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LightCycler® Red 640-N-hydroxysuccinimide ester

Version: 11
Content version: September 2016

For labeling a minimum of 5 × 50 nmol oligonucleotides

Cat. No. 12 015 161 001	1 vial for 5 x 50 nmol oligonucleotides

Store the product at −15 to −25°C.

1. General Information

1.1. Contents

Vial/Bottle	Label	Function/Description	Content
1	LightCycler® Red 640 NHS-ester for ≥250 nmol oligonuc.	▪ Contains a sufficient amount of LightCycler® Red 640-NHS ester for labeling a minimum of 5 × 50 nmol oligonucleotides. ▪ The reagent is supplied as a blue solid.	1 vial

1.2. Storage and Stability

Storage Conditions (Product)

The product is shipped on dry ice.
When stored at −15 to −25°C in a tightly sealed bottle, the product is stable through the expiration date printed on the label.

Vial/Bottle	Label	Storage
1	LightCycler® Red 640 NHS-ester	Store at −15 to −25°C. ⚠ ***Store dry and protected from light.***

1.3. Additional Equipment and Reagents Required

For Oligonucleotide Labeling

- DNA synthesizer
- Standard reagents for oligonucleotide synthesis (tetrazol, etc.)
- Absolute, amine-free dimethylformamide (DMF)
- 5'-Amino modifier
- 3'-Phosphate CPG support
- Standard phosphoramidites
- 0.1 M sodium borate buffer (pH 8.5)

For Ethanol Precipitation of Oligonucleotide

- 3 M sodium acetate buffer (pH 8.5)
- Ice-cold absolute ethanol

For Oligonucleotide Purification by HPLC

- HPLC
- Vacuum centrifuge
- POROS OligoR3 separation medium (PerSeptive Biosystems, Inc., 4.6 × 50 mm column). This separation medium is recommended to obtain optimal purification results.
- 100 mM Triethylammoniumacetate (pH 6.9)
- Acetonitrile

Do not use Reversed Phase (RP) separation media because labeled oligonucleotides tend to stick irreversibly to the RP-material, resulting in lower yields of labeled oligonucleotides.

For Quality Control of HPLC-Purified Oligonucleotides

- Photometer

1.4. Application

The LightCycler® Red 640-NHS ester is used for labeling the 5' end of the downstream probe when HybProbe probes are used as detection format with the respective LightCycler® System. In addition, the 3' end of the probe has to be phosphorylated to avoid elongation during PCR.

2. How to Use this Product

2.1. Before you Begin

Sample Materials

Oligonucleotides, 20 to 30 bp in length, modified at the 3' end by phosphorylation.

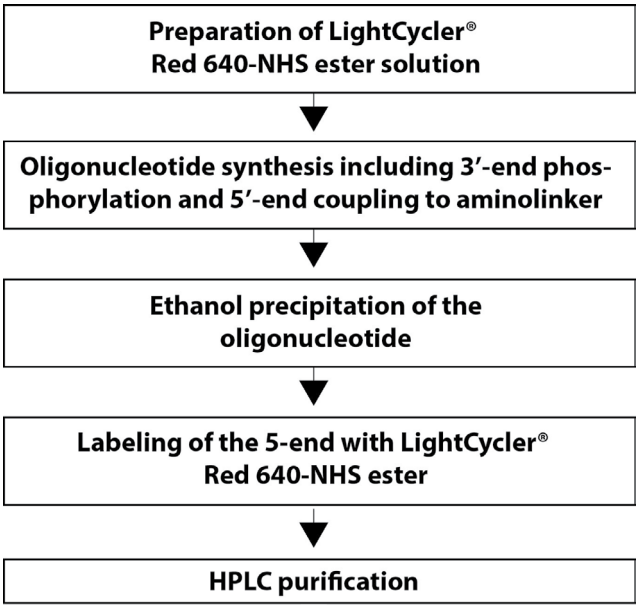
General Considerations

3'-End Labeling of the Upstream Probe with Fluorescein

Label the 3' end of the upstream probe with LightCycler® Fluorescein CPG*. This is essential when dual-color experiments are performed with both the LightCycler® Red 640 and CY5.5-labeled HybProbe probes in a single capillary. Labeling with LightCycler® Fluorescein CPG results in probes with optimized spectral characteristics and superior quality compared to labeling with CPG support material containing a thiourea linkage.

2.2. Protocols

Flowchart



Preparation of the LightCycler® Red 640-NHS Ester Solution

Prepare the LightCycler® Red 640-NHS ester solution as shown below.

