

# Directly tagging PCR primers with fluorescent dyes

Travis C. Glenn

Address: Savannah River Ecology Lab  
Drawer E  
Aiken, SC 29802

Department of Biological Sciences  
University of South Carolina  
Columbia, SC 29208

Telephone: (803) 725-5746 (5881 – lab)  
Fax: (803) 725-3309  
E-mail: Glenn@srel.edu

(803) 777-4606  
(803) 777-4002  
Travis.Glenn@sc.edu

Written: August, 2001

Last Updated: October 2006

Now that you have PCR products for your microsatellite loci, and you know that they are somewhat polymorphic, you are ready to screen large numbers of individuals. The best way to accomplish this is to screen using fluorescently tagged PCR products and an Applied Biosystems genetic analyzer equipped with GeneMapper. The easiest way to obtain a PCR product with a single fluorescent tag is to re-synthesize one primer for each locus, incorporating a fluorescent tag on the 5' end the new primer. This is accomplished by use of the appropriately modified phosphoramidite on the oligosynthesizer (asking for the appropriate 5' modification from IDT or Applied Biosystems).

## About the Dyes

6-FAM = blue , most intense dye

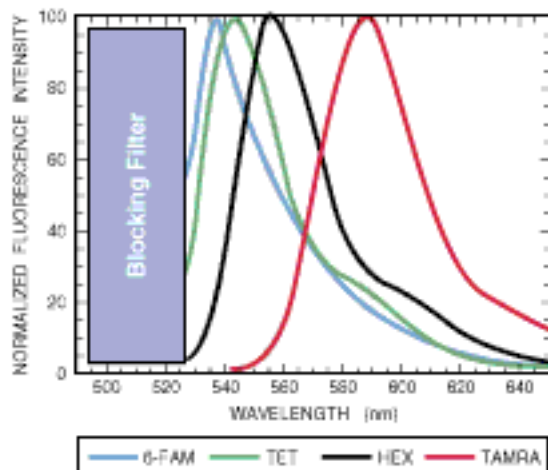
HEX = green

NED = yellow , replacement for TET/TAMRA

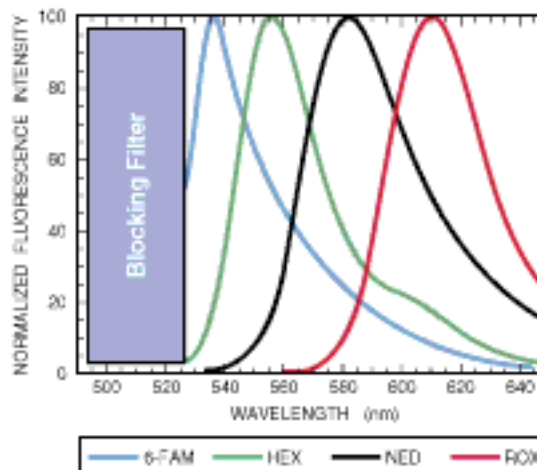
ROX = red, size standard label

TAMRA = red or yellow, alternative size standard label

TET = yellow, least intense dye, strongly overlaps with 6-FAM



First Generation Labels (Do NOT use)



