



SuperScript™ Plus Indirect cDNA Labeling System

**For generating fluorescently labeled cDNA using
Alexa Fluor® dyes for use in microarray screening**

Catalog nos. L1014-04, L1014-05, and L1014-06

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User Manual

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Kit Contents and Storage

Kit Sizes and Modules

All versions of the SuperScript™ Plus Indirect cDNA Labeling System are supplied with a Core Module and a Dye Module. Catalog nos. L1014-05 and L1014-06 also include a Purification Module.

<u>Cat no.</u>	<u>Number of Labeling Reactions</u>	<u>Modules</u>
L1014-04	30	Core and Dye only
L1014-05	10	Core, Dye, and Purification
L1014-06	30	Core, Dye, and Purification

Shipping and Storage

The Core Module and Dye Module are shipped on dry ice, and the Purification Module is shipped at room temperature. Upon receipt, store the components of the Core and Dye Modules at -20°C, and store the components of the Purification Module at room temperature.

Core Module

Store at -20°C.

Item	Components/Concentration	Kit Size	
		10 Rxns	30 Rxns
SuperScript™ III Reverse Transcriptase	400 U/μl in: 20 mM Tris-HCl (pH 7.5) 100 mM NaCl 0.1 mM EDTA 1 mM DTT 0.01% (v/v) NP-40 50% (v/v) glycerol	20 μl	60 μl
5X First-Strand Buffer	250 mM Tris-HCl (pH 8.3, room temp) 375 mM KCl 15 mM MgCl ₂	60 μl	200 μl
Dithiothreitol (DTT)	0.1 M DTT in water	250 μl	250 μl
dNTP Mix	dATP, dGTP, dCTP, dTTP, one aminoallyl-modified nucleotide, and one aminohexyl-modified nucleotide in DEPC-treated water	15 μl	45 μl
2X Coupling Buffer	—	50 μl	300 μl
Anchored Oligo(dT) ₂₀ primer	2.5 μg/μl in DEPC-treated water	20 μl	60 μl
Random hexamer primers	0.5 μg/μl in DEPC-treated water	10 μl	30 μl
DMSO	—	200 μl	750 μl
RNaseOUT™	40 U/μl	10 μl	30 μl
DEPC-treated Water	—	2 ml	2 × 2 ml
Control HeLa RNA	1 μg/μl	20 μl	20 μl

Continued on next page

Kit Contents and Storage, continued

Dye Module

Store at -20°C.

Item	Components/Concentration	Kit Size	
		10 Rxns	30 Rxns
Alexa Fluor® 555 Reactive Dye Pack	60 µg dried-down dye per vial	5 vials	3 × 5 vials
Alexa Fluor® 647 Reactive Dye Pack	60 µg dried-down dye per vial	5 vials	3 × 5 vials

Purification Module

Store at room temperature. This module is included with Catalog Numbers L1014-05 and L1014-06.

Component	Kit Size	
	10 Rxns	30 Rxns
Low-Elution Volume Spin Cartridges (with collection tubes)	2 × 11 columns	6 × 11 columns
Binding Buffer (must be combined with 100% isopropanol to create final buffer; see below)	2 × 5.5 ml	2 × 18 ml
Wash Buffer (must be combined with 100% ethanol to create final buffer; see below)	2 × 2 ml	2 × 5 ml
Amber collection tubes	2 × 11 tubes	6 × 11 tubes

Preparing Binding Buffer with Isopropanol

The Binding Buffer supplied with the Purification Module must be mixed with 100% isopropanol prior to use. Add the amount of isopropanol indicated below directly to the bottle of Binding Buffer to create the final buffer. Be sure to mark the appropriate checkbox on the bottle to indicate that you have added the isopropanol.

	<u>10-rxn kit</u>	<u>30-rxn kit</u>
Binding Buffer	5.5 ml (entire bottle)	18.0 ml (entire bottle)
100% Isopropanol	<u>2.0 ml</u>	<u>6.5 ml</u>
Final Volume	7.5 ml	24.5 ml

Store the Binding Buffer prepared with isopropanol at room temperature.

Preparing Wash Buffer with Ethanol

The Wash Buffer supplied with the Purification Module must be mixed with 100% ethanol prior to use. Add the amount of ethanol indicated below directly to the bottle of Wash Buffer to create the final buffer. Be sure to mark the appropriate checkbox on the bottle to indicate that you have added the ethanol.

	<u>10-rxn kit</u>	<u>30-rxn kit</u>
Wash Buffer	2 ml (entire bottle)	5 ml (entire bottle)
100% Ethanol	<u>8 ml</u>	<u>20 ml</u>
Final Volume	10 ml	25 ml

Store the Wash Buffer prepared with ethanol at room temperature.