1. Dissolve the provided reagent in 1 ml absolute, amine-free dimethylformamide (DMF).

2. Divide the solution into 5 microcentrifuge tubes, 200 µl each.

3. Store the aliquots at −15 to −25°C, dry and protected from light.

Oligonucleotide Synthesis Including 3'-End Phosphorylation and 5'-End Coupling to Aminolinker

The oligonuclotide to be labeled with LightCycler® Red 640 must be modified at the 5' end with a terminal amino group and at the 3' end with a phosphate group.

Oligonucleotide Terminus	Modification
5' end	Introduce the 5'-terminal amino group by performing a reaction between the oligonucleotide and an aminolinker-phosphoramidite in the final oligonucleotide synthesis cycle.
3' end	Introduce the 3'-phosphate group by starting the oligonucleotide synthesis with a corresponding modified CPG support.

Synthesis Procedure

Perform the oligonucleotide synthesis reaction as described below. Refer to the manufacturer's recommendations for appropriate use of the respective reagents.

1. Connect the DNA synthesizer with a 3'-phosphate CPG column. We recommend a 0.2 µmol phosphate CPG support.

2. Dissolve the aminolinker-phosphoramidite in anhydrous acetonitrile according to the manufacturer's recommendations.
   – Attach the vial to the appropriate position of the DNA synthesizer.

3. Program the synthesizer by entering an arbitrary base at the 3' terminus of the oligonucleotide.
   – The 3'-terminal base of the oligonucleotide sequence should be entered as the second base.
   – Set program to "Trityl on".
   – Start the oligonucleotide synthesis.

4. Deprotect the oligonucleotide after cleavage from the CPG support as usual (+50 to +55°C within 5 to 8 hours).

5. Evaporate the solution under vacuum or purify the oligonucleotide by gel filtration.

6. Store the remainder at −15 to −25°C.

Ethanol Precipitation of the Oligonucleotide

Perform the ethanol precipitation as shown below.

1. Dissolve the oligonucleotide (from section **Oligonucleotide Synthesis Including 3'-End Phosphorylation and 5'-End Coupling to Aminolinker**) in 600 µl double-distilled water.

2. Transfer 300 µl of the oligonucleotide solution (corresponding to approx. 50 – 80 nmol from the recommended 0.2 µmol synthesis) in a microcentrifuge tube and add 30 µl sodium acetate buffer (3 M, pH 8.5).

   Store the remainder of the dissolved oligonucleotide at −15 to −25°C for further labeling experiments.

3. Add 0.9 ml ice-cold ethanol, mix well, and store at −15 to −25°C for 2 to 3 hours.

4. Centrifuge at 10,000 × *g* for 15 minutes.
   – Decant the supernatant.

5. Wash the pellet with 100 µl ice-cold ethanol.
   – Centrifuge at 10,000 × *g* for 5 minutes.
   – Decant the supernatant.

6. Store the pellet at −15 to −25°C.

Labeling the 5’ End with the LightCycler® Red 640-NHS Ester

Perform the 5’-end labeling as shown below.
⚠ ***Keep the LightCycler® Red 640-labeled oligonucleotide protected from light.***

1. Dissolve the ethanol-precipitated oligonucleotide (from section **Ethanol Precipitation of the Oligonucleotide**) in 200 µl sodium borate buffer (0.1 M, pH 8.5).

2. Add 200 µl of the LightCycler® Red 640-NHS ester solution (from section **Preparation of the LightCycler® Red 640-NHS Ester Solution**) to the oligonucleotide solution.

3. Store the vial at −15 to −25°C overnight, protected from light.

HPLC Purification

Perform HPLC to separate the labeled oligonucleotide from N-hydroxy-succinimide, unlabeled oligonucleotide, excess dye, and impurities. Perform the purification of the labeled oligonucleotide as shown below.

1. Concentrate the labeling mixture (from section **Labeling the 5’ End with the LightCycler® Red 640-NHS Ester**) in a vacuum centrifuge by heating to 50°C.

2. Dissolve the pellet in 1 ml double-distilled water and apply on a POROS OligoR3 column.

3. HPLC conditions are as follows:

Parameter	Condition
Buffer A	Triethylammoniumacetate (100 mM, pH 6.9)
Buffer B	Triethylammoniumacetate (100 mM, pH 6.9)/acetonitrile (1:1)
Gradient	In 10 minutes from 100% Buffer A to 100% Buffer B
Flow	4 ml/min
Detection	At 260 nm

A typical HPLC elution profile is shown in Figure 1.