2<sup>nd</sup> Generation Labels – still OK

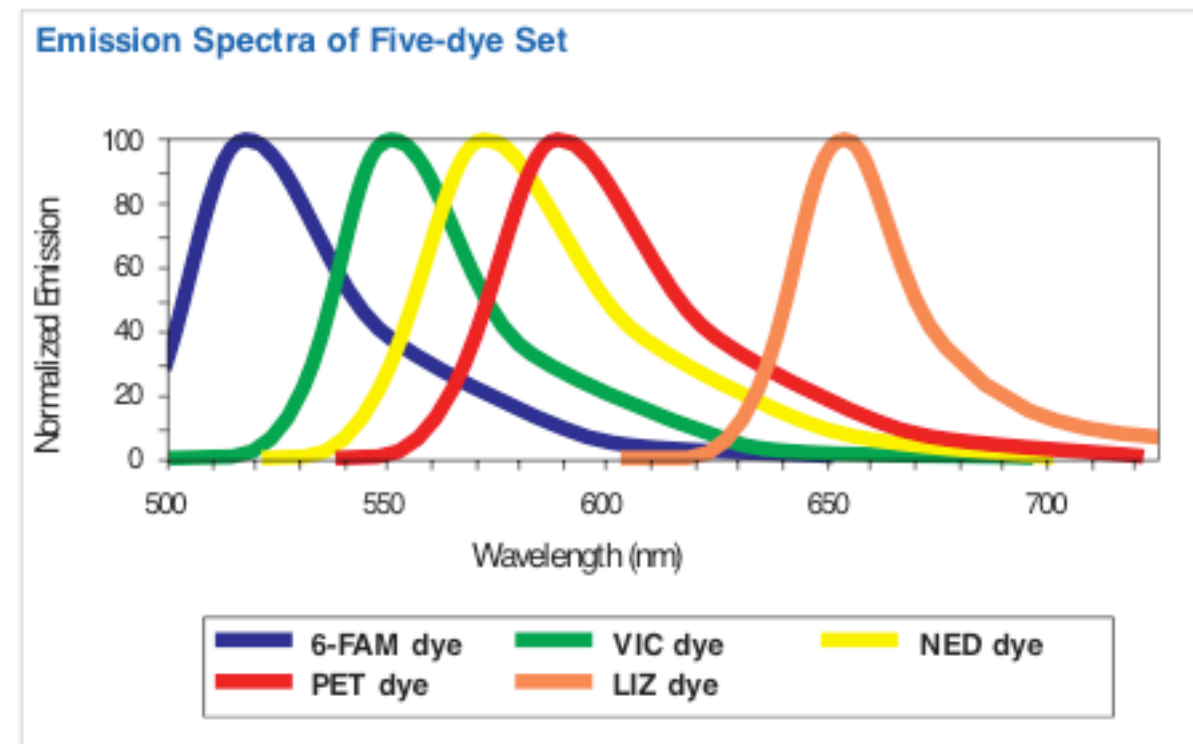
See <http://www.pebio.com/ab/techsupp/tra3b1.html> for review of NED.

The 3<sup>rd</sup> generation dyes were introduced in about 2004, using 5 fluorescent dyes:

---

Dye	Color	
6-FAM	Blue	
VIC	Green	
NED	Yellow	
PET	Red	
LIZ	Orange	Size Standard

---



**Figure 2b.** Shown above are the emission spectra of 6-FAM, VIC, NED, PET, and LIZ dyes used with the AmpF STR Identifier Kit. This dye set was specifically designed to expand the spectral detection range on ABI PRISM genetic analysis instrumentation.

It is possible to mix & match a bit – e.g., using Fam, VIC, NED, and ROX. It is possible to simply use the 2<sup>nd</sup> generation dye set as given above, but then use VIC or HEX as you wish – they overlap sufficiently that the results seem OK (though I wouldn't change along the way for specific loci without checking the results). We use the 4 dye approach on our 3130xl at SREL because it lets us use our ROX size standard (that we make ourselves – with slight modifications from DeWoody et al. (2004 – Biotechniques 37:348-352 [Sept. 04] – the biggest modification is that we pig-tail the primers [i.e., add GTTT to the unlabeled primers – see below for more details on pig-tailing]).

## General Strategy

As you can see from above, there are up to 4 dyes that can be used to label the PCR products. The last dye (red/orange) is used for an internal lane standard. So, you can do 3 or 4 PCR reactions (or multiplex – see below), mix them together regardless of overlapping sizes & determine the sizes of the amplicons for all loci in one lane of the gel/capillary. If you have non-overlapping size distributions for alleles at pairs of loci (i.e., locus 1 has alleles from 100 – 150 bp; and locus 2 has alleles from 175 – 225 bp), then they can be labeled with the same dye & run in the same lane. Using this approach, you can run 6+ loci per lane (note: although it is possible to get up to 12 loci in one lane, it is rarely worth the development time to get more than 5 or 6 running per lane). You can also take the fluorescent characteristics of the dyes into account (e.g., use FAM [the most intense dye] to label the weakest amplifying PCR products).

