[F]dNTP Reagents

Protocol

Applied Biosystems

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Contents

Introduction

The Applied Biosystems fluorescent dNTPs ([F]dNTPs) are dye-labeled deoxyribonucleoside triphosphates that have been developed specifically for the convenient and efficient fluorescent labeling of DNA. Like other nucleotides, [F]dNTPs can be incorporated into both strands of a PCR amplicon at random dC and dT sites and extended by enzymes such as AmpliTaq™ DNA Polymerase and GeneAmp® Thermostable r*Tth* Reverse Transcriptase. The [F]dNTPs consist of either a 2'-deoxyuridine 5'-triphosphate (dUTP) or a 2'-deoxycytidine 5'-triphosphate (dCTP) coupled to one of the following rhodamine dyes:

- [R110] shown as **blue** data in the gels and electropherograms
- [R6G] shown as **green** data in the gels and electropherograms
- [TAMRA] shown as **yellow** data in the gel images and **black** data in the electropherograms

Note	*See Table 11 on page 22 for the spectral properties of the [F]dNTPs. The colors given are those obtained using Filter Set A (ABI 373) or Module A (ABI PRISM 377 and ABI PRISM 310).*

[F]dNTPs are highly concentrated and can be added to the PCR reaction mixture without adjusting the concentration of each unlabeled dNTP.

The use of [F]dNTPs offers the following advantages:

- Provides an alternative to staining and radioactive tagging techniques traditionally used for labeling PCR products.
- Adds flexibility in experimental design, for example, [F]dNTP-labeled PCR products can be used in applications involving restriction enzymes or ligation.
- Increases sensitivity. [F]dNTP-labeled PCR products can be created with more than one fluorescent chromophore, so that you can use smaller PCR volumes.
- Can be used for end labeling and nick translation.

The Applied Biosystems [F]dNTPs are optimized for use with the GeneAmp® PCR instruments and reagents, and for detection of fluorescently-labeled PCR products on the ABI 373 and ABI PRISM 377 DNA Sequencers and ABI PRISM 310 Genetic Analyzer using GeneScan® 672 Software to analyze results. The [F]dNTPs can be viewed on other systems that detect the wavelengths at which the [F]dNTPs emit (for example, the Applied Biosystems LS-50B Luminescence Spectrometer).

Use of [F]dNTPs in PCR

The Applied Biosystems [F]dNTPs are easy to use in PCR. Simply add the [F]dNTP to the PCR reaction mixture before amplification. The amount of [F]dNTP to add is determined empirically and depends on the final concentration of the corresponding unlabeled dNTP in the PCR reaction mixture, the template sequence, and the number of thermal cycles performed during PCR (see Table 1).

For most PCR amplifications, the typical ratio of dNTP to [F]dNTP is between 100:1 and 1000:1. The amount of fluorescence scales linearly with respect to the number of [F]dNTPs incorporated into most PCR-amplified fragments. This means that if you increase the amount of [F]dNTP by a factor of two in PCR, then the signal for a given amount of product increases by a factor of two. The maximum ratio of [F]dNTP to dNTP that can be used in a PCR reaction mixture without adversely affecting the efficiency of DNA amplification is approximately 1:4.

Do not reduce the amount of dTTP or dCTP in relation to the other three nucleotides because this may reduce the overall amplification efficiency of the PCR process. If the amount of [F]dNTP required is smaller than can be reliably pipetted (<0.5–1 µL), then dilute it in either Tris buffer, pH 8.5–9.5, or 1X PCR buffer.

Table 1. Guidelines for the use of [F]dNTPs in PCR

		Concentration (µM)			
Number of template copies	**Number of cycles**	**Each unlabeled dNTP in reaction**	**[R110] or [R6G] dNTP**	**[TAMRA] dNTP**	**Non- labeled dNTPs:[F]dNTPs**
10–100	45	200	2.0	8.0	100:1
3×10^4	25	200	1.0	4.0	200:1
$>10^6$	25	100	0.2	0.8	500–1000:1

Technical Support

You can contact Applied Biosystems for technical support by telephone or fax, by e-mail, or through the Internet. For further information please see Appendix E Technical Support.

Storage and Stability

The Applied Biosystems [F]dNTPs can be stored at –15 to –25 °C for up to one year from the time they are received. The rhodamine dyes have not been shown to be light sensitive. However, you should minimize exposure to light as a precaution. [F]dNTPs can be diluted in 30 mM Tris-HCl, pH 9.5. Diluted [F]dNTPs can be stored for up to one week at –15 to –25 °C.

[F]dNTP Product Configurations

For convenience and flexibility, the [F]dNTPs ([TAMRA]dUTP, [R110]dUTP, [R6G]dUTP, [TAMRA]dCTP, [R110]dCTP and [R6G]dCTP) are available in three package types:

- Each [F]dNTP packaged separately
- A set of three [F]dUTPs or [F]dCTPs: [TAMRA], [R110], and [R6G], packaged together
- A set of three [F]dUTPs or [F]dCTPs: [TAMRA], [R110] and [R6G], packaged with a GeneAmp Kit

The contents of the separately packaged [F]dNTPs are shown in Table 2.

Table 2. Individual Dye-labeled [F]dNTPs

[F]dNTP	nmols/tube	Number of tubes	Volume/tube (µL)	Concentration (µM)	P/N, kit with protocol	P/N, kit without protocol
[TAMRA]dUTP	12	2	30	400	401895	401766
[R110]dUTP	3	2	30	100	401896	401767
[R6G]dUTP	3	2	30	100	401897	401768
[TAMRA]dCTP	12	2	30	400	402794	402173
[R110]dCTP	3	2	30	100	402795	402175
[R6G]dCTP	3	2	30	100	402796	402174

The contents of the [F]dNTP kits packaged as sets of three are provided in Table 3.