4. Start the gradient after appearance of the void volume (first peak).

5. Stop the gradient when the second peak appears (at approx. 20 – 25% of Buffer B).

6. Continue to up to 55 – 65% of Buffer B until the third peak appears; stay isocratic until all of the desired labeled oligonucleotide has come off.

7. Collect the fraction from the third peak.

8. Continue to run the gradient up to 85% of Buffer B (fourth peak), a level at which unreacted dye is eluted.

9. Purge the column with 100% of Buffer B for regeneration.

10. Concentrate the solution from Step 7 using a vacuum centrifuge.
    – Dissolve pellet in 100 µl double-distilled water, then concentrate again in a vacuum centrifuge.
    – Repeat co-evaporation 2 × with double-distilled water, then lyophilize.

11. Store the pellet at −15 to −25°C.

HPLC Elution Profile

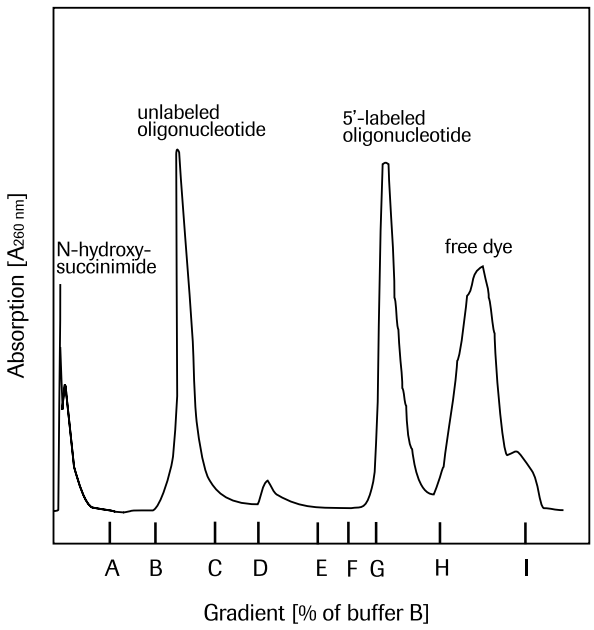


Fig. 1: Typical HPLC elution profile of a purification run showing four peaks. Peak 1 appears with the void volume and represents N-hydroxy-succinimide; peak 2 represents the unlabeled oligonucleotide; peak 3, the 5'-labeled oligonucleotide (slightly blue solution), and peak 4, the free dye (deep blue solution).

The letters (A-I) indicate:
A: Start gradient
B: Stop gradient at 20 – 25% of Buffer B
C: Continue gradient
D: Stop gradient at 35 – 40% of Buffer B
E: Continue gradient
F: 50% of Buffer B
G: Stop gradient at 60 – 65% of Buffer B
H: Continue gradient
I: 100% of Buffer B

Quality Control of HPLC-Purified Oligonucleotides

The 5'-labeled oligonucleotide is characterized by its UV/VIS absorption spectrum in the 200 – 800 nm range. Calculate the yield of labeled oligonucleotide by measuring the $A_{260\ nm}$ units. Follow the steps shown below to determine the yield of the labeled oligonucleotide.

1. Dissolve the pellet (see section **HPLC Purification**) in 1 ml double-distilled water.
   – In a cuvette, add 40 µl of the resulting solution to 760 µl double-distilled water.
   – Measure the extinction at 260 nm.

2. Multiplying the extinction value by a factor of 20 gives the yield in $A_{260\ nm\ units}$ (one $A_{260\ nm}$ unit corresponds to approx. 5 nmol 20-mer oligonucleotide).

3. Store the pellet at −15 to −25°C.

4. Run a UV/VIS absorption spectrum in the 200 – 800 nm range. The resulting spectrum corresponds to Figure 2.

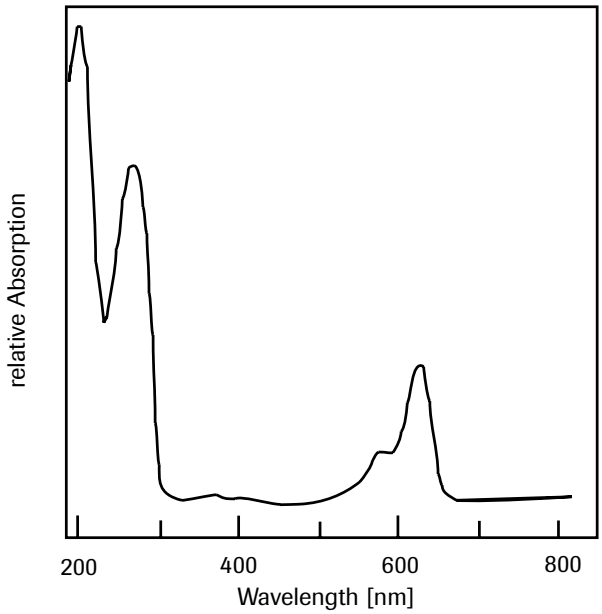


Fig. 2: UV/VIS absorption spectrum.

