

Separation of DNA fragments using an AB 3130 Genetic Analyzer

Russell Johnson

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The sequencing reactions that we ran last week have generated a series of DNA fragments where the new strands of DNA were terminated with the Big Dye terminators. To find the sequence of your DNA, you now need to determine the lengths of all the DNA fragments in each of your samples. Way back in the 1900s this used to be done by pouring acrylamide gels, loading all the samples into wells, running the gel, drying the gel, exposing it to film, and analyzing (by hand) all the bands present in each lane. This was tedious work and was much too slow to allow for sequencing of very large amounts of DNA (such as the human or Arabidopsis genome). Fortunately for you, this analysis can now be done on automated DNA sequencers that separate the fragments using capillary electrophoresis and analyze all of the fragments automatically to give the DNA sequence.

We will use an Applied Biosystems 3130 Genetic Analyzer for our sequencing. This instrument is basically a very fancy (meaning expensive) electrophoresis apparatus that runs four “lanes” at a time. The sample is loaded automatically onto the (−) end of the gel, current is applied, and the DNA bands are detected by a laser as they pass the detection window near the (+) end of the gel. The data for the bands are stored in the computer and can be accessed later. As soon as the first set of four samples is finished, the next set of four will be run etc. until all are finished.

PREPARATION OF SAMPLES

1. Dissolve each of your dried samples in 10 μ L of HiDye formamide. Vortex each sample briefly. Heat at 95° for 2 minutes. Place on ice for 30 s, then spin briefly. Store samples on ice.
2. Place samples into the wells of a 96 well plate, carefully recording which sample is in which well. Cover the plate with a sheet of “plate septa” and spin the plate to ensure that all the liquid is at the bottom of the wells. Store the plate on ice until needed.

RUNNING YOUR SAMPLES ON THE ABI 3130

It is common at large universities to have a central DNA sequencing facility where researchers can send their samples to be analyzed. Typically individual researchers would run their own cycle sequencing reactions and then send the reaction products to the central facility to have the fragments separated and analyzed by electrophoresis. The person in charge of the central DNA sequencing facility would run all the samples that are submitted and then make the data files available to the individual researchers.

We are fortunate at Colby to have such a central DNA sequencing facility where we can send our samples. Our sequencing lab utilizes an Applied Biosystems 3130 sequencer and is managed by Patti Easton. When we have our plate full of samples ready, we can give it to her and she will run the samples for us.

So that we can understand what happens during the process of fragment separation and detection, we will observe the set up, electrophoresis, and data collection. Patti will explain what is going on during these steps.

GETTING THE SEQUENCE DATA

1. On the Windows computer that is connected to the AB 3130, open My Computer and go to the E: drive. Open up the BC378 folder and find the folder with your samples in it. Copy your files onto a memory stick so that you can carry them to another computer.
2. Find a Windows computer (not the one in Arey 204) that has the *Sequence Scanner* application. There are several of these in Olin 236. Copy your sequence files onto the desktop of this computer.
3. Open the *Sequence Scanner* program. Go to **File**→**Import Traces**. In the window that pops up, select the Desktop as the place you will import the files from. Select the (four) files you want to import, click on Add Selected Traces, and then click OK.
4. Double-click on the first sequence file you want to look at. The bottom part of the screen should now display the peaks that were detected by the sequencer.
5. On the very bottom of the screen, click on Raw. This will show you the raw data, exactly as it was collected, before any analysis has been applied. If your sequencing reaction and data collection worked well, you should see strong sharp peaks with amplitude of at least 500 units.
6. On the very bottom of the screen, click on Analyzed. Now you will see what the peaks look like after adjusting the baseline and compensating for the different mobilities of the four dyes. You can use the bar at the bottom to scroll through different parts of your sequence. Above each peak is displayed the base that the computer thinks is correct at that position. Above each “base call”, a colored bar representing the quality value (QV) is displayed. The $QV = -10\log_{10}(Pe)$, where Pe is the probability of error. If the bar is blue, this means the QV for that base is > 20 , which means the $Pe < 1\%$. A full-length blue bar means the QV is at least 60 ($Pe < 0.0001\%$). If you hold the mouse on a colored bar, the computer will tell you the exact QV for that base. Typically, the part of your sequence that is reliable will be the region where all of the bases have a QV > 20 (blue bars). The regions before and after this (with low QVs) should not be trusted.
7. Go to **File**→**Print Setup**. In the window that pops up, leave the default settings as they are, except change Panels per Page to 6. Go to **File**→**Print** and print your electropherogram. It will be best to use the color printer in Olin 236 (137.146.166.37) for this.
8. Follow the same steps for your other three sequences.

Looking at your results.

For each of your sequencing reactions:

1. Indicate on the printout where the pCRII vector sequence ends and the insert sequence begins.
2. At what point does the sequence start to be reliable? Where does the reliable sequence stop?
3. How many bases of reliable sequence were you able to determine?