



Instruction Manual

SuperScript™ Plus Direct cDNA Labeling System

For generating fluorescently labeled cDNA using
Alexa Fluor®-labeled nucleotides for use in
microarray screening

Catalog nos. L1015-04, L1015-05, and L1015-06

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Kit Contents and Storage

Kit Sizes and Modules

All versions of the SuperScript™ Plus Direct cDNA Labeling System are supplied with a Core Module and a Nucleotide Module. Catalog nos. L1015-05 and L1015-06 also include a Purification Module.

<u>Cat no.</u>	<u>Number of Labeling Reactions</u>	<u>Modules</u>
L1015-04	30	Core and Nucleotide only
L1015-05	10	Core, Nucleotide, and Purification
L1015-06	30	Core, Nucleotide, and Purification

Shipping and Storage

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

Core Module

Store components at -20°C.

Item	Components/Concentration	Kit Size	
		10 Rxn	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	200 μl	200 μl
Dithiothreitol (DTT)	0.1 M DTT in water	250 μl	250 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
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

Nucleotide Module Store components at -20°C.

Item	Components/Concentration	Kit Size	
		10 Rxn	30 Rxns
10X Nucleotide Mix with Alexa Fluor® 555-aha-dUTP	Mixture of dCTP, dGTP, dATP, dTTP, and labeled dUTP in 10 mM Tris (pH 8.0), 1 mM EDTA	15 µl	3 × 15 µl
10X Nucleotide Mix with Alexa Fluor® 647-aha-dUTP	Mixture of dCTP, dGTP, dATP, dTTP, and labeled dUTP in 10 mM Tris (pH 8.0), 1 mM EDTA	15 µl	3 × 15 µl

Purification Module Store components at room temperature.

Component	Kit Size	
	10 Rxn	30 Rxns
Low-Elution Volume Spin Cartridges (with collection tubes)	11 columns	3 × 11 columns
Binding Buffer (must be combined with 100% isopropanol to create final buffer; see below)	5.5 ml	18 ml
Wash Buffer (must be combined with 100% ethanol to create final buffer; see below)	2 ml	5 ml
Amber collection tubes	11 tubes	3 × 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™ Direct 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.
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
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
Random primers	9 A ₂₆₀ units	48190-011
UltraPure™ Formamide	500 g	15515-026
UltraPure™ Salmon Sperm DNA Solution	5 × 1 ml	15632-011
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 Direct cDNA Labeling System is a highly robust and efficient system for generating Alexa Fluor®-labeled cDNA for use on microarrays in gene expression studies. It uses SuperScript™ III Reverse Transcriptase in a cDNA synthesis reaction with total RNA or mRNA and fluorescently labeled nucleotides. After cDNA synthesis, the RNA template is hydrolyzed, a purification step removes any unincorporated nucleotides, and the fluorescently labeled cDNA is ready for hybridization to microarrays.

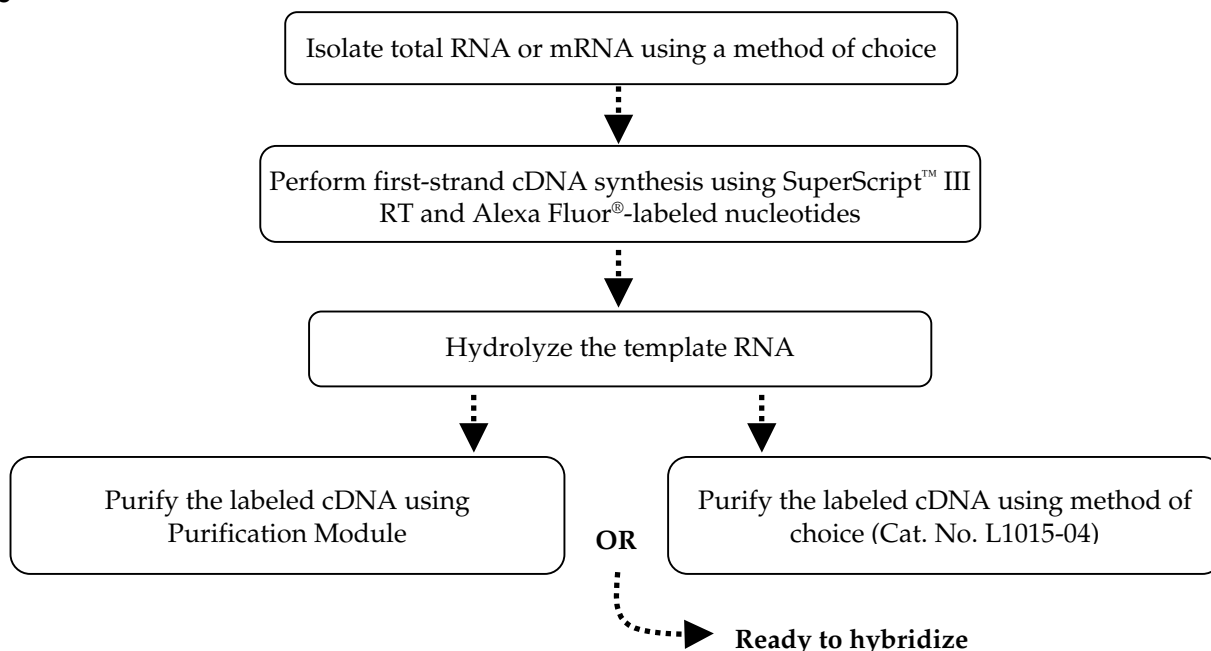
The kit includes optimized nucleotide mixes containing Alexa Fluor® 555-aha-dUTP and Alexa Fluor® 647-aha-dUTP, respectively. It has been optimized for use with 5–20 µg of total RNA or 0.4–2 µg of mRNA as starting material. Lower amounts of starting material may be used, but may result in lower hybridization signals.