Table 3. [F]dNTP Sets

	nmols/tube	# of tubes	Volume/tube (µL)	Concentration (µM)
[F]dUTP (P/N 401894 with protocol, P/N 401765 without protocol)				
[TAMRA]dUTP	12	1	30	400
[R110]dUTP	3	1	30	100
[R6G]dUTP	3	1	30	100
[F]dCTP (P/N 402793 with protocol, P/N 402176 without protocol)				
[TAMRA]dCTP	12	1	30	400
[R110]dCTP	3	1	30	100
[R6G]dCTP	3	1	30	100

The following GeneAmp kits contain the [F]dNTP sets:

- GeneAmp PCR Reagent Kit with AmpliTaq DNA Polymerase and three [F]dUTPs (P/N N808-0220)
- GeneAmp PCR Reagent Kit with AmpliTaq DNA Polymerase and three [F]dCTPs (P/N N808-0223)
- GeneAmp PCR Core Reagents with three [F]dUTPs (P/N N808-0221)
- GeneAmp PCR Core Reagents with three [F]dCTPs (P/N N808-0224)
- GeneAmp Thermostable r*Tth* Reverse Transcriptase RNA PCR Kit with three [F]dUTPs (P/N N808-0222)
- GeneAmp Thermostable r*Tth* Reverse Transcriptase RNA PCR Kit with three [F]dCTPs (P/N N808-0225)

The contents of the GeneAmp kits packaged with [F]dNTP sets are described in Appendix C.

Control Reaction Protocols

The control reaction protocols described here are for demonstrating the uses of the [F]dNTP kits and for providing standard reactions for optimization and troubleshooting.

GeneAmp PCR Control Protocol

PCR amplification of the lambda control DNA template with control primers PC01 and PC02, under the conditions described in Table 4, produces a 500-bp fluorescently-labeled product using [F]dNTPs.

Table 4. Guidelines for the use of [F]dNTPs with lambda control DNA

Target	Number of cycles	Concentration (μM)			Non- labeled dNTPs:[F]dNTPs
		Each unlabeled dNTP in reaction	[R110] or [R6G] dNTP	[TAMRA] dNTP	
Lambda control DNA	25	200	0.5	2.0	400:1, [R110] and [R6G]; 100:1, [TAMRA]

To perform PCR using [F]dNTPs:

1. Dilute the lambda control DNA 1:10 in TE buffer pH 8.0 for a final concentration of 100 ng/mL.
2. Prepare an unlabeled dNTP working stock by combining 125 μL of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP) with 500 μL of deionized water for a final concentration of 1.25 mM each.
3. Combine the following:

Component	Volume (μL) using 10X PCR Buffer[a]	Volume (μL) using 10X PCR Buffer II[a]	Final Concentration
Deionized water	53	47	–
10X PCR Buffer	10	10	1X
$MgCl_2$ (25 mM)	–	6	1.5 mM
dNTP working stock	16	16	200 μM each dNTP
[F]dNTP	0.5	0.5	0.5–2 μM[b]
AmpliTaq DNA polymerase	0.5	0.5	2.5 U
Control Primer #1 PC01	5	5	1 μM
Control Primer #2 PC02	5	5	1 μM
Diluted control DNA	10	10	1 ng
Total Volume	100	100	–

a. GeneAmp Reagent Kit comes supplied with 10X PCR buffer; GeneAmp PCR Core Reagent Kit comes supplied with 10X PCR Buffer II.

b. On the ABI 373, ABI PRISM 377, and ABI PRISM 310, [TAMRA]dNTP fluorescence emission is four times less than that of [R6G]dNTP or [R110]dNTP, so the final [TAMRA] concentration must be four times greater than [R6G] or [R110]. Therefore, the final [TAMRA] concentration is 2.0 μM, and the final [R6G] and [R110] concentrations are 0.5 μM (dNTP:FdNTP/400:1 [R110] or [R6G]; dNTP:FdNTP/100:1 [TAMRA]).

Note *To reduce the PCR reaction volume, reduce all reaction components proportionally.*

4. Program the thermal cycler for linked files with the following parameters shown in Table 5.

Table 5. Thermal Cycler Times and Temperatures for Use With Lambda Control

Thermal Cycler	Tube			Times & Temperatures for this Kit				
	Tube Type	Volume µL/tube (vapor barrier)	Target/ Polymerase/ Primers	Initial Step	Each of 25 Cycles			Final Step
					Melt	Anneal	Extend	
DNA Thermal Cycler or DNA Thermal Cycler 480	GeneAmp PCR Reaction (N801-0180)	100 (oil: 50–100 µL) Range 10–150	1 ng/ 2.5 U/ 1µM each	STEP CYCLE	STEP CYCLE			TIME DELAY
				60 sec. 94 °C 1 cycle	60 sec. 94 °C	60 sec. 37 °C	120 sec. 72 °C	7 min. 72 °C
DNA Thermal Cycler 480	GeneAmp Thin-Walled Reaction (N801-0537) (N801-0611) (N801-0737)	50 (oil: 50–100 µL) Range 10–150	0.1 ng/ 1.25 U/ 0.2 µM each	STEP CYCLE	STEP CYCLE			TIME DELAY
				60 sec. 94 °C 1 cycle	60 sec. 94 °C	120 sec. (combined) 68 °C		7 min. 72 °C
GeneAmp PCR System 9600	MicroAmp™ Reaction (N801-0533) (N801-0540) (N801-0612)	50 (no vapor barrier needed) Range 10–100	0.1 ng/ 1.25 U/ 0.2 µM each	HOLD	CYCLE			HOLD
				60 sec. 94 °C 1 cycle	15 sec. 94 °C	60 sec. (combined) 68 °C		7 min. 72 °C
GeneAmp PCR System 2400	MicroAmp Reaction (N801-0533) (N801-0540) (N801-0612)	50 (no vapor barrier needed) Range 10–100	0.1 ng/ 1.25 U/ 0.2 µM each	HOLD	CYCLE			HOLD
				60 sec. 94 °C 1 cycle	15 sec. 94 °C	60 sec. (combined) 68 °C		7 min. 72 °C

GeneAmp r*Tth* Reverse Transcriptase RNA Control Protocol

When an [F]dNTP is used in the amplification of the positive control pAW109 RNA with control primers DM151 and DM152, under the conditions described in Table 7 on page 8, a 308-bp fluorescently-labeled product will be amplified.