5. Based on the UV/VIS absorption spectrum, calculate the ratios of the extinction values at 620 nm and 260 nm. The approximate ratios are shown below (in relation to oligonucleotide sequence/length).

Oligonucleotide Length	Ratio ($A_{620\ nm}/A_{260\ nm}$)
20-mer	0.60 – 0.65
25-mer	0.43 – 0.55
30-mer	0.35 – 0.45

Labeling Efficiency

The labeling efficiency ranges between 60 – 80%, depending primarily on the length and sequence of the oligonucleotide, as well as the labeling conditions (*e.g.,* pH and DMF quality). The yield of the purified, labeled oligonucleotide is approx. 30 – 50%.

3. Additional Information on this Product

3.1. Test Principle

Spectral Characteristics

LightCycler® Red 640 shows an excitation maximum at 625 nm and an emission maximum at 640 nm (in 2 mM Tris-buffer, pH 8.3).

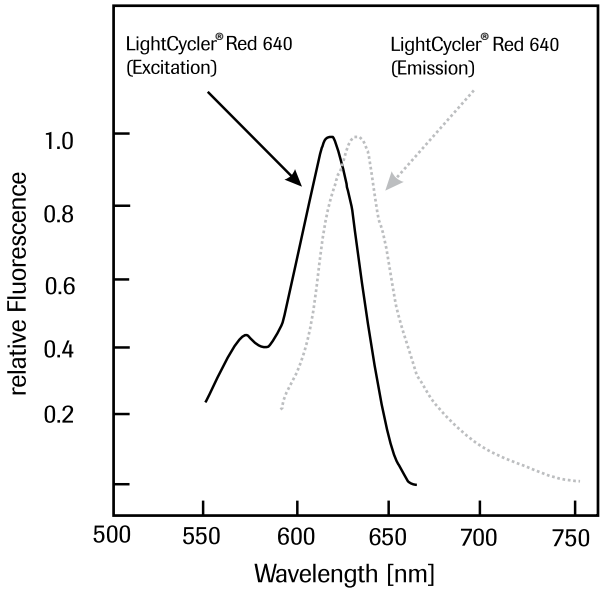


Fig. 3: LightCycler® Red 640 excitation and emission spectra.

Labeling Principle

5'-amino-substituted-3'-phosphorylated oligonucleotides react with the LightCycler® Red 640-NHS ester in a sodium borate buffer/dimethylformamide (DMF) mixture at pH 8.5 – 9.0.

4. Supplementary Information

4.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

4.2. Changes to previous version

Layout changes.
Editorial changes.

4.3. Ordering Information

Roche offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our homepage lifescience.roche.com.

Product	Pack Size	Cat. No.
Instruments		
LightCycler® 2.0 Instrument	1 instrument	03 531 414 001
Reagents , kits		
LightCycler® Control Kit DNA	1 kit, 50 reactions with 20 µl final volume each	12 158 833 001
LightCycler® RNA Master HybProbe	1 kit, 96 reactions of 20 µl final volume each	03 018 954 001
LightCycler® FastStart DNA Master^{PLUS} HybProbe	1 kit, 96 reactions of 20 µl final volume each	03 515 575 001
	1 kit, 480 reactions of 20 µl final volume each	03 515 567 001
	1 kit, 1,920 reactions of 20 µl or 384 reactions of 100 µl final volume each	03 752 178 001
LightCycler® Fluorescein CPG	1 g	03 138 178 001
LightCycler® Red 610-N-hydroxysuccinimide ester	1 vial, for 5 x 50 nmol oligonucleotides	03 561 488 001
LightCycler® Multiplex RNA Virus Master	1 kit, (20 µl), 200 reactions of 20 µl final volume each	06 754 155 001
	1 kit, (20 µl), 1,000 reactions of 20 µl final volume each	07 083 173 001

4.4. Trademarks

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4.5. License Disclaimer

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4.6. Regulatory Disclaimer

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4.7. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

4.8. Contact and Support

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