10min (final extension. Taq adds an 'A' base at the end of every product, and this helps ensure that absolutely every product has that 'A' so they're all the same length, since that's what we'll be looking at)
7. 4°C for forever (just keeps it from degrading prior to running on a gel)

Note on annealing temperatures: they typically vary from primer to primer, but I'm hoping that all our primers will amplify prettily at 55C.

Now we'll check to make sure we got PCR product; if we can see it on agarose, we'll see it on the ABI PRISM 310 Genetic Analyzer.

Running PCR products on an agarose gel

Mix up a 1-2% agarose gel:

In a 500mL Erlenmeyer flask:

100uL TAE buffer

1-2g of agarose (not metaphor unless you follow their protocol)

Heat in the microwave for about 2min, watching that it doesn't boil over. Take it out using hot gloves and make sure it's all completely dissolved – no floaties, sparklies, or anything like that.

Add 5uL EtBr stock solution (10mg/mL)

Wait for it to cool but not solidify (alternatively, hold under faucet 'till cool enough to handle)

Put tape on either side of a gel tray, put in combs, and pour gel to about 0.7cm deep

Wait for gel to set (15-30min)

Remove tape and gel combs, place in gel rig containing TAE buffer with wells closest to the black electrode

Loading samples

5uL PCR product

1-2uL loading dye

mix on parafilm or in another tube, load into bottom of well taking care not to puncture bottom of well.

You'll also want to include a lane of ladder, so you can tell how long your fragments are. Use 'PCR Marker' by Promega. The sizes are posted on a lab bench somewhere, online, or you can look at the product insert that I left in the 'ladders and loading dye' box in the door of the big freezer.

Ladder: 5uL ladder, 1uL loading dye (if ladder is clear- if ladder is green/blue, no need to add loading dye).

Run gel by turning the machine on to 70V. You can let the gel run 'till the blue band is about 1/2-way across the gel.

Visualizing samples with EtBr

EtBr: We have to use EtBr (or SyberGreen) to visualize the DNA. We can see the loading dye, but not the DNA itself until we put something with it that'll make it visible. EtBr is the most common dye, though there are others. It intercalates in the major groove of the DNA – maybe you can find a picture of this on the web? Dunno. Anyway, it sticks into the DNA and preferentially binds there. It'll be in the gel, but concentrated in DNA strands, so we'll see it when we whack it with UV light.

Taking pictures (I prefer using the Typhoon, see below)

1. Go to rm 615 using green key. Put gel in specified EtBr area. Turn on printer, monitor, blue box above monitor, and 'white light' on the big white box.
2. Put your gel on the transilluminator that's in the white box
3. Look at the picture on the monitor. Try to get a picture that's as big as possible, and of course focused. Focusing and zooming are controlled on the camera that sticks into the big white box.
4. You may want to set the frame intervals to a low setting while setting this up so that you can see what's going on in close-to-real time. Do this by dialing to about 10 on the blue box that's above the monitor.
5. When you think you have your gel nicely placed, close the door of the big white box, turn off the white light, and turn on the UV light switch. Boost the frame intervals to about 40-90, until you see nice bright bands.
6. When ready to print, hit the 'print' button on the printer.
7. Pack up, turn everything off, and go home. Oh – take your gel with you and put it in the gel waste container in our hood.

Visualizing samples on the Typhoon Phosphoimager

On the 5th floor in one of Niles Lehman's rooms is a big phosphorimager. Here are some general instructions on how to use it. For more detailed information, I strongly suggest you read the protocol books located in that same room.

For visualization

1. Place your gel on the glass plate, with the wells along the LH side of the plate. Note the grid numbers/letters that designate the size of your gel. Scan as small as possible of an area.
2. Login (Cruzan, piriqueta)
3. Open the Typhoon software
4. Highlight the grid area that corresponds with your gel
 - a. Optional: change PMT to 400 if you expect really bright bands on your gel
5. Hit 'scan'
6. Tell it where to store your gel picture (C:\Data\Cruzan lab\your folder)
7. Wait while it scans your picture. Once it's done scanning, your picture is saved. Make sure there are no red lines on the picture – that means the image is saturated, and you'll get an inaccurate quantification estimate

For quantification (this may be slightly off... if in doubt, read the manual or ask someone in the know)

1. Open 'ImageQuant' software
2. Go to 'file' and 'open' and open the gel file you just made
3. Using the box tool, draw a box around the band on the size standard that you want to use to quantify.
4. Copy that box (cntrl-C) and past it onto each of your sample bands (cntrl-V, arrows or mouse to move them). All the boxes need to be the exactly the same size, as the computer doesn't know where the band is.
5. Also place another box in a representative blank area – this will be used for background correction (not absolutely necessary, but nice to do here as you'll have to do it eventually anyway)
6. Go to 'analyze' and 'background correction'. A box will open up. Select 'object' and select the number of the box corresponding to your background box. Hit 'set'.
7. Go to 'analyze' again, no need to close the previous box, and select 'volume report'. Select all the samples and hit 'report'
8. A report will appear with numbers in it. If you double-click on any box, it will automatically open an excel file. You can use this file to convert raw numbers to ng if you know your sample control ng amount. Wheel!