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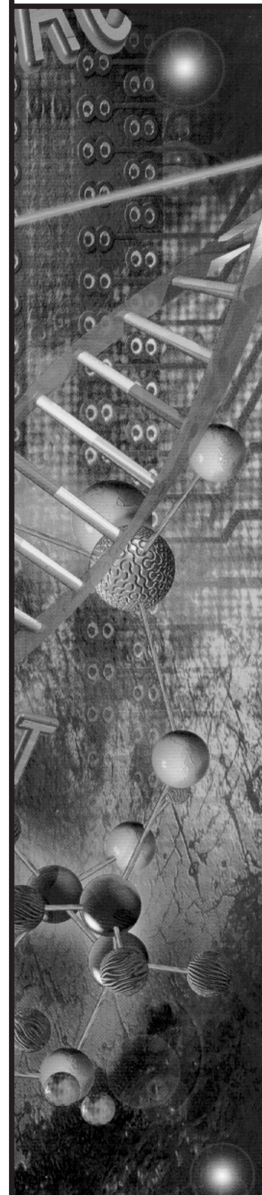
DNA Synthesis With IRDye® 800 Phosphoramidite

Part #4000-33



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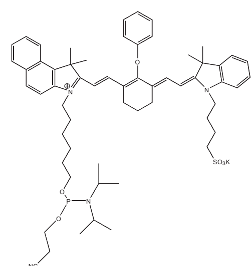
Principle and Scope

The phosphoramidite of the Near IR fluorescent dye, IRDye® 800, can be used to label DNA molecules prepared in a DNA synthesizer. The dye is attached to the 5' end of the protected, support-bonded oligonucleotide via fast deprotection phosphoramidite chemistry. On syntheses at 200 nmol scale, typical crude yields of dye-labeled oligonucleo-tides are 65 to 100 nmol. Standard phosphoramidites and deprotection conditions cannot be used. This protocol was developed on an Applied Biosystems 8909 DNA synthesizer and works well using the Polygen DNA Synthe-sizer. For best results on other systems, protocol modifi-cation may be required.