Table 6. Guidelines for the use of [F]dNTPs with pAW109 RNA and primers DM151 and DM152

		Concentration (μM)			
Target	**Number of cycles**	**Each unlabeled dNTP in reaction**	**[R110] or [R6G] dNTP**	**[TAMRA] dNTP**	**Non- labeled dNTPs:[F]dNTPs**
Control RNA	30–35	40	0.5	2.0	80:1, [R110] and [R6G]; 20:1, [TAMRA]

To perform the reverse transcriptase protocol:

1. Prepare an unlabeled dNTP working stock by combining 125 μL of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP) with 500 μL autoclaved ultrafiltered deionized water for a final concentration of 1.25 mM each.
2. Combine the following to make the reverse transcriptase mixture (RT):

Component	**Volume (μL)**	**Final Concentration**
Autoclaved deionized ultrafiltered water	7.8	–
10X r*Tth* Reverse Transcriptase Buffer	2	1X
$MnCl_2$	2	1 mM
dNTP working stock	3.2	200 μM
r*Tth* DNA polymerase	2	5 U
Primer DM152	1	0.75 μM
pAW109 RNA	2	10^6 copies
Total volume per sample	20	–

3. Mix briefly and spin in microcentrifuge to collect the sample.
4. If using an Applied Biosystems DNA Thermal Cycler or a DNA Thermal Cycler 480, overlay the samples with 50 μL of mineral oil.
5. Incubate in the thermal cycler at 70 °C for 5–15 minutes. Store the sample on ice until ready to use.

To perform PCR using [F]dNTPs:

1. Combine the following to make the PCR master mix (PCR):

Component	Volume (µL)	Concentration in Combined (RT & PCR) Reaction Mix
Autoclaved deionized ultrafiltered water	64.5	–
10X Chelating Buffer	8	1X
$MgCl_2$ Solution	6	1.5 mM
Primer DM151	1	0.15 µM
[F]dNTP	0.5	–[a]
Total volume	80	–

a. Because [TAMRA]dNTP fluorescence emission is four times less than that of[R6G]dNTP or [R110]dNTP on the ABI 373, ABI PRISM 377, and ABI PRISM 310, the final [TAMRA] concentration must be four times greater than [R6G] or [R110]. Therefore, the final [TAMRA] concentration is 2.0 µM, and the final [R6G] and [R110] concentrations are 0.5 µM.

2. Add an aliquot of 80 µL of the PCR master mix (PCR) to the reverse transcriptase reaction.
3. Program your thermal cycler for linked files with the following parameters shown in Table 7.

Table 7. Thermal Cycler Times and Temperatures for use with pAW109 RNA control

Thermal Cycler	Tube				Examples of Times and Temperatures				
	Tube Type	Volume in µL/tube			Reverse Transcription	First Step	Melt	Anneal/ Extend	Final Step
		RT[a]	PCR[a]	Vapor Barrier					
DNA Thermal Cycler or DNA Thermal Cycler 480	GeneAmp PCR Reaction (N801–0180)	20	80	50–100 µL oil (P/N 0186-2302)	STEP CYCLE	STEP CYCLE	Each of 35 Cycles STEP CYCLE		TIME DELAY
					5–15 min. 70 °C	120 sec. 95 °C	60 sec. 95 °C	60 sec. 60 °C	7 min. 60 °C
GeneAmp PCR System 9600	MicroAmp Reaction (N801–0533) (N801–0540) (N801–0612)	20	80	(no vapor barrier needed)	HOLD	HOLD	Each of 35 Cycles CYCLE		HOLD
					5–15 min. 70 °C	60 sec. 95 °C	10 sec. 95 °C	15 sec. 60 °C	7 min. 60 °C
GeneAmp PCR System 2400	MicroAmp Reaction (N801–0533) (N801–0540) (N801–0612)	20	80	(no vapor barrier needed)	HOLD	HOLD	Each of 35 Cycles CYCLE		HOLD
					5–15 min. 70 °C	60 sec. 95 °C	10 sec. 95 °C	15 sec. 60 °C	7 min. 60 °C

a. RT = reverse transcriptase master mix; PCR = PCR master mix.

Detection of [F]dNTP-Labeled PCR Products

Using ABI PRISM DNA Sequencers with GeneScan Software

This section contains guidelines for detecting [F]dNTP-labeled PCR products using the ABI 373 or ABI PRISM 377 DNA Sequencer or the ABI PRISM 310 Genetic Analyzer with GeneScan 672 software. Refer to your user's manual and *GeneScan 672 Software User's Manual* (P/N 902842) for more detailed information.

Considerations for Loading PCR Products

When the ratio of dNTP to [F]dNTP used in the PCR ranges from 50–500:1, then the typical amount of [F]dNTP-labeled PCR product to analyze will range from 0.125–0.5 µL.

The exact loading amount needs to be determined empirically because several factors can influence the number of [F]dNTP molecules actually incorporated into the PCR product. Some of these factors include the ratio of dNTP to [F]dNTP, the sequence of the template, the starting copy number of the template, and thermal cycling conditions.

When analyzing an [F]dNTP-labeled PCR product for the first time, determine the correct amount of PCR product to load by titrating the amount loaded in several samples.

If the PCR product has been overloaded, it will be difficult to distinguish the signals of the [F]dNTP-labeled PCR products from interfering fluorescent species on the ABI 373 and ABI PRISM 377 gels and ABI PRISM 310 electropherograms. If these excess fluorescent signals are observed, try loading one-fifth to one-tenth the amount of PCR product or use one of the methods described in "Fluorescent By-products, Unincorporated [F]dNTPs, and Their Removal" on page 15 to remove them.

Typical volumes that are loaded in square-tooth wells on the ABI PRISM 377 and ABI 373 are shown in Table 8 on page 10. To load more of the PCR product, concentrate and desalt it before loading. On the ABI PRISM 310, you can change the sample injection conditions. Refer to the *ABI Prism 310 Genetic Analyzer GeneScan Chemistry Guide* (P/N 903560) for detailed instructions.