Accessory Products

Additional Products

Many of the reagents in the SuperScript™ Indirect cDNA Labeling System, as well as additional reagents that may be used with this system, are available separately from Invitrogen. Ordering information is provided below.

Product	Quantity	Catalog no.
SuperScript™ Indirect cDNA Labeling System	10 reactions	L1014-01
	30 reactions	L1014-02
	30 reactions (w/o purification module)	L1014-03
RNase AWAY™ Reagent	250 ml	10328-011
PureLink™ Micro-to-Midi Total RNA Purification System	50 reactions	12183-018
PureLink™ 96 RNA Purification System	384 reactions	12173-011
TRIzol® Reagent	100 ml	15596-026
	200 ml	15596-018
Micro-FastTrack™ 2.0 mRNA Isolation Kit	20 reactions	K1520-02
FastTrack® 2.0 mRNA Isolation Kit	6 reactions	K1593-02
	18 reactions	K1593-03
FastTrack® MAG Micro mRNA Isolation Kit	12 reactions	K1580-01
FastTrack® MAG Maxi mRNA Isolation Kit	6 reactions	K1580-02
RNaseOUT™ Recombinant Ribonuclease Inhibitor	5000 units	10777-019
PureLink™ PCR Purification System	50 reactions	K3100-01
	250 reactions	K3100-02
Alexa Fluor® 555 reactive dye decapack	10 vials	A32756
Alexa Fluor® 647 reactive dye decapack	10 vials	A32757
Alexa Fluor® 555 and Alexa Fluor® 647 reactive dye decapacks	2 × 10 vials	A32755
Yeast tRNA	25 mg	15401-011
	50 mg	15401-029
Human Cot-1 DNA®	500 µg	15279-011
Mouse Cot-1 DNA®	500 µg	18440-016
UltraPure™ Formamide	500 g	15515-026
UltraPure™ Salmon Sperm DNA Solution	5 × 1 ml	15632-011
Random primers	9 A ₂₆₀ units	48190-011
UltraPure™ DEPC-treated water	4 × 1.25 ml	10813-012
UltraPure™ 10% SDS solution	4 × 100 ml	15553-027
UltraPure™ 20X SSC	1 L	15557-044
UltraPure™ 20x SSPE	1 L	15591-043

Overview

Introduction

The SuperScript™ Plus Indirect cDNA Labeling System is a highly efficient system for generating fluorescently labeled cDNA for use on microarrays in gene expression studies. It uses an aminoallyl-modified nucleotide and an aminoethyl-modified nucleotide together with other dNTPs in a cDNA synthesis reaction with SuperScript™ III Reverse Transcriptase. After a purification step to remove unincorporated nucleotides, the amino-modified cDNA is coupled with a monoreactive, N-hydroxysuccinimide (NHS)-ester fluorescent dye included in the kit—either Alexa Fluor® 555 succinimidyl ester or Alexa Fluor® 647 succinimidyl ester. A final purification step removes any unreacted dye, and the fluorescently labeled cDNA is ready for hybridization to microarrays.

This system uses 5–20 µg of total RNA or 0.4–2 µg of mRNA as starting material. Catalog nos. L1014-05 and L1014-06 include a Purification Module containing Low-Elution-Volume Spin Cartridges that yield a highly pure, highly concentrated sample.

Advantages of the System

- Optimized reagents and protocol ensure highly robust and reproducible labeling reactions
 - SuperScript™ III Reverse Transcriptase in the first-strand synthesis reaction ensures high specificity and high yields of cDNA, as well as more full-length cDNA
 - Use of two amino-modified nucleotides in the cDNA synthesis reaction results in a greater incorporation of fluorescent dye, an even distribution of fluorescent signal, and higher signal intensity with small amounts of starting material
 - Alexa Fluor® dyes provide higher correlation coefficients, signal intensities, and signal-to-background ratios than other labeling dyes
 - System includes all major reagents and materials for preparing Alexa Fluor®-labeled cDNA
-

Advantages of SuperScript™ III Reverse Transcriptase

SuperScript™ III Reverse Transcriptase is an engineered version of M-MLV RT with reduced RNase H activity and increased thermal stability. The enzyme can be used to synthesize first-strand cDNA from total RNA or mRNA at temperatures up to 55°C, providing increased specificity, higher yields of cDNA, and more full-length product than other reverse transcriptases.