Structure and Properties

Fig. 1. Structure of IRDye 800 Phosphoramidite

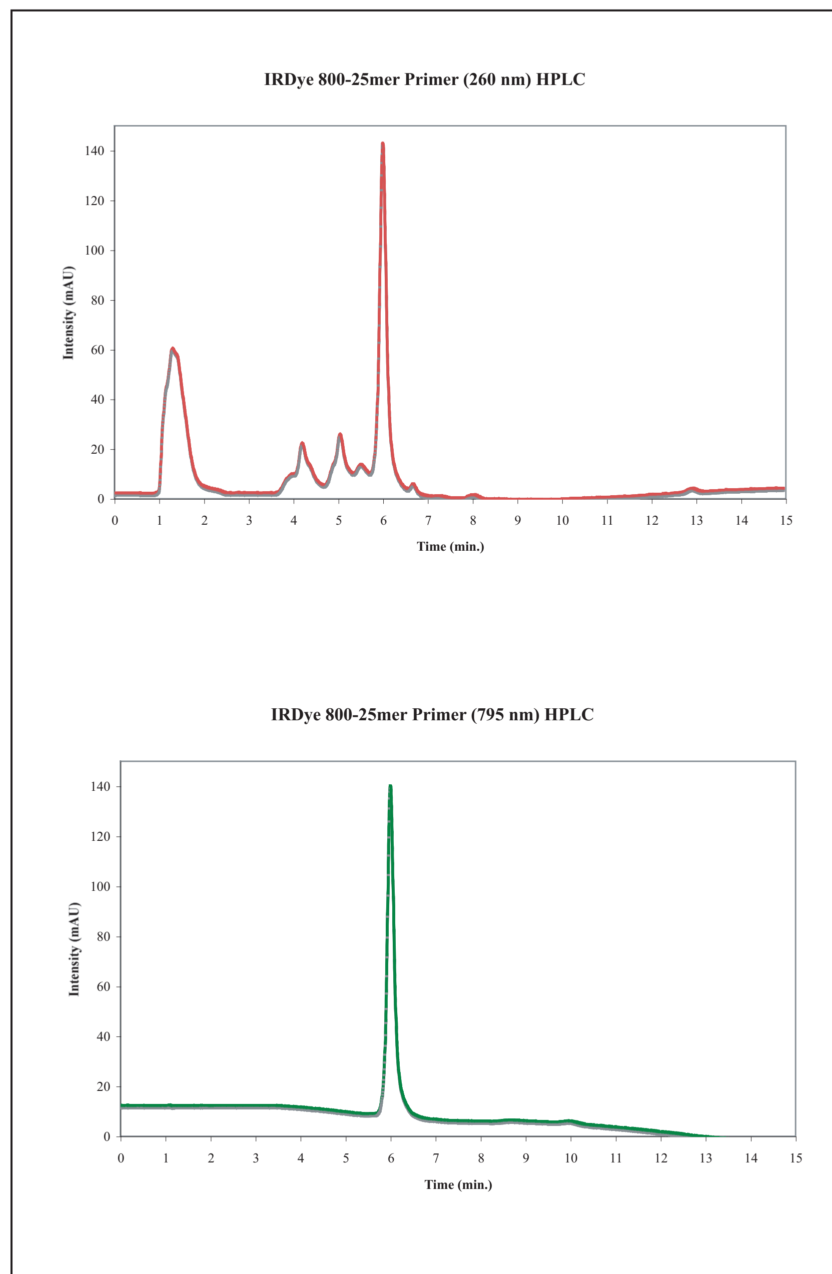


IRDye 800 Phosphoramidite	
Absorption Max (λ_{max}, methanol)	787 nm
Absorption Max (aqueous oligonucleotide)	795 nm
Molar Absorptivity (ε, methanol)	270,000
Emission Max (methanol)	812 nm
Emission Max (aqueous oligonucleotide)	819 nm
Molecular Weight	999.30

Equipment and Reagents

- IRDye 800 phosphoramidite (LI-COR Biosciences, 4000-33)
- Polygen DNA-Synthesizer or Equivalent
- CPG column/beads, 500A, 200 nanomole (Proligo A322004-04, C322004-01, G322004-01, T321010-00, A302001-01, C302001-01, G302001-01, T301001-01 or equivalent)
- A, C, G, and T phosphoramidites (Proligo A112081-12, C112081-12, G112081-12, T111081-12 or equivalent)
- Synthesis Reagents: Oxidizing solution, Capping reagent solutions, Deblocking reagent solution, Activator solution, Acetonitrile (Glen Research 40-4132-52, Proligo L870020-06, L850020-4N, L820090-06, L880020-06, L810045-06 or equivalent)
- Ammonium Hydroxide
- Microcentrifuge, standard and filter tubes
- Gradient HPLC system, reverse phase C18, for product analysis and purification, if desired. Acetonitrile (HPLC grade), triethylammonium

Fig. 3 Typical Analytical HPLC of Crude Oligonucleotide



5. Primer Analysis and Purification by HPLC

The crude oligonucleotides are sufficiently pure for many purposes. Their purities can be checked with analytical HPLC, and preparative HPLC can be used when highest purity is needed. It is convenient to monitor at least two wavelengths with the HPLC detector. Detection at 795 nm shows just the dye-labeled components in the sample, while 260 or 280 nm will also reveal unlabeled products, such as the failure sequences of the synthesis. These HPLC conditions can be used for analysis and purification.

Solvents:	A = 0.1 M triethylammonium acetate in 4% acetonitrile/96% water
	B = 0.1 M triethylammonium acetate in 80% acetonitrile/20% water
Column:	C18, 5 µm, 300 Å, 10 × 100 mm
Flow:	3.4 ml/min
Gradient:	30–40% B over 0.3 minutes
	40-100% B from 0.3 – 8 minutes
	100% B for 2 minutes
	100-30% B for 5 minutes

Analytical HPLC. Dilute 1 µl of the filtered, deprotected oligonucleotide solution from step 3 with 99 µl of sterile, pure water. Inject 10 µl of the diluted solution for the analytical run. Typical chromatograms for both wavelengths are shown in Figure 3 for an oligonucleotide prepared with standard deprotection chemistry. The main peak (~6 min) is the desired product. Synthesis failure sequences are more hydrophyllic, so these components elute earlier.

Preparative HPLC. If LC purification is desired, all or part of the filtered, deprotected oligonucleotide solution from step 3 is directly injected. Collect appropriate fractions and remove the solvents and the triethylammonium acetate buffer in a vacuum drying unit.

6. Solution Makeup

Dissolve the oligonucleotide in 1x TE (10 mM Tris, 1 mM EDTA, pH 7.4) to the desired concentration. For a 1.0 µM (1.0 pmol/µL) solution, dilute the dye labeled oligonucleotide to obtain 0.27 absorbance units

Precautions

1. The oxidizing solution supplied by Glen Research (40-4132-52 or equivalent*) should be used. Harsher oxidizer solutions supplied by some manufacturers are not recommended.
2. All phosphoramidites are sensitive to moisture and oxygen, and the handling procedures described here are good practice in general. No special modifications of your standard procedures for similar products are likely to be necessary for IRDye 800 phosphoramidite. Protect the product from air and moisture. Use only dry, oxygen-free acetonitrile ("DNA synthesis grade") for preparation of the amidite solutions.
3. For best results, use the entire solution of IRDye 800 amidite within a day. There is some loss of coupling efficiency of the solutions on standing. If the solutions will not be used within a day, place the bottle in a jar with some Drierite and store at -20°C to prolong the working life of the amidite.
4. Flushing the lines of the synthesizer with acetonitrile (or methanol followed by acetonitrile) is generally sufficient to remove any traces of the dye.
5. The ammonia solution used in the deprotection of the oligonucletides should be handled in a fumehood.