Table 8. Typical Loading Volumes for Square-tooth Wells on the ABI 373 and ABI PRISM 377

DNA Sequencer	Configuration	Volume
ABI 373	24 well	5 µL
ABI 373	36 well	3.5 µL
ABI PRISM 377	34 well	1.5 µL
ABI PRISM 377	24 well	2.5 µL

Multicolor Detection of PCR Products in a Single Loading

With the ABI PRISM multicolor fluorescence automated detection systems, multiple fluorescently-labeled PCR products can be detected in a single loading. Several individual optimized PCR amplification products can be combined (pooled) and loaded into one gel lane or injected simultaneously into a capillary. The fragments not overlapping in size can be labeled with the same [F]dNTP, and fragments with overlapping sizes can be labeled with differently colored [F]dNTPs. Initially, PCR products generated by each primer pair should be analyzed individually to verify that PCR amplification conditions are adequate and to determine the volumes of each product that should be added to each pool thereafter.

Loading PCR Products onto the ABI 373 or ABI PRISM 377 Gels

Note *The procedure for preparing the PCR product for loading on a denaturing gel differs from the procedure for preparing the PCR product for loading on a native gel.*

Denaturing Systems

1. Prepare the Loading Cocktail by combining:
   - 2.5 µL formamide
   - 0.5 µL blue dextran (50 mm EDTA, 50 mg/mL blue dextran)
   - 0.5 µL size standard (GeneScan-350 [ROX], GeneScan-500 [ROX], GeneScan-1000 [ROX] or GeneScan-2500 [ROX])

   A Master Mix can be prepared in advance based on these ratios. The Master Mix can be stored at 2–6 °C for one to two weeks.
2. Mix 1.5 µL of product or pooled product with 3.5 µL Loading Cocktail.
3. Heat at 95 °C for three minutes in a capped tube.
4. Chill on ice.
5. Load each mixture onto an individual gel lane.

Native Systems

1. Prepare the Loading Cocktail by combining:
   - 2.5 µL 2x loading buffer
   - 0.5 µL size standard (GeneScan-350 [ROX], GeneScan-500 [ROX], GeneScan-1000 [ROX] or GeneScan-2500 [ROX])

   A Master Mix can be prepared in advance based on these ratios. The Master Mix can be stored at 2–6 °C for one to two weeks.
2. Mix 2.0 µL of product or pooled product with 2.5 µL Loading Cocktail.
3. Load each mixture onto an individual gel lane.

For most applications, you should not denature PCR products by heating prior to gel loading if analysis is performed on either a non-denaturing (native) polyacrylamide gel matrix or a non-denaturing agarose gel matrix.

Preparing PCR Products for Injection into the ABI PRISM 310 Capillary

Note	*For detailed information on analysis procedures, see the* GeneScan Analysis User's Manual. *For information on instrument settings and modifications to run modules, see the* ABI Prism 310 Genetic Analyzer User's Manual.

Denaturing Systems

1. Combine 12 µL of deionized formamide with 1 µL of appropriately diluted PCR product or pooled product in an ABI PRISM 310 Genetic Analyzer sample tube.
2. Add 0.5 µL of the GeneScan-350 [ROX], GeneScan-500 [ROX], GeneScan-1000 [ROX] or GeneScan-2500 [ROX] size standard.
3. Close the tube with a septum.
4. Heat for two minutes at 95 °C in a capped tube.
5. Chill on ice.
6. Prepare the autosampler. (See instructions for preparing the autosampler in the *ABI PRISM 310 Genetic Analyzer User's Manual.)*
7. Place sample tubes in the appropriate positions on the autosampler.

Native Systems

1. Combine 12 µL of deionized water with 1 µL of appropriately diluted PCR product or pooled product in an ABI PRISM 310 Genetic Analyzer sample tube.
2. Add 0.5 µL of the GeneScan-350 [ROX], GeneScan-500 [ROX], GeneScan-1000 [ROX] or GeneScan-2500 [ROX] size standard.
3. Close the tube with a septum.
4. Prepare the autosampler. (Refer to instructions for preparing the autosampler in the *ABI PRISM 310 Genetic Analyzer User's Manual.)*
5. Place sample tubes in the appropriate positions on the autosampler.

The Matrix File

Although the [F]dNTPs fluoresce at different wavelengths, there is some overlap in the emission spectra. It is necessary to correct for this overlap (or filter cross-talk) before analyzing data with GeneScan 672 analysis software. To accomplish this, a mathematical matrix needs to be created and stored as a matrix file. When data is analyzed, the appropriate matrix is applied to the data to subtract out any emission overlap.

A matrix file must be created when a different set of dyes, different run conditions, or different gel types are used for the first time. Therefore, before analyzing samples labeled with the [F]dNTPs, you must make a new matrix.

Note	*When preprocessing a gel to use in making a matrix, make sure you de-select the matrix file in the pre-process menu, and that you do not include the primer peak or any other peaks that may be off-scale. Also, make sure there are at least three peaks in each color for the region you are using to make the matrix. (If necessary, use more than 1000 points so that 3–5 peaks can be analyzed.)*

[F]dNTP Matrix Standards

To make a new matrix, use the [F]dNTP Matrix Standard Kit (P/N 402792). These standards are used to generate the "multicomponent matrix" required for four-color fluorescent fragment detection using GeneScan 672 software on the ABI 373 and ABI PRISM 377 DNA Sequencers and the ABI PRISM 310 Genetic Analyzer. The analysis software uses the multicomponent matrix to automatically analyze signal from the different-colored [F]dNTP-labeled DNA samples and a ROX-labeled size standard.

This matrix kit contains one tube each of the following:

- Taq G-term, [R110]/Blue dye
- Taq A-term, [R6G]/Green dye
- Taq T-term, [TAMRA]/Yellow dye
- [ROX]/Red dye Matrix standards

Note *Cap color does not correspond to dye color.*

Each tube contains sufficient standard to load ten lanes. The standards are formulated in a blue loading buffer for convenience in gel loading. The DNA concentration per labeled fragment is 0.25 nM for [R110] (Taq G-term) and [R6G] (Taq A-term), 1 nM for [TAMRA] (Taq T-term) and 4 nM for [ROX]. The standards are buffered in 1X TBE containing 7 mM EDTA (pH 8.5). They are stable for six months at 2–8 °C (avoid freeze-thaw cycles).

Thoroughly mix the contents of each standard tube before use by vortexing, and centrifuge briefly to collect the liquid on the bottom of the tube.