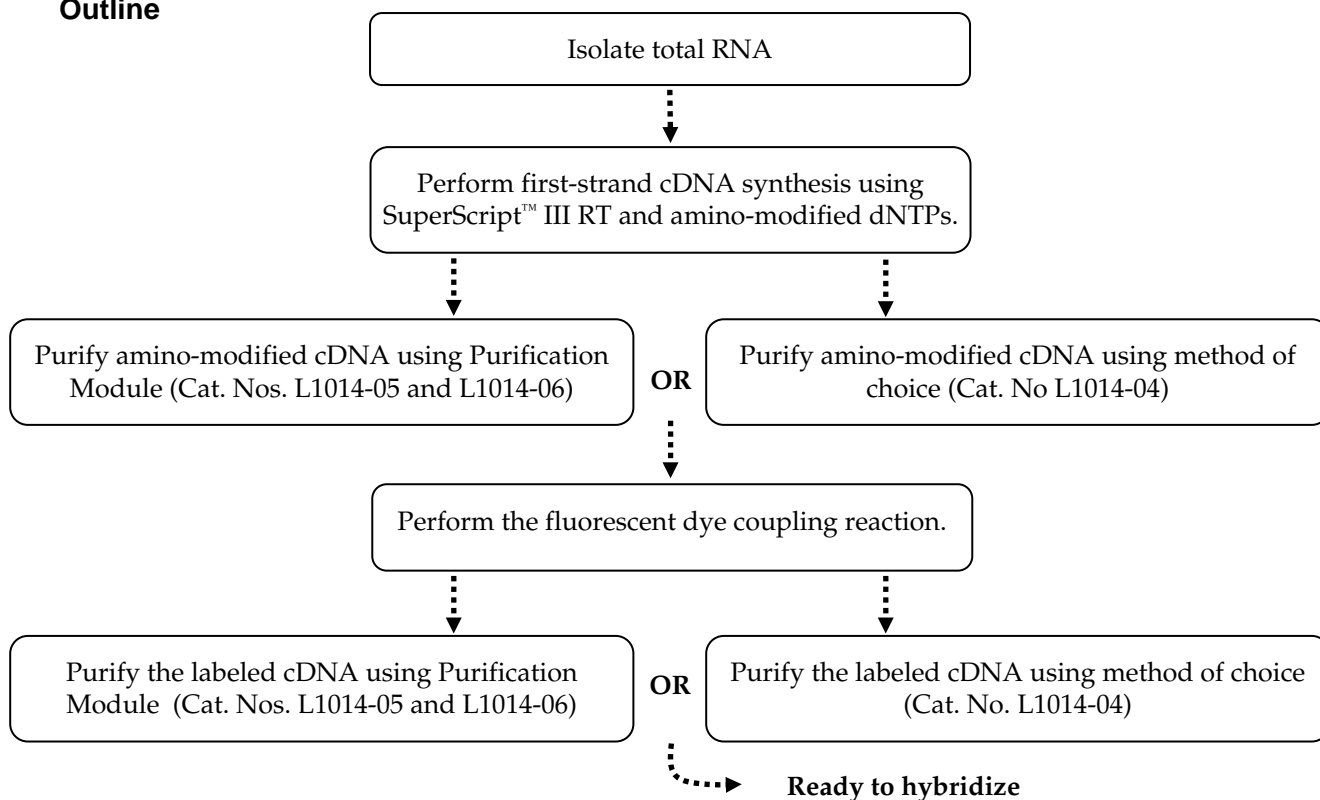
The SuperScript™ III RT in this kit is provided at an optimal concentration and used at an optimal temperature for incorporating amino-modified nucleotides in first-strand cDNA synthesis.

Continued on next page

Overview, continued

Experimental Outline

The flow chart below outlines the experimental steps of the system:



Alexa Fluor® 555 and Alexa Fluor® 647 Reactive Dyes

The Alexa Fluor® 555 and Alexa Fluor® 647 dyes included in this kit are compatible with commonly used microarray scanners, and provide greater signal correlation (R^2) values than the spectrally similar Cy™3 and Cy™5 dye pair, improving the resolution of two-color microarray gene expression assays. The exceptionally bright Alexa Fluor® dyes are also insensitive to pH and are highly water-soluble. The table below shows the excitation and emission maxima and color of each dye:

<u>Dye</u>	<u>Excitation/Emission (nm)</u>	<u>Color</u>
Alexa Fluor® 555	555/565	Orange Fluorescent
Alexa Fluor® 647	650/670	Far-Red Fluorescent

Anchored Oligo(dT)₂₀

Anchored oligo(dT)₂₀ primer is a mixture of 12 primers, each consisting of a string of 20 deoxythymidylic acid (dT) residues followed by two additional nucleotides represented by VN, where V is dA, dC, or dG, and N is dA, dC, dG or dT.

The VN “anchor” allows the primer to anneal only at the 5' end of the poly(A) tail of mRNA, providing more efficient cDNA synthesis for labeling applications.