## Getting Fluorescently Tagged Primers

The oldest & now “cheap” way to go is to use FAM or HEX to label the primers. Phosphoramidites with these three dyes are widely available, thus most commercial companies that make oligos (primers) can make a primer modified with one of these dyes. We use IDT (<http://www.idtdna.com/>) for small orders of primers. They charge an additional \$75 per primer that is modified with one of the ABI dyes. Since the fluorescent tag is a set price, you are almost always best advised to get the 0.2  $\mu$ M scale synthesis. If we are getting large numbers of primers made at once, then we will get the phosphoramidites (~\$300 per bottle – makes about 8 oligos at 0.2  $\mu$ M scale), and have them synthesized at the Univ. of South Carolina.

The new & better way to is to use FAM, VIC, and NED to label the primers. You can get the FAM primers as noted above. VIC & NED are only available from Applied Biosystems. You can order primers online from Applied Biosystems (<http://www.appliedbiosystems.com>). They charge \$75 for 10k pmol; \$125 for 80k pmol and \$185 for 300k pmol of primers up to 50 bases. The smallest scale will allow a few hundred PCRs, the middle scale a few thousand, and the largest many thousands. Order what you expect to use within a year or 3. For typical projects they're slightly overpriced, but you'll make up the difference by having better labeling & an easier time dealing with the data.

---

## Which primer should I label?

If you have already screened the primer pairs via a universal 5' sequence tag, then the answer is really easy – take off the tag sequence & replace it with a fluorescent dye.

## Other Considerations

It is difficult to get <30% +A products when using Taq; so you're probably better off to “drive” amplicons to the +A state.

To have the highest proportion of amplicons with A's added, the reverse primers should start with G. Along these same lines, there is the strategy of “PIG-tailing” the unlabeled primer with **GTTT**CTT to increase the odds of of an A being added to almost 100% (Brownstein et. al.

1996). Some researchers simply add GTTT and get good efficiency (e.g., Traxler et al. 2000). We almost always add GTTT to the primer that is not labeled with the fluorescent dye.

To have the highest proportion of amplicons without A's added, the reverse primer should end in T, ideally TTT. Alternatively, you can use something other than Taq. For example, Life Technologies sells a polymerase (GENOTYPE Tsp) that will not put on A's.

---

Here is some old information (from 2001) that still may be useful to people with old equipment.

## Recommended Fluorescent Dye and Filter Set Combinations

### Dye and Virtual Filter Set Combinations

The following are the recommended dye and virtual filter set combinations to be used with labeled primers for GeneScan analysis.

#### ABI Prism 377 DNA Sequencer and ABI Prism 310 Genetic Analyzer

<u>Applications</u>	<u>Dye Set</u>	<u>Virtual Filter Set</u>
AFLP Kits I and II for Plant Mapping	5-FAM (B), JOE (G), TAMRA (Y), ROX (R)	A
ABI Linkage Mapping Set and Stock Mark Kits	6-FAM (B), TET (G), 6-HEX (Y), TAMRA (R)	C
Custom-labeled primers for fragment analysis	6-FAM (B), 6-HEX (G), NED (Y), ROX (R)	D
AFLP Microbial Identification Kit and Human Identification Profiler Kits	5-FAM (B), JOE (G), NED (Y), ROX (R)	F

The letters in parenthesis represent the color of each dye in the gel display.

B = blue, G = green, Y = yellow, R = red

---

You must make a new matrix if you switch to one of the new dye sets that include NED. Matrices are dye set, instrument, and run condition dependent. As such, matrices must be remade when any of these conditions change. The following table lists the matrix dyes required for NED fluorescent dye sets.

**Table 1.** Matrix dyes required

<u>Dye Set</u>	<u>Matrix Dyes Required</u>	<u>Part Number</u>
6-FAM, HEX, NED, ROX	NED Matrix Standard	402996
	Amidite Matrix Standards Kit (use 6-FAM, HEX, and ROX only)	401546
	NED Matrix Standard	402996
5-FAM, JOE, NED, ROX	Primer Matrix Standards Kit (use 5-FAM, JOE, and ROX only)	401114

---