

Protocol

Part 1: Running your gel

You will use a 1% agarose gel, running nine samples and a lane of molecular weight marker (DNA ladder).

1. Add 20 ul of sterile water to the 5 ul PCR aliquots you stored after Mod1 Day2.
2. Add 2.5 ul loading dye to the PCR aliquots as well as to the pCX-NNX digested by XbaI and/or EcoRI, and the PCR product digested by XbaI/EcoRI. Loading dye contains xylene cyanol as a tracking dye to follow the progress of the electrophoresis (so you don't run the smallest fragments off the end of your gel!) as well as glycerol to help the samples sink into the well.
3. Flick the eppendorf tubes to mix the contents then quick spin them in the microfuge to bring the contents of the tubes to the bottom.
4. Load the gel in the order shown in the table below.
To load your samples, draw 25 ul into the tip of your P200. Lower the tip below the surface of the buffer and directly over the well. You risk puncturing the bottom of the well if you lower the tip too far into the well itself (puncturing well = bad!). Expel your sample into the well. Do not release the pipet plunger until after you have removed the tip from the gel box (or you'll draw your sample back into the tip!).
5. Once all the samples have been loaded, attach the gel box to the power supply and run the gel at 125V for no more than 45 minutes.
6. You will be shown how to photograph your gel and excise the relevant bands of DNA.

Lane	Sample	Volume to load
1*	Uncut pCX-NNX	5 ul
2**	pCX-NNX XbaI (large volume reaction***)	5 ul
3**	pCX-NNX EcoRI (large volume reaction***)	5 ul
4**	pCX-NNX XbaI (small volume reaction***)	5 ul
5**	pCX-NNX EcoRI (small volume reaction***)	5 ul
6	pCX-NNX XbaI + EcoRI	25 ul
7	DNA Ladder	5 ul
8	PCR Product XbaI + EcoRI	25 ul
9	PCR Product Uncut	25 ul
10	PCR no-template-control	25 ul

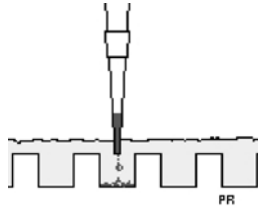
* Prepare a sample using your original stock of pCX-NNX.

Use 1 ul of the original stock and add water and loading buffer to get a total of 5 ul.

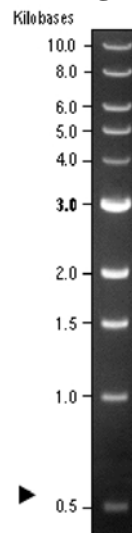
(Loading buffer is 5X)

** These samples will be provided for you by the T.A.

*** In the large volume reaction, about 400 ul of the total reaction medium was used while 25 ul was used in the small volume reaction. We will see the effect of this volume difference on the efficiency of the restriction enzyme reactions.



Loading a gel



The 1Kb markers should look something like this. Note how the 3.0 Kb band is brighter than the rest.

Part 2: Isolating/Purifying DNA

To purify your DNA from the agarose, you will use another kit sold by Qiagen. Again the reagents have uninformative names and their contents are proprietary. Like the PCR Clean-up kit, the Agarose Purification kit requires binding the DNA to a spin-column with a silica-gel membrane, washing away salts and eluting the DNA from the membrane.

1. Estimate the volume of your gel slices by weighing them.
2. Add 3 volumes of **QG** for every 1 volume of agarose. (The maximum advised volume is 550 ul.)

3. Incubate at 50°C for 10 minutes, until the agarose is completely dissolved. Every few minutes, you can remove your tubes from the 50°C heat to flick the contents. This will help dissolve the agarose.
4. Add 125 ul of isopropanol to each eppendorf tube.
5. Get two QIAquick columns and two collection tubes from the teaching faculty. Label the spin-column (not the collection tubes!) either “bkb” or “frag” then pipet the appropriate dissolved agarose mixture to the top. Microfuge the column in the collection tube for 60 seconds. **The maximum capacity of the QIAquick columns is 800 uL!** If you have more than 800 uL in your mixture, you will need to repeat this step.
6. Discard the flow-through in the sink and replace the spin-columns in their collection tubes. Add 750 ul of **PE** to the top of the column and spin as before.
7. Discard the flow-through in the sink and replace the spin-columns in their collection tubes. Add nothing to the top but spin for 60 seconds more to dry the membrane.
8. Trim the cap off two new eppendorf tubes and label the sides with your team color, the date, and either “bkb” or “frag.” Place the spin-column in the correct trimmed eppendorf tube and add 30 ul of **EB** to the center of the membrane.
9. Allow the columns to sit at room temperature for one minute and then spin as before. The material that collects in the bottom of the eppendorf tubes is your purified plasmid backbone or insert, ready to be ligated.

Part 3: Evaluate recovery

Ligations generally work best when there is a 1:4 ratio of backbone to insert. You will ligate your fragments next time, but before you do, run a small amount out of the purified products on a gel. This will allow you

- to check that you purified your DNA from the agarose
- to assess the quality of the DNA (that it's not degraded or contaminated)
- to adjust the amount of backbone and fragment in your ligations for an ~1:4 ratio.

1. Move 5 ul of each sample into an eppendorf tube labeled “purif bkb” or “purif frag.” Add 15 ul sterile water to each tube and 2 ul loading dye. Use a colored label to identify your team color and give the samples to the TA. The TA will run a 1% agarose gel for you and the result will be posted. You should examine the image before coming to lab next time (see “for next time” section).

2. Freeze the remainder of your purified DNAs at -20°C.

DONE!