Preparing Matrices

To generate the [F]dNTP matrix on the ABI 373 and ABI PRISM 377:

Set instrument to Filter Set A or Module A and run each of the matrix standards under the same conditions to be used for experimental samples. Refer to your instrument's user manual for gel preparation instructions and electrophoresis conditions.

Denaturing systems

1. In a separate tube for each standard, combine 2.5 µL of standard with 2.5 µL of deionized formamide.
2. Heat the mixtures at 90 °C in a capped tube for two minutes to denature.
3. Store on ice until loading.
4. Use a separate lane for each standard, and load alternate lanes, leaving the intervening lanes empty.
5. Load the appropriate amount (see Table 8 on page 10) of the standard/formamide mix per lane.

Note *DNA must not be stored in formamide for more than a few hours.*

Native systems

1. In a separate tube for each standard, combine 2.5 µL of standard with 2.5 µL of deionized water.
2. Store on ice until loading.
3. Use a separate lane for each standard, and load alternate lanes, leaving the intervening lanes empty.
4. Load the appropriate amount (see Table 8 on page 10) of the standard / water mix per lane.

To generate the [F]dNTP matrix on the ABI PRISM 310:

Refer to the *ABI PRISM 310 Genetic Analyzer GeneScan Chemistry Guide* for polymer preparation instructions and electrophoresis conditions.

Denaturing systems

1. In the ABI PRISM 310 collection software / injection list assign the GS Short Denatured A Module to each of the matrix standard samples, applying the same conditions as will be used for experimental samples.
2. In a separate tube for each standard, combine 1 µL of standard with 12 µL of deionized formamide.
3. Heat the mixtures at 90 °C for two minutes to denature and quick chill on ice.
4. Store on ice until loading.
5. Prepare the autosampler. Refer to instructions for preparing the autosampler in the *ABI PRISM 310 Genetic Analyzer User's Manual.*
6. Place sample tubes in the appropriate positions on the autosampler.

Native systems

1. In the ABI PRISM 310 collection software / injection list assign the GS Native A Module to each of the matrix standard samples, applying the same conditions as will be used for experimental samples.
2. In a separate tube for each standard, combine 1 µL of standard with 12 µL of deionized water.
3. Prepare the autosampler. Refer to instructions for preparing the autosae tuectwhe *ABI PRISM 310 Genetic Analyzer User's Manual.*
4. Place sample tubes iwhe appropriate positions on the autosampler.

Appendix A Troubleshooting

Table 9. Observations and Recommended Actions

Observation	Possible Cause	Recommended Action
Signal of product very weak or absent	Insufficient PCR product loaded	Load more of the PCR product on the gel or capillary. If necessary, concentrate the sample with Microcon-30 or Centricon-30
	Not enough [F]dNTP added to PCR reaction	Rerun PCR with more [F]dNTPs
Signal is very strong with streaks and extra bands	Overloaded gel or capillary	Load 5–10 times less PCR product on the gel or capillary
		Change injection conditions (ABI PRISM 310)
		Decrease the amount of [F]dNTPs used in the PCR
		Purify the PCR product
Split peaks	Base composition of target DNA (split peaks are sequence specific, especially if the AT content is <35% or >65%.)	Use a native gel to analyze the PCR product

Fluorescent By-products, Unincorporated [F]dNTPs, and Their Removal

In some cases, fluorescent interferences migrate into the gel or capillary. They are often easy to recognize because they give rise to signals in specific regions of the gel or electropherogram. The two types of species usually seen are reaction by-products and excess, unincorporated [F]dNTPs.

By-products are related to thermal cycling conditions and template purity. Optimizing your PCR conditions lessens their generation, but both they and unincorporated [F]dNTPs can be removed easily if they appear. Some of these species are shown in Table 10 on page 16.

Table 10. Interfering fluorescent species

Type of gel or capillary	Species	Migration pattern
373/377 denaturing gel[a]	[TAMRA]dUTP by-product	220 bp, diffuse band
	[R110]dUTP by-product	220 bp, diffuse band
	[R6G]dUTP by-product	250 bp, diffuse band
	unincorporated [F]dNMPs	~90 bp
	unincorporated [F]dNDPs	~65 bp
	unincorporated [F]dNTPs	~30 bp
	unincorporated [F]dUs (after CIP treatment)	300–350 bp, large, diffuse spot
373/377 native gel	unincorporated [F]dNTPs	1200 bp, discrete, tight bands

a. 6% polyacrylamide/8M urea

Calf Intestinal Alkaline Phosphatase Treatment

If the PCR products contain less than 100 bases, unincorporated [F]dNTPs may co-migrate with the band of interest. The migration of these [F]dNTPs can easily be changed by removing their phosphate groups through the use of a phosphatase, such as calf intestinal alkaline phosphatase (CIP). The resulting fluorescent nucleosides migrate as a large diffuse spot at approximately 300–350 bases on a 6% polyacrylamide / 8M urea gel. The CIP treatment does not affect the dye-labeled PCR product or the dUTP reaction by-products.

Calf intestinal alkaline phosphatase (10,000 Units / mL) can be purchased from New England Biolabs. The following conditions work well for PCR products generated by conditions similar to either of the GeneAmp control reactions. Other conditions may require the addition of more enzyme units.

To treat PCR products with CIP:

1. Add 1 µL of CIP solution to 100 µL of PCR product. For smaller reaction volumes, dilute enzyme proportionally.
2. Incubate at 37 °C for 30 minutes.
3. Analyze PCR product as usual.

If fluorescent by-product signals occur at other locations that interfere with your product signals, you can use one of the following methods to eliminate the problem:

- Decrease the amount of PCR product loaded onto the gel
- Add less [F]dNTP to the PCR

You can also remove the unwanted material by one of the following methods:

- Centricon-30 or MicroCon-30 ultrafiltration columns (Amicon)
- CentriSep columns (Princeton Separations)
- Phenol:chloroform extraction

Centricon-30 Use with [F]dNTP-Labeled PCR Products

To purify PCR products with a Centricon-30:

1. Load the following into the retentate reservoir:
   - Entire PCR sample
   - Enough deionized water to fill the retentate reservoir
2. Spin at 3000 × g for ten minutes in a fixed angle rotor.
3. Flip the retentate reservoir over into a clean collection tube.
4. Spin two minutes at 270 × g to recover the PCR product. Typical recovery is 40 µL.
5. Bring up the retentate to any desired volume, for example, the original PCR volume loaded.