Continued on next page

Overview, continued

Materials Supplied by the User

In addition to the kit components, you should have the following items on hand before using the SuperScript™ Indirect cDNA Labeling System.

- Vortex mixer
 - Microcentrifuge
 - Aerosol resistant pipette tips
 - Water baths or incubator
 - 1 N NaOH
 - 1 N HCl
 - Sterile microcentrifuge tubes
 - 100% Isopropanol
 - 100% Ethanol
 - 75% Ethanol
-

Control Reaction

We recommend performing the labeling procedure using the Control HeLa RNA included in the system to determine the efficiency of the labeling reaction. The section on **First-Strand cDNA Synthesis** (page 6) describes how to set up the control reaction and page 14 has equations for calculating the efficiency of the labeling procedure.

Product Qualification

This kit was verified in replicate labeling reactions using 10 µg of Control HeLa RNA, 2 µl of 2.5 µg/µl anchored oligo(dT)₂₀ primer, and amino-modified dNTP mix for cDNA synthesis. For the coupling step, Alexa Fluor® 555 or Alexa Fluor® 647 dyes were used. After purification, the labeled cDNA was scanned to read the full absorbance spectrum from 240–800 nm. The amount of coupled dye was calculated using the formulas on page 14. In addition, each reaction was run on a 1.2% E-Gel to determine the quality of the product.

Methods

Isolating RNA

Introduction

High-quality, intact RNA is essential for full-length, high-quality cDNA synthesis. In this step, you isolate total RNA or mRNA using a method of choice.



Important

The quality of the RNA is critical for successful labeling and hybridization. The presence of contaminants in the RNA may significantly increase background fluorescence in your microarrays. Carefully follow the recommendations below to prevent RNase contamination.

General Handling of RNA

When working with RNA:

- Use disposable, individually wrapped, sterile plasticware.
- Use aerosol resistant pipette tips for all procedures.
- Use only sterile, new pipette tips and microcentrifuge tubes.
- Wear latex gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin.
- Use proper microbiological aseptic technique when working with RNA.
- Dedicate a separate set of pipettes, buffers, and enzymes for RNA work.
- Microcentrifuge tubes can be taken from an unopened box, autoclaved, and used for all RNA work. RNase-free microcentrifuge tubes are available from several suppliers. If it is necessary to decontaminate untreated tubes, soak the tubes overnight in a 0.01% (v/v) aqueous solution of diethylpyrocarbonate (DEPC-treated), rinse the tubes with sterile distilled water, and autoclave the tubes.

You can use RNase AWAY™ Reagent, a non-toxic solution available from Invitrogen (see page vii), to remove RNase contamination from surfaces. For further information on controlling RNase contamination, see Ausubel, *et al.*, 1994, and Sambrook, *et al.*, 1989.

Isolating RNA

This system is optimized for use with 5–20 µg total RNA or 0.4–2 µg of mRNA. Lower amounts of starting material may be used, but may result in lower hybridization signals.

To isolate total RNA, we recommend the PureLink™ Micro-to-Midi Total RNA Purification System, TRIzol® Reagent, or (for high-throughput applications) the PureLink™ 96 RNA Purification System. To isolate mRNA, we recommend the FastTrack® 2.0 mRNA Isolation Kits or the FastTrack® MAG mRNA Isolation Kits. Ordering information is provided on page vii.

After you have isolated the RNA, check the quality of your RNA preparation as described on the following page.

Continued on next page

Isolating RNA, continued

Checking the RNA Quality

To check RNA quality, analyze 500 ng of RNA by agarose/ethidium bromide gel electrophoresis. You can use a regular 1% agarose gel or a denaturing agarose gel (Ausubel *et al.*, 1994). For total human RNA using a regular agarose gel, mRNA will appear as a smear from 0.5 to 9 kb, and 28S and 18S rRNA will appear as bands at 4.5 kb and 1.9 kb, respectively. The 28S band should be twice the intensity of the 18S band. If you are using a denaturing gel, the rRNA bands should be very clear and sharp.

If you do not load enough RNA, the 28S band may appear to be diffuse. A smear of RNA or a lower intensity 28S band with an accumulation of low molecular weight RNA on the gel are indications that the RNA may be degraded, which will decrease the labeling efficiency. If you do not detect any RNA, you will need to repeat RNA isolation. Refer to the **Troubleshooting** section on page 15.

Storing RNA

After preparing the RNA, we recommend that you proceed directly to **First-Strand cDNA Synthesis** on page 6. Otherwise, store the RNA at -80°C .

First-Strand cDNA Synthesis

Introduction

After you have isolated RNA and checked the quality of your RNA preparation, you are ready to synthesize cDNA.

