[F]dNTP protocol – December 15, 2004

Many of us will be designing a lot of microsatellite primers in the near future. To be certain of the quality of a locus prior to use, it should be screened first by genotyping a number of individuals (usually 20-50) to determine whether null alleles might be present, etc. Labeled primers currently run about $70-100. As such, using [F]dNTPs for initial screening is a much cheaper alternative – you simply don't have to guess as to whether a locus might be good prior to taking the "labeled primer plunge."

What you'll need:

- ChromaTide® Rhodamine Green™-5-dUTP (catalog # C7629 from Molecular Probes, Inc., now owned by Invitrogen). It currently costs $272.00 and comes as 1000uM in 25ul volume. A **dilution of 1:600** ((serial dilution of 1:20, then 1:30 in ddH_2O) works fine for labeling PCR products. Since you can dilute 1:600 and use 1μl of this per PCR, you get approximately 15,000 labeled reactions per tube. In other words, you can label and screen as many as 300 loci (@ 50 individuals/locus) for **less than $1 per locus**.

- Normal PCR reagents: 10X PCR buffer, $MgCl_2$, unlabeled primers, Taq polymerase

- Optional: SAP, Exonuclease I, and SAP reaction buffer

PCR Protocol

Set up a PCR like you normally would, but add 1μl of diluted [F] dUTP to it. For example, I usually do a 10μl reaction for genotyping:

dNTPs (1mM)	2.0μl	
*[F] dUTP (67nM)	1.0μl	(*1:600 dilution of original stock from Molecular Probes)
10X PCR buffer	1.0μl	
$MgCl_2$ (50mM)	0.4μl	
Forw. primer (10μM)	0.4μl	
Rev. primer (10μM)	0.4μl	
Taq (5U/μl)	0.12μl	
DNA ($\approx$100ng)	1.0μl	
ddH_2O	3.68μl	

Cook your PCRs as per your optimized conditions.

Optional step – SAP cleanup

Prior to genotyping, it may be helpful to perform a SAP cleanup of your PCR products. This is particularly true if your fragments are short (ex. 90-120bp) enough that they can be obscured by primer dimer peaks and other unincorporated dye. See Figure 1 for a comparison of products run with and without SAP cleanup.

For each reaction, prepare a cocktail as follows:

SAP	0.25µl
Exonuclease I	0.25µl
SAP buffer*	0.5µl

Add 1.0µl directly to each PCR reaction and incubate at:

37° for 25 minutes
80° for 15 minutes
4° forever (storage temp. following reaction)

Genotyping protocol

- Prepare a cocktail for each reaction as follows. The tubes provided by ABI work best, but you can also use regular 0.5ml PCR tubes. If you choose the latter, be sure to cut off the caps and use the rubber septa (caps) provided by ABI. Note: The septa can be washed and reused. If you do use regular PCR tubes, be aware that the depth is not quite the same as the ABI tubes and the machine is more likely to “miss” samples when trying to inject.

  15µl formamide
  1.0µl** TAMRA 500 size standard
  1.0µl PCR products (straight or SAP cleaned)

- Denature @ 95° for 2 minutes (be sure not to clamp the bonnet down because the septa will stick!).

- Quench on ice for 2 minutes.

- Load into the 310 in the appropriate order.

Setup of ABI 310

- Double click 310 Collection icon on desktop.
- Select "new" from File menu > select GeneScan Sample Sheet, 48 tube.
- Input your sample names in the appropriate order.
- Click on the "color present" box next to blue (the PCR product color) and black (the color of the TAMRA). This simply tells the machine what colors to look for.
- Set the size standard to yellow (click on box next to "yellow" - a black diamond indicates the size standard color). Note: This setting is different than what you would use for labeled primers.
- Save the sample sheet. Note: You cannot make changes once it is saved.
- Select "new" from the File menu > select GeneScan Injection List.
- When the injection list appears, select the sample sheet that you just saved. Note: It may take awhile for it to appear… just be patient.
- For the first sample, click on the arrow below MODULE and select "GS STR POP 4 (1 mL) A," then click on MODULE to highlight the column, go to the Edit menu, and select "fill down." This will copy the chosen setting to every sample.
- In a similar fashion, set the matrix file to, "Mike's GS A for [F]dNTPs" and the analysis parameters to, "Mike's long analysis param" for all samples. The most critical settings are the module and matrix file – if these aren't right, your samples won't be read correctly, if at all.
- Check to be sure that the size standard is set to "TAMRA 500" for all samples.
- Click on the green arrow to start the run.