Note	*Centricon-30s hold a total volume of 2 mL.*

MicroCon-30 Use with [F]dNTP-Labeled PCR Products

To purify PCR products with a MicroCon-30:

1. Load the following into the retentate reservoir:
   - 450 µL deionized water
   - ≤50 µL PCR sample
2. Spin at 14000 × g for twelve minutes (13,000 rpm in an Eppendorf 5415C Centrifuge).
3. Flip the retentate reservoir over into a clean collection tube.
4. Spin one minute at 14000 × g to recover the PCR product. Typical recovery is 1–3 µL.
5. Bring up the retentate to any desired volume, for example, the original PCR volume loaded.

Note	*MicroCon-30s hold a total volume of 500 µL.*

CentriSep Purification of Extension Products

To purify PCR products with a CentriSep spin column:

1. Gently tap the CentriSep column to cause the gel material to settle to the bottom.
2. Remove the column stopper and add 750 mL of deionized water.
3. Replace stopper and invert the column several times to mix.
4. Allow the gel to hydrate for at least 30 minutes at room temperature. Hydrated columns can be stored for a few days at 2–6 °C. Longer storage is not recommended. Allow columns that have been stored at 2–6 °C to warm to room temperature before use. Remove any air bubbles by inverting the column and allowing the gel to settle.
5. Remove the upper-end cap first and then remove the lower-end cap. Allow the column to drain completely, by gravity. If flow does not begin immediately, apply gentle pressure to the column with a pipet bulb.
6. Insert the column into the wash tube provided.
7. Spin in a variable-speed microcentrifuge at 750 × g for two minutes to remove the interstitial fluid.

Note *You can use the following formula to find the correct rpm at which to spin the column:*

$$\text{rpm} = (g/11.8)^{1/2} \times (1/r)^{1/2} \times 1000$$

where g is the recommended force and r is the radius of the centrifuge (in cm).

---

8. Remove the column from the wash tube and insert it into a sample collection tube.
9. Carefully add the PCR reaction mixture (maximum 40 µL) on the top of the gel material. Use each column only once.
10. Spin in a variable-speed microcentrifuge at 750 × g for two minutes. If using a centrifuge with a fixed angle rotor, place the column in the same orientation as it was for the first spin. This is important because the surface of the gel will be at an angle in the column after the spin.

Phenol/Chloroform Extraction

Phenol/chloroform extraction provides an inexpensive alternative to spin column separation. The recoveries are also good with this method. The following reagents are required:

- Deionized H_2O
- Chloroform, 100 µL/reaction (Applied Biosystems P/N 400459)
- Phenol:H_2O:chloroform, at room temperature, 200 µL/reaction (Applied Biosystems P/N 400765)
- 2 M sodium acetate, pH 4.5, 15 µL/reaction (Applied Biosystems P/N 400884)
- 100% ethanol, at room temperature, 300 µL/reaction
- 70% ethanol, at room temperature, ~500 µL/reaction

Note *Vortex the phenol:H_2O:chloroform reagent before using.*

To purify PCR products by phenol/chloroform extraction:

1. At the end of thermal cycling, bring PCR reaction volume up to 100 µL with deionized H_2O.
2. Add 100 µL of chloroform to dissolve the oil (alternatively, this oil can be removed with a pipet, in which case you do not need to add the chloroform).
3. Extract the PCR with 100 µL of phenol:H_2O:chloroform reagent.
4. Vortex and centrifuge the sample, then remove and discard the lower organic phase.
5. Re-extract the aqueous layer with a second 100 µL aliquot of the phenol:H_2O:chloroform reagent.
6. Vortex and centrifuge the sample for one minute, then transfer the aqueous upper layer to a clean tube.
7. Precipitate the extension products by adding 15 µL of 2 M sodium acetate, pH 4.5, and 300 µL of 100% ethanol.
8. Centrifuge the mixture for 15 minutes at room temperature. Wash the pellet with 70% ethanol, then dry.
9. Bring sample up to original volume in deionized water.

Appendix B Spectral Properties of [F]dNTPs

The laser in the ABI 373 and ABI PRISM 377 DNA Sequencers and ABI PRISM 310 Genetic Analyzer emits at 488 and 514 nm. [R110]dNTPs and [R6G]dNTPs absorb light much more strongly at 488 and 514 nm than do [TAMRA]dNTPs (see Figures 1 and 3). Therefore, [TAMRA]dNTP is supplied at four times greater concentration than [R110]dNTP or [R6G]dNTP. This concentration difference optimizes detection of the [F]dNTP-labeled PCR products on the ABI 373/672. The spectral properties of the [F]dNTPs are shown in the following figures.