Before Starting

The following materials are supplied by the user:

- 5–20 µg total RNA or 0.4–2 µg mRNA
- 1 N NaOH
- 1 N HCl
- Water baths, heating block, or incubator set at 46°C and 70°C
- Ice
- 1.5-ml RNase-free microcentrifuge tubes

The following materials are supplied in the kit:

- Anchored Oligo(dT)₂₀ primer
 - Random hexamers (for mRNA starting material only)
 - dNTP mix, including amino-modified nucleotides
 - 5X First-Strand buffer
 - 0.1 M DTT
 - RNaseOUT™
 - SuperScript™ III RT
 - DEPC-treated water
 - 10 µg of Control HeLa RNA per reaction; optional, see page 3
-



Note

RNaseOUT™ Recombinant RNase Inhibitor has been included in the system to safeguard against degradation of target RNA due to ribonuclease contamination of the RNA preparation.

Continued on next page

First-Strand cDNA Synthesis, continued

First-Strand cDNA Synthesis Reaction

The following procedure is designed to convert 5–20 µg of total RNA or 0.4–2 µg of mRNA into first-strand cDNA.

Note: If you are setting up a control reaction (recommended for first-time users), use 10 µl of the Control HeLa RNA supplied in the kit (1 µg/µl).

1. Mix and briefly centrifuge each component before use.
2. Prepare each reaction as follows in a 1.5-ml RNase-free tube:

<u>Component</u>	<u>Volume</u>
5–20 µg total RNA or 0.4–2 µg mRNA	X µl
Anchored Oligo(dT) ₂₀ Primer (2.5 µg/µl)	2 µl
Random hexamers (only if using mRNA)	1 µl *
DEPC-treated water	to 18 µl

*For mRNA, use **both** anchored oligo(dT)₂₀ and random hexamers. For total RNA, use **only** 2 µl of anchored oligo(dT)₂₀.

3. Incubate tubes at 70°C for 5 minutes, and then place on ice for at least 1 minute.
4. Add the following to each tube on ice:

<u>Component</u>	<u>Volume</u>
5X First-Strand buffer	6 µl
0.1 M DTT	1.5 µl
dNTP mix (including amino-modified nucleotides)	1.5 µl
RNaseOUT™ (40 U/µl)	1 µl
SuperScript™ III RT (400 U/µl)	<u>2 µl</u>
Final Volume	30 µl

5. Mix gently and collect the contents of each tube by brief centrifugation. Incubate tube at 46°C for 2–3 hours. **Note:** A 3-hour incubation results in 20–30% higher cDNA yield than a 2-hour incubation.

After incubation, proceed directly to **Alkaline Hydrolysis and Neutralization**, below.

Hydrolysis and Neutralization

After cDNA synthesis, immediately perform the following hydrolysis reaction to degrade the original RNA:

1. Add 15 µl of 1 N NaOH to each reaction tube from Step 5, above. Mix thoroughly.
2. Incubate tube at 70° C for 10 minutes.
3. Add 15 µl of 1 N HCl to neutralize the pH and mix gently.

Proceed to **Purifying the First-Strand cDNA** on the next page.

Purifying the First-Strand cDNA

Introduction

Catalog nos. L1014-05 and L1014-06 include a Purification Module developed for use with the system. Follow the procedure in this section to purify the amino-modified first-strand cDNA using this module.

Catalog no. L1014-04 does not include a Purification Module. Use your preferred purification method, and then continue to **Coupling with Fluorescent Dye** on page 10.



Note

The PureLink™ PCR Purification System (K3100-01 and K3100-02) has been tested with this kit, and is recommended if you are using catalog no. L1014-04. Ordering information is provided on page vii.

Before Starting

The following items are supplied by the user:

- Microcentrifuge
- Vortex mixer

The following items are supplied in the Purification Module:

- DEPC-treated water
 - Low-Elution Volume Spin Cartridges preinserted into collection tubes
 - Amber collection tubes
 - Binding Buffer (prepared with isopropanol as described on page vi)
 - Wash Buffer (prepared with ethanol as described on page vi)
-

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Purifying the First-Strand cDNA, continued

Purification Procedure

Use the following procedure to purify the first-strand cDNA using the components of the Purification Module (Cat nos. L1014-05 and L1014-06).

