Confirmation of Restriction & Ligation

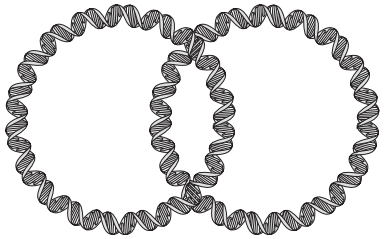
Using Agarose-Gel Electrophoresis

It is important, at this stage of our experimental procedure, that we confirm *Bam*H I and *Hin*d III have digested the original pKAN-R and pARA plasmids and the restriction fragments have been ligated together by DNA ligase. This lab will provide evidence that we have recombinant DNA molecules.

Gel electrophoresis is a procedure commonly used to separate fragments of DNA according to their molecular size. Like the dyes you separated in Lab 1, DNA fragments will migrate through the agarose maze. DNA, because of the phosphate groups, is negatively charged and will move toward the positive (red) electrode. Because it is easier for small molecules to move through the agarose matrix, they will migrate faster than the larger fragments. Picture a group of cross-country runners who are racing through a dense tropical rain forest. All other factors being equal, the shorter runners will be able to navigate through the tangle of overhanging vines and dense foliage faster than the taller runners. So, smaller DNA fragments will move through the tangle of agarose molecules faster than the longer fragments.

We'll take all of our plasmid samples: digested, undigested and ligated, and use electrophoresis to separate these pieces. You might have predicted that your uncut plasmids would produce only a single DNA band; there's no reason why you would think otherwise. However, it is likely that two or three bands will appear in the undigested plasmid lanes. The reason for this is that plasmids isolated from cells exist in several forms. One form of plasmid is called "**supercoiled**." You can visualize this form by thinking of a circular piece of plastic tubing that is twisted. This twisting or supercoiling results in a very compact molecule, one that will move through the gel very quickly for its size.

A second plasmid form is called a "**nicked-circle**" or "**open-circle.**" Often a plasmid will experience a break in one of the covalent bonds located in its sugar-phosphate backbone along one of the two nucleotide strands. Repeated freezing and thawing of the plasmid or other rough treatment can cause the break. When this break occurs, the tension stored in the supercoiled plasmid is released as the twisted plasmid unwinds. This circular plasmid form will not move through the agarose gel as easily as the supercoiled form. Although it is the *same* size, in terms of base pairs, it will be located closer to the well that the supercoiled form.



The last plasmid form we are likely to see is called the "**multimer.**" When bacteria replicate plasmids, the plasmids are often replicated so fast that they end up linked together like links in a chain. If two plasmids are linked, the multimer will be twice as large as a single plasmid and will migrate very slowly through the gel. In fact, it will move slower than the nicked-circle. Your pKAN-R – and pARA – samples, then, *may* each have three bands that appear in the gel. Starting closest to the well, you might observe a multimer, followed by a nicked-circle band and, finally, a fast traveling supercoiled band.

We will use a special staining technique that permits us to see the fragments embedded within the gel, then make a photographic record of your gel to document this important step.

Materials

REAGENTS

Plasmid samples:
- K-, K+
- A-, A+
- Ligated plasmid ("LIG" tube)

0.8% agarose gel
5x loading dye
1x SB (or 0.5x TBE)
DNA size marker (25 ng/µL)

EQUIPMENT & SUPPLIES

P-20 micropipette and tips
1.5 mL microfuge tubes
Electrophoresis apparatus
Power supply
Marker pen
Plastic microfuge tube rack

Methods

1 Collect the five plasmid samples and the DNA marker from your teacher and place them in your plastic tube rack. You should have *six* tubes.

2 Obtain five clean 1.5 mL microfuge tubes and label them as follows: **A-**, **A+**, **K-**, **K+**, and **L**. The microfuge tube with the marker should already be labeled.

3 The following table summarizes plasmid sample preparation for electrophoresis. See "**Hints**" before setting up these tubes.

Tube	dH_2O	Loading dye	K+	K-	A-	A+	LIG	Total volume
A+	4µL	2µL	–	–	–	4µL	–	10µL
A–	4µL	2µL	–	–	4µL	–	–	10µL
K–	4µL	2µL	–	4µL	–	–	–	10µL
K+	4µL	2µL	4µL	–	–	–	–	10µL
L	3µL	2µL	–	–	–	–	5µL	10µL

Hints:

- For example, to the tube labeled "A-," add 4μL of pARA-, 4μL of dH_2O and 2μL of loading dye. The loading dye should be located in your plastic microfuge tube rack next to the dH_2O tube.

