Fragment Analysis Information

Our facility has a service to carry out fluorescent DNA fragment analysis--this can include analyzing labeled products generated by AFLP, STR, or even DNAse footprinting or primer extension. We recommend using the Genemarker software from Soft Genetics for analysis of samples run on capillary instrumentation. Because of the different types of experimental manipulations and output that fall under the heading "fragment analysis," we have numerous options for sample preparation, drop off, and computer analysis.

Generation of labeled fragments
All the PCR reactions are run in your lab--we don't do that here in the facility. New users are encouraged to run a sample of their reaction products on an agarose gel to insure that something is there before bringing it to our facility. Many of the amplification procedures can be tricky.
Fluorophores:
Our ABI instrumentation is spectrally calibrated to detect a number of fluorophores. On the 3730, our highest output machine (16 96 well plates/day, meaning shorter turnaround times in general for dropped off samples) we can run filter set D or G5 samples. These include the dyes 6-FAM (blue), HEX (green), NED (yellow), and ROX (red). ROX is reserved for the internal MW standard run in every sample. FAM and HEX are commercially available dyes. NED primers are only available from Applied Biosystems. A 5th dye called LIZ is also available from ABI.

FAM labeled primers are a good place to start because that label is the most robust (it's the brightest, and most stable) and generally speaking we can synthesized cheaper than the other fluorophores. It is unwise to go back and forth between instruments, so all fragment analysis projects will be run on the 3730 only. For each instrument the internal consistency is excellent--run to run will give highly reproducible data.

Sample Preparation

Following PCR, 2 ul of the amplified products are mixed with 10ul of formamide and 0.3ul MW standard, then heat denaturated and loaded onto the machine. A critical fact to note is that the samples for all the machines are loaded onto the capillaries using electrokinetic injection, meaning an electric field is applied to bring the sample to the capillary. This method is very sensitive to components in the sample, particularly salts/buffers of any kind, excess primer, or other abundant contaminants such as primer dimers or very short PCR products. Such unwanted components can inhibit injection of your sample and/or the size marker, resulting in non-analyzable data and a wasted sample. From what we see with most users, 2 ul straight out of an amplification rarely gives good data. There are 2 ways to get around this problem. The simplest is to dilute the PCR reaction in water then add diluted sample to the formamide. We recommend trying a couple of dilutions at least--1:5 and 1:20 are good places to start. The other method is to do a magnetic bead or column cleanup of the PCR products following amplification. While we have done magnetic bead cleanup of PCR products in the past, we are no longer offering that service nor do we recommend it due to potential loss

of fragments. WE WILL NOT ACCEPT RESPONSIBILITY FOR POOR RESULTS ON UNCLEANED/UNDILUTED SAMPLES.
Another critical factor is the formamide used for dilution- NOT ALL COMMERCIAL FORMAMIDES WILL WORK! We use the ABI Hi-Dye formamide; we know that deionized formamide from Sigma also works. If you want to use another brand of formamide, contact us first.
Our primary goal is to generate good data for our users and we want to make every effort to insure that samples look good. Therefore, for users just beginning their projects, we can run a number of samples for free so you can test cleanup and/or dilution parameters. The first step (after reading this of course) is to talk with us about what you have, and we'll suggest what to do next.
Sample Drop Off
OK, you now know how to get good amplification, how to treat your samples and you want to drop stuff off for us to process. It all happens on the sequencing drop off computer. If necessary, we'll log you in as a new user (takes about a minute) so when you come over you'll be recognized by the computer. There are various options you can select for processing of GeneScan samples.
1. If you have cleaned up your samples, or diluted them appropriately, select “GeneScan Plate” from the pull down menu and enter the number of plates.
In the comments field, we also need you to write out the following info:
a) Which dyes are used to label your samples (this is VERY IMPORTANT).
b) The maximum fragment size expected or required. Analysis is commonly limited to fragments of about 600 bp or below, but we can go higher upon consultation. This is due to both labeled standard and software limitations. We currently keep a size standards on hand: Chimerx’s GeneFlo-Rox 625. For most samples the GeneFlo-625 is the best option.
c) The type of samples that you are dropping off (AFLP, Microsatellite, etc.)
Additional sample submission guidelines:
a) Samples must be in v-bottom 96 well format. No 0.5 or 1.5 ml tubes.
b) We do not accept samples covered with oil. If that’s how the PCR was done, please transfer them or remove all the oil.
c) Don’t dry down your samples.
d) We strongly recommend you include at least one sample representing a negative control and one well representing a positive control. That will help insure the samples were loaded correctly.
e) While we load only 2ul of sample, we want at least 10 ul to insure proper pipetting of material. It also allows us to do a rapid rerun if necessary.
2. An inexpensive way to carry out fragment analysis is using the "Ready-To-Go", or RTG-Genescan, option. To take advantage of this, mix the appropriately cleaned or diluted PCR samples with formamide and MW standard in the correct plate. We can provide you with the plate and foil to use. Then enter the number of plates under RTG GeneScan.

Data Output

Here's where it gets interesting! In our opinion, and of many in the fragment analysis community, analysis software options are limiting. It's also a rapidly changing environment, so what's true today may not be a year from now as options increase. Currently the data generated from our

Applied Biosystems 3730 are in PC format, and we initially analyze it using a program called Genemarker.
We look at representative files (each file represents one sample) from each run to make sure the MW marker ran correctly and generates a nice standard curve, then pass the files on to you. You then have several options to handle the files:
a) GeneMapper from ABI is a very expensive program ($10K or so)
b) Genemarker from SoftGenetics can be purchased.
c) You can download a FREE (PC based) program called Genographer. There are step by step instructions, a zipped MW standard file for commonly used standards, and a windows shortcut with a modified command line. Genographer creates a virtual gel image from your imported files, lets you click on the gel image to define bins, then bins the data. Setting up the bins the first time is relatively painful, but once done you can add subsequent files into this template easily. As with the other programs, we will instruct you in its use in order to analyze your data. It is a free program, and you do get what you pay for, so it may not be sufficient to solve all your problems but it can be a good place to start.
d) Peak Scanner is another option, and is only PC based. This is also freeware.
e) PeakStudio from the Fodor lab has been successful in some labs. Again freeware.