1. Add 700 μ l of Binding Buffer (prepared with isopropanol as described on page vi) to the reaction tube containing the first-strand cDNA from **Hydrolysis and Neutralization**, Step 3, page 7. Vortex briefly to mix.
2. Each Low-Elution Volume Spin Cartridge is preinserted into a collection tube. For multiple reactions, clearly label each collection tube, and then load the cDNA/Binding Buffer solution directly onto the Spin Cartridge.
3. Centrifuge at $3,300 \times g$ in a microcentrifuge for 1 minute. Remove the collection tube and discard the flow-through.
4. Place the Spin Cartridge in the same collection tube and add 600 μ l of Wash Buffer (prepared with ethanol as described on page vi) to the column.
5. Centrifuge at maximum speed for 30 seconds. Remove the collection tube and discard the flow-through.
6. Place the Spin Cartridge in the same collection tube and centrifuge at maximum speed for 30 seconds to remove any residual Wash Buffer. Remove the collection tube and discard.
7. Place the Spin Cartridge onto a new **amber** collection tube (supplied in the kit).
8. Add 20 μ l of DEPC-treated water to the center of the Spin Cartridge and incubate at room temperature for 1 minute.
9. Centrifuge at maximum speed for 1 minute to collect the purified first-strand cDNA. **The eluate contains your purified cDNA.**

Proceed directly to **Coupling with Fluorescent Dye** on the next page.

Coupling with Fluorescent Dye

Introduction

After cDNA synthesis and purification, you are ready to couple the amino-modified cDNA with Alexa Fluor® dye.

Before Starting

The following items are supplied by the user:

- Microcentrifuge
- Vortex mixer

The following items are provided in the kit:

- DMSO
 - 2X Coupling Buffer
 - Alexa Fluor® 555 Reactive Dye Pack (60 µg per vial) /
Alexa Fluor® 647 Reactive Dye Pack (60 µg per vial)
-



Important

Fluorescent dyes are sensitive to photobleaching. When preparing the reaction, be careful to minimize exposure of the dye solution to light. The dye coupling reaction must be incubated in the dark.



DMSO is hygroscopic and will absorb moisture from the air. Water absorbed from the air will react with the NHS ester of the dye and significantly reduce the coupling reaction efficiency. Keep the DMSO supplied in the kit in an amber screw-capped vial at -20°C, and let the vial warm to room temperature before opening to prevent condensation.

Coupling Procedure

Follow the steps below to couple Alexa Fluor® dye to the amino-modified first-strand cDNA. Use only the DMSO provided with this kit.

1. Dry the purified first-strand cDNA from Step 9, page 9, in a speed vac at medium heat until the volume is reduced to 3 µl.
2. Add 5 µl of 2X Coupling Buffer to the tube.
3. Add 2 µl of DMSO directly to a vial of Alexa Fluor® Reactive Dye to resuspend the dye. Vortex thoroughly and then spin briefly to collect the contents.
4. Add the DMSO/dye solution to the tube from Step 2 and vortex to mix thoroughly.
5. Incubate the tube at room temperature in the dark for 1–2 hours. Reaction can be stored overnight if necessary.

Proceed to **Purifying the Fluorescently Labeled cDNA** on the next page.

Purifying the Fluorescently Labeled cDNA

Introduction

Catalog nos. L1014-05 and L1014-06 include a Purification Module developed for use with the system. Follow the procedure below to purify the labeled cDNA using this module.

Catalog no. L1014-04 does not include a Purification Module. Use your preferred purification method instead of the following procedure, and then continue to hybridization.



Note

The PureLink™ PCR Purification System (K3100-01 and K3100-02) has been tested with this kit, and is recommended if you are using catalog no. L1014-04. Ordering information is provided on page vii.

Before Starting

The following items are supplied by the user:

- Microcentrifuge
- Vortex mixer

The following items are supplied in the Purification Module:

- DEPC-treated water
 - Low-Elution Volume Spin Cartridges pre-inserted into collection tubes
 - Amber collection tubes
 - Binding Buffer (prepared with isopropanol as described on page vi)
 - Wash Buffer (prepared with ethanol as described on page vi)
-

Purification Procedure

Use the following procedure to purify the labeled cDNA using the components of the Purification Module (Cat nos. L1014-05 and L1014-06).

1. Add 700 μ l of Binding Buffer (prepared with isopropanol as described on page vi) to the reaction tube containing the labeled cDNA from **Coupling Procedure**, Step 5, page 10. Vortex briefly to mix.
2. Each Low-Elution Volume Spin Cartridge is preinserted into a collection tube. For multiple reactions, clearly label each collection tube, and then load the cDNA/Binding Buffer solution directly onto the Spin Cartridge.
3. Centrifuge at $3,300 \times g$ in a microcentrifuge for 1 minute. Remove the collection tube and discard the flow-through.
4. Place the Spin Cartridge in the same collection tube and add 600 μ l of Wash Buffer (prepared with ethanol as described on page vi) to the column.
5. Centrifuge at maximum speed for 30 seconds. Remove the collection tube and discard the flow-through.
6. Place the Spin Cartridge in the same collection tube and centrifuge at maximum speed for 30 seconds to remove any residual Wash Buffer. Remove the collection tube and discard.