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 produces high yields of cDNA, greater incorporation of fluorescent nucleotides, and higher signal-to-background ratios with small amounts of starting material
 - Alexa Fluor® dyes provide higher correlation coefficients, signal intensities, and signal-to-background ratios than other labeling dyes
 - Simplified purification protocol reduces the time of the overall procedure
 - Provides a complete solution for fluorescent labeling of cDNA
-

Experimental Outline

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



Continued on next page

Overview, continued

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 labeled nucleotides in first-strand cDNA synthesis.

Alexa Fluor® 555 and Alexa Fluor® 647-labeled Nucleotides

The labeled 5-aminohexylacrylamido-dUTP (aha-dUTP) nucleotides included in the kit are modified with a unique hexylacrylamide linker, which serves as a spacer between the nucleotide and the dye. This spacer reduces interactions between the nucleotide and the dye, resulting in brighter conjugates.

The Alexa Fluor® 555 and Alexa Fluor® 647 dyes used to label the nucleotides 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
- 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.

Control RNA

Control HeLa RNA is included in the kit to help you determine the efficiency of the labeling procedure. We recommend that you perform the complete labeling procedure using 10 µg (10 µl) of the Control HeLa RNA if you are a first-time user of the system.

Equations for calculating the efficiency of the labeling procedure are provided on page 11.

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™ Plus Direct cDNA Labeling System.

- 5–20 µg total RNA or 0.4–2 µg mRNA starting material
 - Vortex mixer
 - Microcentrifuge
 - Aerosol resistant pipette tips
 - Water baths, incubator, or thermal cycler
 - 0.1 M NaOH
 - 0.1 M HCl
 - 0.5 or 1.5-ml RNase-free microcentrifuge tubes
 - 100% Isopropanol
 - 100% Ethanol
-

Product Qualification

This kit was verified using 10 µg total HeLa or Human Placenta RNA in replicate labeling reactions with Alexa Fluor® 555-aha-dUTP and Alexa Fluor® 647-aha-dUTP. After purification, the labeled cDNA was scanned to read the full absorbance spectrum from 240–800 nm.

The amounts of incorporated nucleotides were calculated using the formulas on page 11. In addition, the length of the labeled product was determined by agarose gel electrophoresis.

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), 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 12.

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 items are supplied by the user:

- 5–20 µg total RNA or 0.4–2 µg mRNA per reaction
- 0.1 M NaOH
- 0.1 M HCl
- Incubator, water bath, or thermal cycler set at 46°C and 70°C
- Ice
- 0.5-ml or 1.5-ml RNase-free microcentrifuge tubes

The following items are supplied in the kit:

- Anchored Oligo(dT)₂₀ primer
 - Random hexamers (for mRNA starting material only)
 - 10X Nucleotide Mix with Alexa Fluor® 555-aha-dUTP / 10X Nucleotide Mix with Alexa Fluor® 647-aha-dUTP
 - 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 2
-



Important

Fluorescent nucleotides are sensitive to photobleaching. When preparing the reaction, be careful to minimize exposure of the fluorescent nucleotides and labeled DNA to light. We recommend that you use amber reaction tubes and/or wrap reaction tubes in foil to protect against light exposure.



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 labeled first-strand cDNA. Lower amounts of starting material may be used, but may result in lower hybridization signals.