* Recipe: 4.3 g iodine, 90 ml water, 4 ml pyridine, and 900 ml THF

Procedure

1. Prepare a 0.1 M Solution of the IRDye 800 Phosphoramidite in Acetonitrile

a. Add 1.00 ml of acetonitrile to the 100 µmol package to make the solution. It is good practice to provide a source of dry nitrogen or argon gas to displace the solvent that will be removed from the acetonitrile bottle. A needle connected to a stream of the gas and a bubbler is a convenient way to do this. Another approach is to use a second syringe barrel filled with dessicant for the vent, although this does not remove oxygen. Use a similar setup to allow for the escape of the gas displaced when the acetonitrile is added to the phosphoramidite vial.

b. Choose a clean, dry syringe and needle of appropriate size. Pierce the seal of the acetonitrile reagent bottle with the needle and slowly withdraw slightly more than the desired amount of solvent.

c. Do the next steps rapidly to minimize exposure of the acetonitrile to air. Remove the syringe and needle from the acetonitrile bottle. Invert the syringe and force any air bubbles out the needle. Push the excess acetonitrile from the syringe into an appropriate waste container. Inject the acetonitrile into the sealed IRDye 800 phosphoramidite vial. Remove the needles from the seals, and allow the IRDye 800 phosphoramidite to dissolve. Swirl the solution occasionally until no undissolved particles are observed on the sides of the vial.

2. DNA Synthesis Program

The basic synthesis protocol for your synthesizer can be used until the last step involving the dye amidite. A longer coupling time for the IRDye 800 phosphoramidite is recommended. A 5-10 minute increase in standard coupling time will improve final yields. Since the IRDye 800 phosphoramidite does not contain any trityl group, a "DMT On" mode should be used to avoid unnecessary exposure of the dye to the detritylation reagent.

3. Primer Deprotection

Note: All work with ammonium hydroxide (NH_4OH) should be performed in a fume hood. Methylamine deprotection methods do not work with IRDye phosphoramidites.

Place CPG beads from the synthesis column in a microcentrifuge tube (1.5–2.0 mL capacity). Add 400 µL of cold NH_4OH to each tube. Close tubes and mix the contents thoroughly on a vortex mixer. Allow the samples to stand in the dark for 1 hour at room temperature. Add an additional 400 µL of cold NH_4OH to each tube, close and mix thoroughly. Allow the samples to stand an additional 30 minutes in the dark at room temperature. Strip off the aqueous ammonia in a vacuum drying unit, then resuspend the dry pellet in 200 µL of sterile, ultrapure water. Decant solution from the beads or use a syringe filter to remove CPG beads from the solution and transfer to a new tube.

4. Measurement of Crude Synthesis Yield (Optional)

Dilute 1.0 µL of the filtered primer solution with 199.0 µL of sterile ultrapure water. Obtain the UV/vis absorption spectrum of the resulting solution (see Figure 2). The crude yield can then be calculated from the absorbance value at 795 nm (A_{795}), the absorptivity (270 µL / nmol / cm), the path length (b cm), the dilution factor (200) and the total primer solution volume (200 µL):

$$\text{Yield (nmol)} = A_{795} / (270 \times b) \times 200 \times 200$$

For example, a measured absorbance of 0.46 in a 1 cm cell would correspond to a crude yield of 68 nmol.

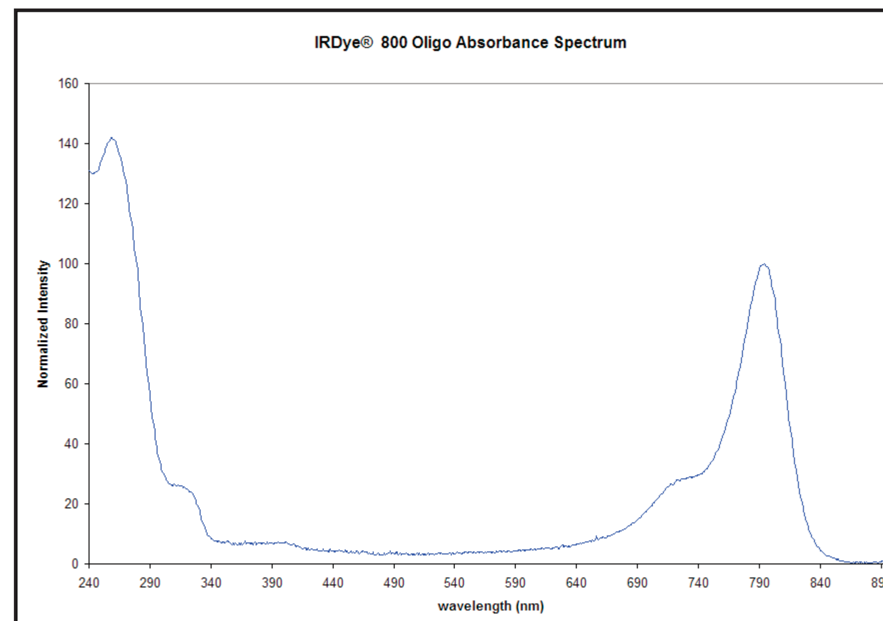


Fig. 2 Typical UV/Vis Spectrum