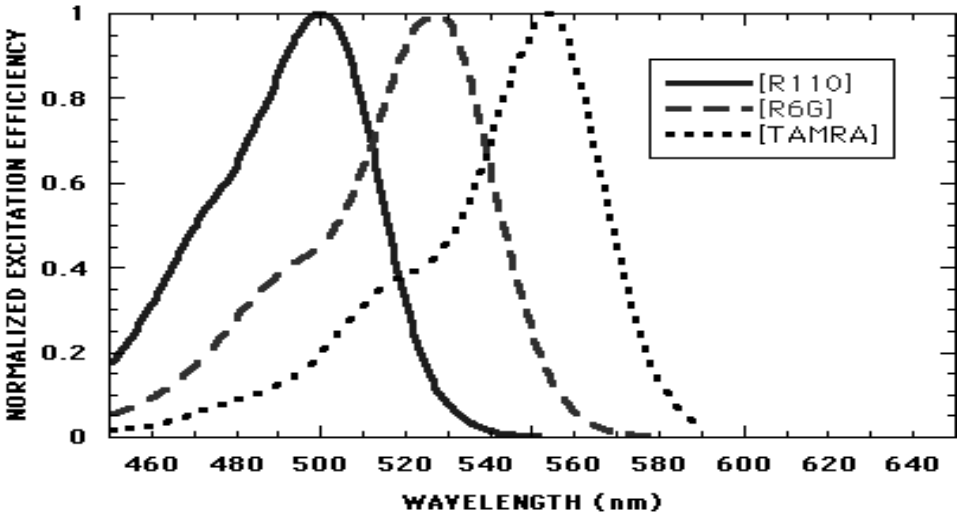

Figure 1. Excitation Spectra of [F]dCTPs

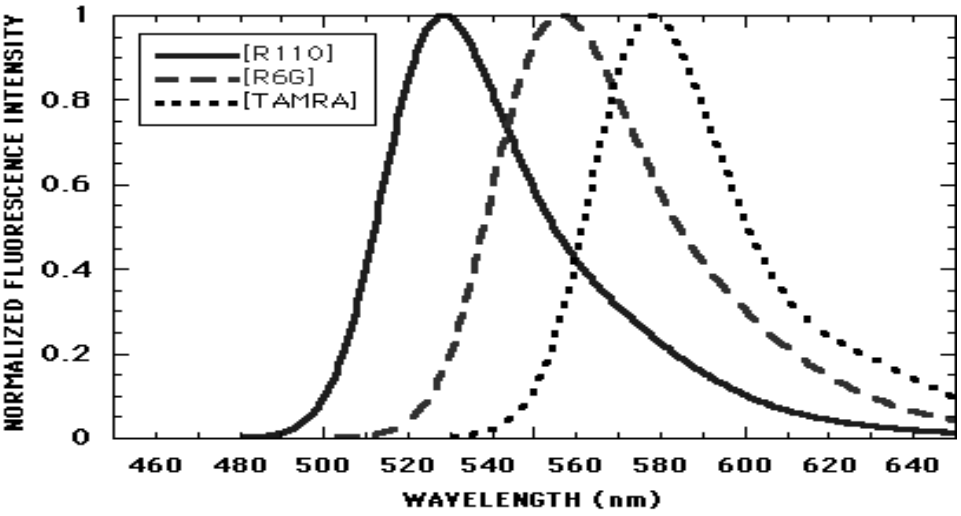

Figure 2. Emission Spectra of [F]dCTPs

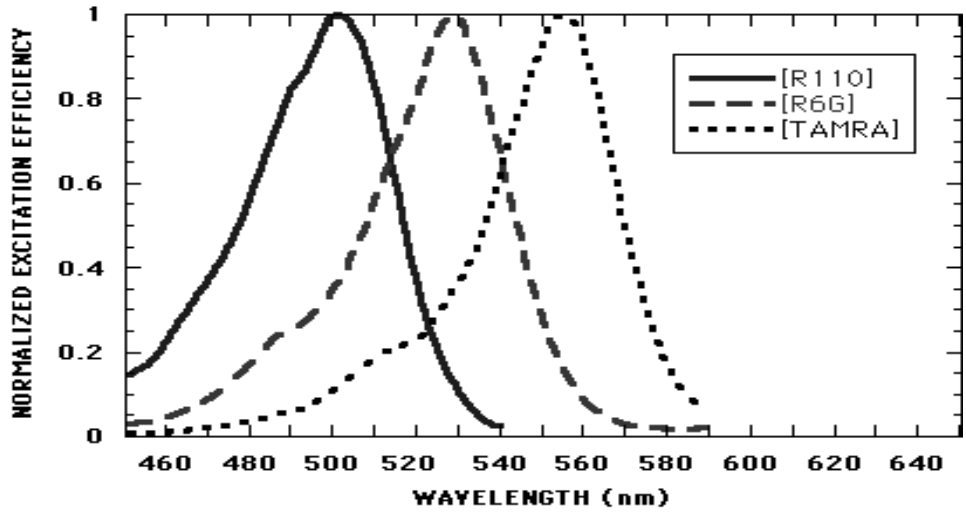

Figure 3. Excitation Spectra of [F]dUTPs

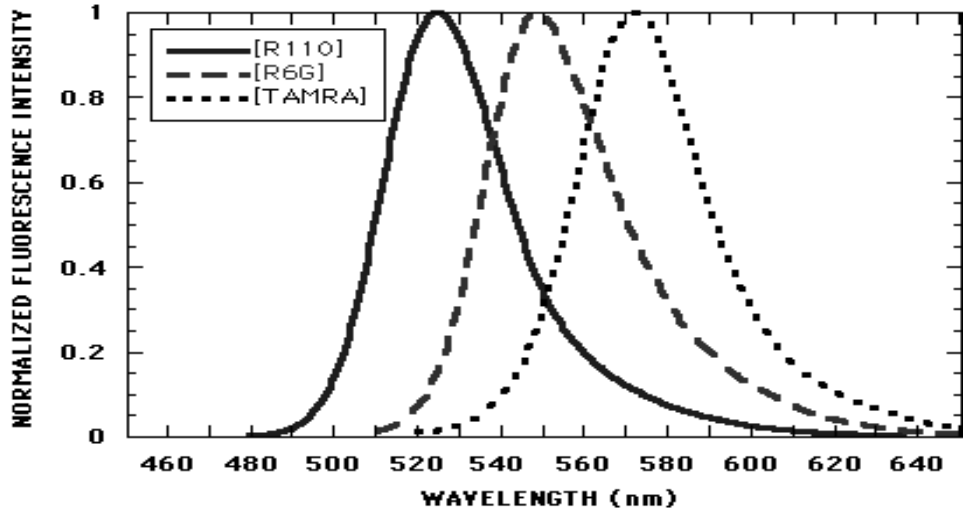

Figure 4. Emission Spectra of [F]dUTPs

The spectral properties of the [F]dNTPs are summarized in Table 11.

Table 11. [F]dNTP Spectral Properties

Components	Spectral Properties[a]	
	Excitation λ max (nm)	Emission λ max (nm)
[R110]dUTP	502	530
[R6G]dUTP	528	555
[TAMRA]dUTP	552	580
[R110]dCTP	500	529
[R6G]dCTP	527	556
[TAMRA]dCTP	553	578

a. Measured in 30 mM Tris buffer at pH 9.5.

Appendix C GeneAmp PCR Reagent Kit Configurations

The contents of each GeneAmp Kit coupled with three [F]dNTP are provided in Tables 12, 13, and 14.