- If you study this table, you'll see that you can add water to all five tubes, then add the loading dye to all of the tubes without changing the tip. Then, **dispense the plasmid sample into each tube, changing the tip each time to avoid contamination.**

- **Save the *"LIG"* tube** that contains your ligated plasmid; there should be about 10μL remaining in this tube.
  Important: Return the "LIG" tube to the collection rack, at the front of the room, as you will need it for the next lab.

- Centrifuge all samples to pool the reagents at the bottom of each tube. Be certain that the tubes are placed in a balanced configuration.

4 Prepare the gel and electrophoresis box to receive these plasmid samples.

- Be certain the gel wells are oriented closest to the negative (black) electrode.

- Pour the 1x SB buffer (or 0.5x TBE) over the gel until there are no visible "dimples" breaking the surface of the buffer over the wells. It's important that the gel be completely under the SB buffer. However, you don't want so much buffer in the box to allow the electrical current to run through the buffer and not the gel.

5 Take your plasmid samples and marker to the gel, along with your pipette and tips.

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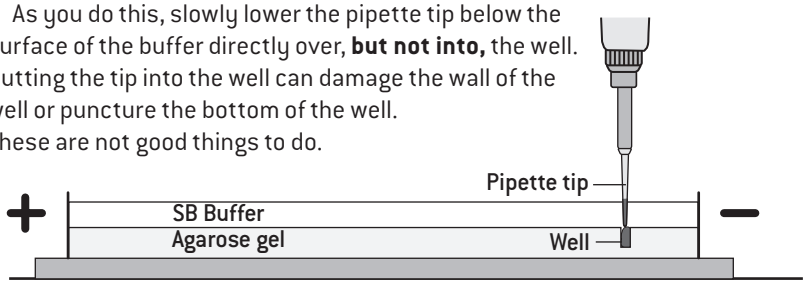
6 You will share this gel with another group.
Unless your teacher has you load your samples in a different pattern, load your samples in the order indicated below. Follow the loading directions that begin with step seven. If you load your sample in a different order, **be certain to record it in your notebook for later reference.**

7 Using a clean tip, set your P-20 micropipettor to 10 μL. Aspirate 10 μL of your "DNA size marker" and **slowly** dispense it into the well.

- As you do this, slowly lower the pipette tip below the surface of the buffer directly over, **but not into,** the well. Putting the tip into the well can damage the wall of the well or puncture the bottom of the well.
These are not good things to do.



- Use two hands to steady the pipette. **Slowly** dispense the sample by pushing to the first stop of the pipette. Because of the loading dye, the sample will have a greater density than the electrophoresis buffer. This will allow the sample to **sink** into the well.

- **Important:** While holding the button on the first stop, slowly remove the pipette tip from the gel box. If you've loaded your sample correctly, the well will be filled with a blue-colored solution.

8 Continue this procedure with the plasmid samples, following the order indicated on page 4.3. Change the tip for each sample. If you choose to load your samples in a different order, be certain to record the sample order in your notebook.

9 Close the gel box lid tightly over the electrophoresis chamber. Connect the electrical leads to the power supply. Be certain that both leads are connected to the same channel (same side) with the negative (black) to negative (black) and positive (red) to positive (red).

10 On the power supply, set the voltage to 130-135v.

11 After two or three minutes, look at your gel and be certain that the purple dye (bromophenol blue) is moving toward to positive electrode. If it's moving in the other direction—toward the negative (black) electrode—check the electrical leads to see whether they are plugged into the power supply correctly.

12 Be certain that you return your "LIG" tube to the front of the room. This tube should contain your recombinant plasmids and will be used for the next lab.

13 Your teacher will explain what to do with your gels, **so listen carefully.** If your lab time is short, you may not have sufficient time to complete the electrophoresis. The yellow dye will need to run just to the end of the gel, about 40–50 minutes.

Conclusions

These questions are to be answered after you've had an opportunity to analyze your gel photograph.

1 How did your actual gel results compare to your gel predictions?

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2a Are there any bands, appearing in your gel photo, that are not expected?

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2b What could explain the origin of these unexpected bands?

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3a Do you see evidence of the three plasmid forms in the uncut lanes?

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3b Is there evidence of more than one form of multimer?

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4 Why are the ligated plasmids so close to the well?

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5 Two of the 702 bp pKAN-R fragments, *rfp* gene fragments, may form a circularized fragment because each end of the fragments terminates in *Bam*H I and *Hin*d III sticky ends. Is there evidence of a circularized 1404 bp fragment in the ligated lane?

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