What is Fluorescent Fragment Analysis?

**Fluorescent  Fragment Length Analysis** is used to determine genotypes and assess loss of heterozygosity and microsatellite instability. This technique involves using fluorescent primers during amplification to label PCR products. These products are then separated by capillary electrophoresis. Users are responsible for providing fluorescently-labeled PCR products which are run together with sizing standards on the 3730 Genetic Analyzer.

**Instrumentation** The facility performs this analysis on the 3730 capillary platform. Running time is two hours for 96 samples up to 750 bases in size. The ABI GeneScan™ platform has several advantages over conventional methods. These include running individual lane sizing standards so lane-to-lane migration variation is eliminated. This instrument also provides increased sensitivity of detection, which allows the use of less DNA. This can be important when working with limited tumor samples. Also, PCR-based tests are easy to standardize and automate so the results are very reproducible.

**What does GeneScan™ do?** The ABI Prism GeneScan™ software allows fluorescently labeled PCR products which have been separated by size during electrophoresis to be rapidly and accurately sized.

The optimum size for this kind of analysis is 100-400 bases. The size standard used for the analysis is ROX 350 or ROX 500. Please do not label your primers with ROX or Liz dyes.

**What is GeneMapper™?** This is the software that allows for the accurate determination of the length of fluorescently labeled PCR fragments. This technology will allow multiplexing of many different fragments in one lane and so allow for rapid screening of multiple loci. The software can be used to determine the size, height and area under the allele peak. This information can then be used to determine the size of an allele, whether it shows microsatellite instability or whether an allele has been lost.

**What dyes can I use?** Options for primer labels include 6FAM, NED, VIC or HEX and PET. The common multiplexes done in this laboratory are NED, 6FAM and VIC. Only one primer should be fluorescently labeled. Primers should be labeled at the 5' end of the primer

Custom kits and pre-optimized kits are available from Life Technologies.

What are Microsatellites?

Microsatellites are highly informative markers found in the genome. They are defined as tandem repeats of two- to 10-bp units and may be present as perfect or imperfect repeats, e.g., CAGCAGCAGCAG. Repetitive regions are highly polymorphic across populations but tend to be conserved within an individual and their family and, therefore, act as informative molecular markers. The number of repeats found in each individual are highly variable with as few as two, or as many as 50, copies in each microsatellite unit. The most commonly used microsatellite markers are dinucleotide, trinucleotide and tetranucleotide repeats. These genetic markers can be used as valuable tools in researching the fields of molecular population genetics, medical genetics, forensic DNA research and evolutionary biology.

Primers are constructed from the DNA flanking microsatellite regions as the adjacent DNA is usually conserved. During PCR the regions containing the microsatellite are amplified. The PCR products that are fluorescently labeled are then separated by size using capillary or gel electrophoresis. The size of the PCR product can then be used to identify the number of repeats.

What is Multiplexing?

When individual PCR reactions are each labeled with a different dye and mixed prior to running on the instrument, many PCR products can be run and sized in one lane.

This allows for high throughput microsatellite screening. In this facility as many as five independently amplified PCR products have been multiplexed successfully.  Commercial kits are available with which as many as 10-16 loci can be multiplexed.

A second option for multiplexing involves mixing multiple primer sets with one template and simultaneously co-amplifying all the products. This requires a lot more optimization, as all the markers may not amplify with equal efficiency during a reaction.

We strongly recommend that you consider multiplexing when submitting a large number of samples.

What are Pull-Up Peaks?

When the signal from the PCR products is too high, the instrument software can no longer correct for the spectral overlap that exists for a dye set. Ideal rfu values should remain between 200-2000. This means that other colored small peaks will appear under the position of one strong peak. This will create errors in data interpretation. PCR products submitted for this service should have concentrations of 0.5-2 ng/µl.

Special Consideration for Fluorescently Labeled Primer Design

**Steps for Fluorescent Fragment Analysis**

* Guidelines for good primer design  a. Design forward and reverse primers so primers have comparable Tm.  b. Blast primer sequence to make sure there is only one target for the primer.  c. Design the reverse primer with a tail. This is very important in making accurate allele calls as taq polymerase randomly incorporates an adenosine at the 3' end of the template during PCR. This is frequently referred to as the 'Plus A' phenomenon. The addition of the sequence tail GTTTCTT on the 5' end of the reverse primer is one way to force the "+A" reaction to completion (Browstein et al.,1996). This will reduce difficult data interpretation that is caused by trying to discriminate the +A allele from the true allele.  d. Also adding a 30-minute final extension at 70˚C at the end of PCR will promote the completion of 'Plus A' addition.
* Optimize PCR by performing a magnesium chloride titration and varying the annealing temperature.
* Quantitate the PCR product. As little as 0.5-1 ng of product will give a strong signal. It is also important to remember that too much product will cause the CCD camera to saturate, and the software will no longer be able to correct for spectral overlap. This will result in what is known as 'pull-up peaks'.

**Critical Factors** The following factors are crucial to the success of detecting small mobility factors:

* *Primer labeling*  ABI /Life technologies recommends using 5' end-labeled primers Please label only ONE of your primers
* *Size standard*  We use an internal standard that ranges from 35-500 bp. As a result, we strongly recommend that your PCR products be between 50-500 bp in size  The internal standard that our software uses to analyze the fragment lengths is labeled with ROX (red) so we do not recommend using this label for your primers
* *Control DNA*  We also recommend that you submit PCR product from an individual with a known genotype. This will help serve as a troubleshooting device for the customer as well as the service. It allows for monitoring gel-to-gel variability as well as providing information on the effectiveness of the PCR amplification.