* The cocktail for 1mL of SAP buffer is as follows. The $MgCl_2$ is the same stuff that comes with the Invitrogen Taq that we use. I am not sure how critical it is to use this buffer, but it worked very well when I used it (see Figure 1).

1M Tris-HCl (pH 8.0)	20μl
$MgCl_2$ (50mM)	200μl
ddH_2O	780μl
	=1 ml

Thoroughly mix by vortexing.

** This volume may be reduced as long as the size standard peaks clearly readable by the machine.)

One final note: Although [F]dNTPs are much cheaper for screening, you still want to order labeled primers for data collection **once you know a locus is "good**." The benefits of using labeled primers are:

1. Per sample, they are very inexpensive for data collection.
2. You can label primers for different loci with different colored dyes and multiplex them in the same GeneScan run.
3. If you're persistent enough, you might even be able to multiplex them in a single PCR reaction and really save yourself some time! The best I ever got to work was 5 loci in a single reaction: 3 that were non-overlapping (in terms of allele length) labeled with HEX (black), 1 labeled with FAM (blue), and 1 labeled with TET (green).

GOOD LUCK!

Mike

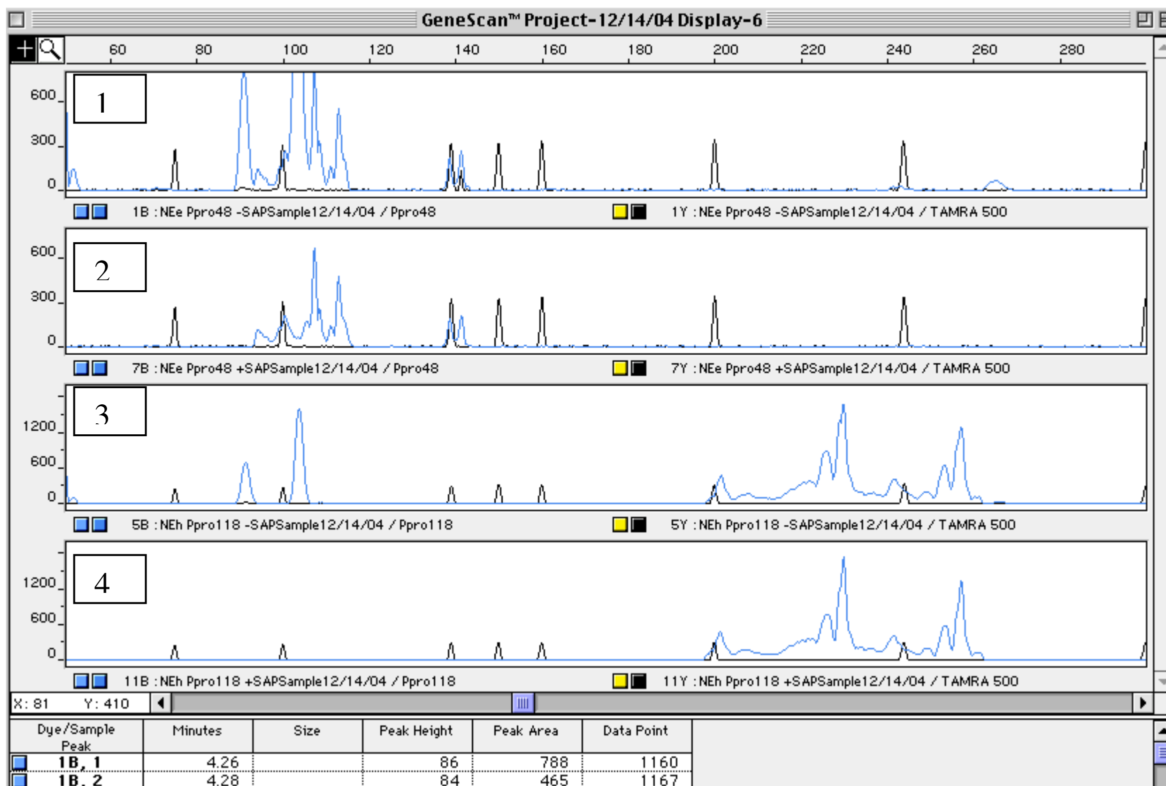

Figure 1. Chromatograms for four [F]dNTP labeled samples. PCR products are blue and TAMRA size standard peaks are black. Samples 1 and 2 are the same except that #1 is straight PCR product and #2 is SAP cleaned. The same is true for samples 3 (straight product) and 4 (SAP cleaned). Numbers 1 and 2 are a dinucleotide locus and the individual is a107/113 heterozygote. Numbers 3 and 4 are a tetranucleotide locus and the individual is a 229/257 heterozygote.