Procedure continued on next page.

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Purifying the Fluorescently Labeled cDNA, continued

Purification Procedure, continued

Procedure continued from previous page.

7. Place the Spin Cartridge onto a new **amber** collection tube (supplied in the kit).
8. Add 20 μ l of DEPC-treated water to the center of the Spin Cartridge and incubate at room temperature for 1 minute.
9. Centrifuge at maximum speed for 1 minute to collect the purified cDNA. **The eluate contains your purified labeled cDNA.**

The sample may be stored at -20° C for up to one week prior to hybridization. Avoid freeze/thawing. To determine the efficiency of the labeling reaction, proceed to **Assessing Labeling Efficiency** (page 14).

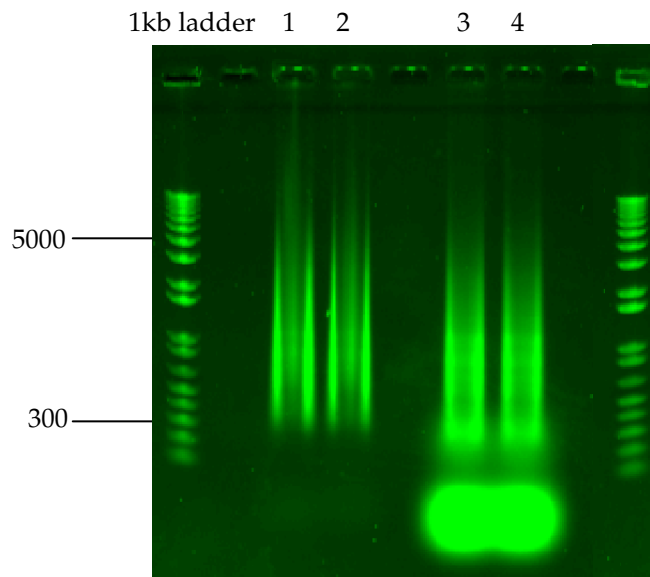
Note on cDNA Purity

Because of the high purity of the cDNA from the Low-Elution Volume Spin Cartridges included with catalog nos. L1014-05 and L1014-06, the yield and picomole dye incorporation calculations will be more accurate than with other purification methods.

In the 1.2% E-Gel below, Lanes 1 and 2 contain Alexa Fluor[®] 555-labeled cDNA purified using the Low-Elution Volume Spin Cartridges, and Lanes 3 and 4 contain Alexa Fluor[®] 555-labeled cDNA purified using columns from another manufacturer. The labeled cDNA appears as smear from 500–5,000 bp.

The large band at the bottom of Lanes 3 and 4 is unincorporated dye that was not removed by the other manufacturer's purification column. Such material would be included in the picomole dye incorporation calculations, resulting in an incorporation level that is higher than theoretically possible.

For this reason, we strongly recommend using the purification columns provided with catalog nos. L1014-05 and L1014-06.



Hybridization

Hybridization

After purification, you are ready to use the labeled cDNA in any application of choice, including glass microarray hybridization. Follow the preparation and hybridization instructions for your specific application.

Appendix

Assessing Labeling Efficiency

Introduction

You can use UV/visible spectroscopy scanning to measure the amount of labeled cDNA and dye incorporation. The expected amounts using the Control HeLa RNA provided in the kit are shown below.

Calculating the Results

To calculate the amount of labeled cDNA using a UV/visible spectrophotometer:

1. Transfer a volume of purified, labeled cDNA from step 9, page 12, to a clean cuvette. Use an appropriate volume for your spectrophotometer. Add DEPC-treated water to the cDNA if you need to increase the volume of the eluate for your spectrophotometer.

Note: The labeled DNA must be purified as described on page 11 before scanning, as any unincorporated dye will interfere with the detection of labeled DNA.

2. Blank the spectrophotometer using DEPC-treated water, and then scan the sample at 240–800 nm. Wash each cuvette thoroughly between samples.
3. Calculate the yield of cDNA using the following formula:

$$\text{cDNA (ng)} = (A_{260} - A_{320}) \times 37 \text{ ng}/\mu\text{l} \times \text{volume in } \mu\text{l}$$

4. Calculate the amount of fluorescent dye using the following formulas:

$$\text{Alexa Fluor}^{\text{®}} 555 \text{ (pmole)} = (A_{555} - A_{650}) / 0.15 \times \text{volume in } \mu\text{l}$$

$$\text{Alexa Fluor}^{\text{®}} 647 \text{ (pmole)} = (A_{650} - A_{750}) / 0.24 \times \text{volume in } \mu\text{l}$$