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. In a 1.5- or 0.5-ml RNase-free tube, add the following:

<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 15 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 tube at 70°C for 10 minutes, and then place on ice for at least 1 minute.
4. Add the following to the tube on ice:

<u>Component</u>	<u>Volume</u>
5X First-Strand buffer	6 µl
0.1 M DTT	3 µl
10X Nucleotide Mix with Alexa Fluor® 555-aha-dUTP <i>or</i> 10X Nucleotide Mix with Alexa Fluor® 647-aha-dUTP	3 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. **Note:** After addition of the labeled nucleotides, be careful to minimize exposure of the tube to light.
6. Incubate tube at 46°C in the dark for 3 hours. **Note:** A 2-hour incubation is sufficient for generating high-quality labeled cDNA with high levels of picomole incorporation; however, a 3-hour incubation will result in 10–20% greater incorporation of labeled nucleotides and more full-length cDNA.

After incubation, proceed directly to **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 0.1 M NaOH to each reaction tube from Step 6, above. Mix thoroughly.
2. Incubate tube at 70° C for 30 minutes.
3. Add 15 µl of 0.1 M HCl to neutralize the pH and mix gently.

Proceed to **Purifying the Labeled cDNA** on the following page.

Purifying the Labeled cDNA

Introduction

Catalog nos. L1015-05 and L1015-06 include a Purification Module developed for use with the system. Follow the procedure below to purify your labeled cDNA using this module.

Catalog no. L1015-04 does not include a Purification Module. Use your preferred method of cDNA purification 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. L1015-04. Ordering information is provided on page vii.

Before Starting

The following items are supplied by the user:

- Microcentrifuge

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 cDNA using the components of the Purification Module (Catalog nos. L1015-05 and L1015-06).

1. Add 700 µl of Binding Buffer (prepared with isopropanol as described on page vi) to the reaction tube containing the labeled cDNA from **Hydrolysis and Neutralization**, Step 3, page 7.
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 µl 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 the next page.

Continued on next page

Purifying the Labeled cDNA, continued

Purification Procedure, continued

Procedure continued from the 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 labeled cDNA. **The eluate contains your purified labeled cDNA.**

The sample can 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 11).



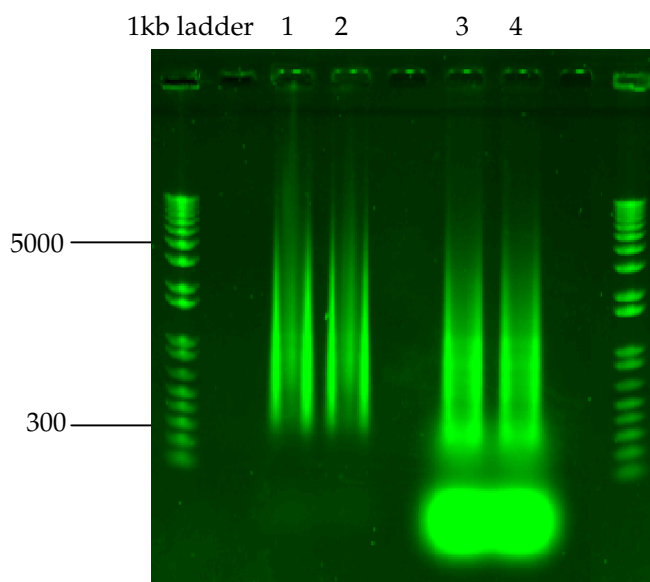
Note

Because of the high purity of the cDNA from the Low-Elution Volume Spin Cartridges included with catalog nos. L1015-05 and L1015-06, the yield and picomole dye incorporation calculations will be more accurate than with other purification methods.

For example, the 1.2% E-Gel below shows purification results from an indirect labeling method. 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. L1015-05 and L1015-06.



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 9, 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 8 before scanning, as any unincorporated labeled nucleotides 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 cDNA</u>	<u>Incorporated Labeled Nucleotides</u>	<u>Dyes Molecules/ 100 Bases</u>
≥ 400 ng	≥ 30 pmole	≥ 1.0

If you do not obtain these amounts, see **Troubleshooting** on page 12.

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.
28S band is diminished or low molecular weight RNA appears in the gel	RNA is degraded	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 HeLa RNA provided in the kit.
	Concentration of template RNA is too low	Increase the concentration of template RNA. Use at least 10 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). Test for the presence of inhibitors by mixing 1 µg of Control HeLa 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.
	Reagents were not properly mixed before use.	Repeat the procedure, being careful to briefly vortex and centrifuge each reagent before use.
Amount of incorporated labeled nucleotides in the control reaction is low and/or fluorescence of labeled cDNA is low	cDNA has been lost in the purification step	Measure the amount of cDNA produced by the Control RNA before and after purification. Follow the purification procedure without modifications.
	Reaction tubes have been exposed to light	Avoid direct exposure of the labeling reaction to light. Use the amber tube provided in the kit for collection of the final product.
	Inefficient labeling due to improper purification	Follow all purification steps carefully and without modification.
	Starting amount of RNA is too low	Increase the amount of starting RNA

Purchaser Notification

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Ribonuclease
Inhibitor**

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