Table 12. GeneAmp PCR Reagent Kit with three [F]dNTPs: [F]dUTP, P/N N808-0220; [F]dCTP, P/N N808-0223

Reagent	Volume	Description
AmpliTaq DNA Polymerase	50 µL	1 tube containing 5 U/µL, 250 U
10X PCR Buffer	1.4 mL	1 tube of 100 mM Tris-HCl, pH 8.3 (at room temperature), 500 mM KCl, 1.5 mM $MgCl_2$, and 0.01% (w/v) gelatin (solution has been autoclaved)
dATP	320 µL	1 tube each of 10 mM of dATP, dTTP, dGTP, and dCTP in sterile, deionized water at pH 7.0
dTTP	320 µL	
dGTP	320 µL	
dCTP	320 µL	
Lambda control DNA template[a]	100 µL	1 tube of 1 µg/mL of lambda DNA
Control primer 1	50 µL	1 tube of 20 µM primer, 25 nucleotides in length
Control primer 2	50 µL	1 tube of 20 µM primer, 25 nucleotides in length
[TAMRA]dNTP	30 µL	12 nmol
[R110]dNTP	30 µL	3 nmol
[R6G]dNTP	30 µL	3 nmol

a. The control lambda DNA is also available as an individual product (P/N N808-0008).

Table 13. GeneAmp PCR Core Reagents with three [F]dNTPs: [F]dUTP, P/N N808-0221; [F]dCTP, P/N N808-0224

Reagent	Volume	Description
AmpliTaq DNA Polymerase	50 µL	1 tube containing 5 U/µL, 250 U
10X PCR Buffer II	1.4 mL	1 tube of 100 mM Tris-HCl, pH 8.3 (at room temperature), 500 mM KCl, and 0.01% (w/v) gelatin (solution has been autoclaved)
dATP	320 µL	1 tube each of 10 mM of dATP, dTTP, dGTP, and dCTP in sterile, deionized water at pH 7.0
dTTP	320 µL	
dGTP	320 µL	
dCTP	320 µL	
25 mM $MgCl_2$	1.4 mL	1 tube of 25 mM $MgCl_2$ solution
[TAMRA]dNTP	30 µL	12 nmol
[R110]dNTP	30 µL	3 nmol
[R6G]dNTP	30 µL	3 nmol

Table 14. GeneAmp Thermostable r*Tth* Reverse Transcriptase RNA PCR with three [F]dNTPs: [F]dUTP, P/N N808-0222; [F]dCTP, P/N N808-0225

Reagent	Volume	Description
r*Tth* Reverse Transcriptase	200 µL	1 tube containing 2.5 U/µL, 500 U
10X Chelating Buffer	1.4 mL	100 mM Tris-HCl, 1.0 M KCl, pH 8.3, 7.5 mM EGTA, 0.5% (w/v) Tween 20, and 50% (v/v) glycerol)
10X r*Tth* Reverse Transcriptase Buffer	400 µL	100 mM Tris-HCl, 900 mM KCl, pH 8.3
dATP	320 µL	1 tube each of dATP, dTTP, dGTP, and dCTP at 10 mM concentration in autoclaved, deionized, ultrafiltered water, titrated with NaOH to pH 7.0
dTTP	320 µL	
dGTP	320 µL	
dCTP	320 µL	
pAW109 control RNA	50 µL	1 tube of 5000 copies/ µL of RNA transcribed from pAW109
Primer DM 151	50 µL	1 tube of 15 µM DM152 primer (downstream)
Primer DM 152	50 µL	1 tube of 15 µM DM151 primer (upstream)
10 mM $MnCl_2$	400 µL	1 tube of 10 mM $MnCl_2$ solution
25 mM $MgCl_2$	1.4 mL	1 tube of 25 mM $MgCl_2$ solution
[TAMRA]dNTP	30 µL	12 nmol
[R110]dNTP	30 µL	3 nmol
[R6G]dNTP	30 µL	3 nmol

Appendix D Materials and Equipment Not Supplied

The following items in Tables 15 and 16 may be required in addition to the reagents supplied in the [F]dNTP kits. Equivalent sources may be acceptable where noted. This list does not include equipment or reagents required for the synthesis of primers or for DNA extraction.

Table 15. Reagents and Materials

Reagent/Material	Source
[F]dNTP Matrix Standard Kit	Applied Biosystems (P/N 402792)
GeneScan-350 [ROX], GeneScan-500 [ROX], GeneScan-1000 [ROX], or GeneScan-2500 [ROX] size standards	Applied Biosystems (P/N 401735, 401734, 401098, or 401100, respectively)
2X loading buffer	Applied Biosystems (P/N 401144)
Blue Dextran	Sigma (P/N D5751)
Calf intestinal alkaline phosphatase	New England Biolabs (P/N 290S)
Disodium ethylenediaminetetraacetic acid dihydrate (Na_2EDTA)	Sigma (P/N E4884) Gibco BRL (P/N SS7SUA)
Formamide	Applied Biosystems (P/N 400596)
Mineral Oil	Applied Biosystems (P/N 0186-2302)

Table 16. Equipment

Equipment	Source
ABI 373 or ABI PRISM 377 DNA Sequencer, or ABI PRISM 310 Genetic Analyzer	Applied Biosystems (P/N 373-01, 903526, or 903953)
GeneScan 672 software	Applied Biosystems (P/N 672-90)
Centricon-30 ultrafiltration columns	Perkin-Elmer, Norwalk, CT (P/N N930-1381)
MicroCon-30 ultrafiltration columns	Amicon (P/N 42410)
CentriSep columns	Princeton Separations (P/N PSR00100)
GeneAmp Thin Walled Reaction Tubes	Applied Biosystems, Norwalk, CT (P/N N801-0537)
MicroAmp Reaction Tubes	Applied Biosystems, Norwalk, CT (P/N N801-0540, N801-0533, N801-0580)
DNA Thermal Cycler 480	Applied Biosystems, Norwalk, CT (P/N N801-0100, N801-0101, N801-0102)
GeneAmp PCR System 9600	Applied Biosystems, Norwalk, CT (P/N N801-0001, N801-0002, N801-0003)
GeneAmp PCR System 2400	Applied Biosystems, Norwalk, CT (P/N N803-0001, N803-0002, N803-0003)

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