5. Calculate the base-to-dye ratio using the following formulas:

$$\begin{aligned} \text{Base/dye ratio for Alexa Fluor}^{\text{®}} 555 = \\ \{(A_{260} - A_{320}) - [(A_{555} - A_{650}) \times 0.04]\} \times 150,000 / (A_{555} - A_{650}) \times 8,919 \end{aligned}$$

$$\begin{aligned} \text{Base/dye ratio for Alexa Fluor}^{\text{®}} 647 = \\ \{(A_{260} - A_{320}) - [(A_{650} - A_{750}) \times 0]\} \times 239,000 / (A_{650} - A_{750}) \times 8,919 \end{aligned}$$

The number of dye molecules per 100 bases is calculated using the formula:

$$100 / (\text{base/dye ratio})$$

Expected Amounts Using Control DNA

If you prepare a control reaction using 10 μg of Control HeLa RNA as starting material, the following amounts are expected:

<u>Labeled DNA</u>	<u>Incorporated Dye</u>	<u>Dyes Molecules/100 Bases</u>
$\geq 250 \text{ ng}$	$\geq 24 \text{ pmole}$	≥ 2.50

If you do not obtain these amounts, see **Troubleshooting** on page 15.

Troubleshooting

Problem	Cause	Solution
28S and 18S bands are not observed after isolation of total RNA and agarose gel electrophoresis	Too little RNA loaded on the gel.	Be sure to load at least 250 ng of RNA for analysis.
	RNA is degraded due to RNase activity.	Follow the guidelines on page 4 to avoid RNase contamination. Use a fresh sample for RNA isolation.
Yield of cDNA is low	Temperature too high during cDNA synthesis.	Perform the cDNA synthesis at 46° C.
	Incorrect reaction conditions used.	Verify that all reaction components are included in the reaction and use reagents provided in the system. Verify the reaction conditions using the control RNA provided in the kit.
	Concentration of template RNA is too low.	Increase the concentration of template RNA. Use at least 5 µg of total RNA or 0.4 µg of mRNA.
	Poor quality RNA used or RNA is degraded.	Check the quality of your RNA preparation (see page 5). If RNA is degraded, use fresh RNA.
	RNase contamination.	Use the RNaseOUT™ included in the kit to prevent RNA degradation.
	RT inhibitors are present in your RNA sample.	Inhibitors of RT include SDS, EDTA, guanidinium chloride, formamide, sodium phosphate and spermidine (Gerard, 1994). Remove inhibitors from your RNA sample by performing an additional 70% ethanol wash after ethanol precipitation during RNA isolation and purification. Test for the presence of inhibitors by mixing 1 µg of control RNA with 25 µg total RNA or 1 µg mRNA and compare the yields of first-strand synthesis.
	Improper storage of SuperScript™ III RT.	Store the enzyme at -20°C.
	Concentration of NaOH and/or HCl used in the hydrolysis and neutralization reaction is incorrect. This affects the pH of the reaction and therefore may affect binding to the column.	Verify the concentration of NaOH and HCl, and repeat the reaction if necessary
	cDNA has been lost during purification	Measure the amount of cDNA in the control reaction before and after purification. Follow the purification procedure without modifications.

Continued on next page

Troubleshooting, continued

Problem	Cause	Solution
Amount of coupled dye in the control reaction is low (< 24 pmoles) and/or fluorescence of labeled cDNA is low	Reaction tubes have been exposed to light	Avoid direct exposure of the labeling reaction to light. Use amber tube provided in the kit for collection of the final product.
	Dye solution has been exposed to light	Repeat the labeling reaction with a fresh mixture of dye, being careful to avoid direct exposure to light.
	DMSO used to prepare dye mixture was contaminated with water.	Prepare a new mixture of dye using fresh DMSO. Carefully follow the instructions for storing and handling DMSO in the Caution on page 10.
	Inefficient labeling due to improper purification	Follow all purification steps carefully and without modification.
	2X Coupling Buffer was not stored properly	Store 2X Coupling Buffer at -20°C.

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Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our website (www.invitrogen.com).

Corporate Headquarters:

Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail: tech_support@invitrogen.com

Japanese Headquarters:

Invitrogen Japan
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Tel: 81 3 5730 6509
Fax: 81 3 5730 6519
E-mail: jpinfo@invitrogen.com

European Headquarters:

Invitrogen Ltd
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK
Tel: +44 (0) 141 814 6100
Tech Fax: +44 (0) 141 814 6117
E-mail: eurotech@invitrogen.com

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Corporate Headquarters

Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008
T: 1 760 603 7200
F: 1 760 602 6500
E: tech.service@invitrogen.com

For country-specific contact information visit our web site at www.invitrogen.com