SuperScript™ Indirect cDNA Labeling System

For generating fluorescently labeled cDNA to use in microarray screening

Catalog nos. L1014-01, L1014-02, and L1014-03
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Kit Contents and Storage

Kit Sizes

The SuperScript™ Indirect cDNA Labeling System is supplied with either a Core Module and a Purification Module, or a Core Module only. Note that the Core Module contains the labeling components.

<table>
<thead>
<tr>
<th>Cat no.</th>
<th>Number of Labeling Reactions</th>
<th>Modules</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1014-01</td>
<td>10</td>
<td>Core and Purification</td>
</tr>
<tr>
<td>L1014-02</td>
<td>30</td>
<td>Core and Purification</td>
</tr>
<tr>
<td>L1014-03</td>
<td>30</td>
<td>Core only</td>
</tr>
</tbody>
</table>

Shipping and Storage

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

Core Module

The components of the Core Module should be stored at -20°C.

<table>
<thead>
<tr>
<th>Item</th>
<th>Components/Concentration</th>
<th>Kit Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>SuperScript™ III Reverse Transcriptase</td>
<td>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</td>
<td>10 Rxns 30 µl 30 Rxns 75 µl</td>
</tr>
<tr>
<td>5X First-Strand Buffer</td>
<td>250 mM Tris-HCl (pH 8.3, room temp) 375 mM KCl 15 mM MgCl₂</td>
<td>1020 µl 1020 µl</td>
</tr>
<tr>
<td>Dithiothreitol (DTT)</td>
<td>0.1 M DTT in water</td>
<td>270 µl 270 µl</td>
</tr>
<tr>
<td>dNTP Mix</td>
<td>dATP, dGTP, dCTP, dTTP, one aminoallyl-modified nucleotide, and one aminohexyl-modified nucleotide at optimal concentrations in DEPC-treated water</td>
<td>20 µl 60 µl</td>
</tr>
<tr>
<td>2X Coupling Buffer</td>
<td>—</td>
<td>100 µl 300 µl</td>
</tr>
<tr>
<td>Anchored Oligo(dT)₂₀ primer</td>
<td>2.5 µg/µl in DEPC-treated water</td>
<td>30 µl 75 µl</td>
</tr>
<tr>
<td>Random hexamer primers</td>
<td>0.5 µg/µl in DEPC-treated water</td>
<td>15 µl 45 µl</td>
</tr>
<tr>
<td>Glycogen</td>
<td>20 mg/ml</td>
<td>30 µl 75 µl</td>
</tr>
<tr>
<td>DMSO</td>
<td>—</td>
<td>200 µl 750 µl</td>
</tr>
<tr>
<td>RNaseOUT™</td>
<td>40 U/µl</td>
<td>15 µl 45 µl</td>
</tr>
<tr>
<td>DEPC-treated Water</td>
<td>—</td>
<td>2 ml 6 ml</td>
</tr>
<tr>
<td>3 M Sodium Acetate</td>
<td>pH 5.2</td>
<td>1 ml 2 ml</td>
</tr>
<tr>
<td>Control RNA Ladder (0.24-9.5 Kb)</td>
<td>0.5 µg/µl in DEPC-treated water</td>
<td>10 µl 10 µl</td>
</tr>
</tbody>
</table>

Continued on next page
Kit Contents and Storage, continued

**cDNA Labeling Purification Module**

The components of the Purification Module should be stored at room temperature. This module is included with Catalog Numbers L1014-01 and L1014-02.

<table>
<thead>
<tr>
<th>Item</th>
<th>Components/Concentration</th>
<th>Kit Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loading Buffer (you must add 100% isopropanol to create the final buffer; see below)</td>
<td>4.3 ml</td>
<td>13 ml</td>
</tr>
<tr>
<td>Wash Buffer (you must add 100% ethanol to create the final buffer; see below)</td>
<td>8.33 ml</td>
<td>25 ml</td>
</tr>
<tr>
<td>S.N.A.P.™ Columns</td>
<td>22 cols</td>
<td>62 cols</td>
</tr>
<tr>
<td>Clear Collection Tubes</td>
<td>22 tubes</td>
<td>62 tubes</td>
</tr>
<tr>
<td>Amber Collection Tubes</td>
<td>11 tubes</td>
<td>31 tubes</td>
</tr>
</tbody>
</table>

**Preparing Loading Buffer with Isopropanol**

The Loading Buffer supplied in each Purification Module must be mixed with 100% isopropanol prior to use. The Loading Buffer plus isopropanol is stable for at least six months at room temperature.

Add the amount of isopropanol indicated below directly to each bottle of Loading Buffer. Be sure to mark the appropriate checkbox on the bottle to indicate that you have added the isopropanol.

<table>
<thead>
<tr>
<th>Item</th>
<th>10-Rxn Kit</th>
<th>30-Rxn Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loading Buffer</td>
<td>4.3 ml (entire bottle)</td>
<td>13 ml (entire bottle)</td>
</tr>
<tr>
<td>100% Isopropanol</td>
<td>10.0 ml</td>
<td>30 ml</td>
</tr>
<tr>
<td>Final Volume</td>
<td>14.3 ml</td>
<td>43 ml</td>
</tr>
</tbody>
</table>

**Preparing Wash Buffer with Ethanol**

The Wash Buffer supplied in each Purification Module must be mixed with 100% ethanol prior to use. The Wash Buffer plus ethanol is stable for at least six months at room temperature.

Add the amount of ethanol indicated below directly to each bottle of Wash Buffer. Be sure to mark the appropriate checkbox on the bottle to indicate that you have added the ethanol.

<table>
<thead>
<tr>
<th>Item</th>
<th>10-Rxn Kit</th>
<th>30-Rxn Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash Buffer</td>
<td>8.33 ml (entire bottle)</td>
<td>25 ml (entire bottle)</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>25.00 ml</td>
<td>75 ml</td>
</tr>
<tr>
<td>Final Volume</td>
<td>33.33 ml</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

**Product Qualification**

This kit was verified in a standard labeling reaction using 0.5 µl of 1 µg/µl Control RNA Ladder, 2 µl of 2.5 µg/µl anchored oligo(dT)20 primer, 1 µl of 0.5 µg/µl random hexamers, and dNTP mix for cDNA synthesis. For the coupling step, Cy3™ or Cy5™ was used. After purification, the labeled cDNA was scanned to read the full absorbance spectrum from 240–800 nm, using dH2O as a blank. The amount of coupled dye was calculated using the formulas on page 10.
Overview

Introduction

The SuperScript™ 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 aminohexyl-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. 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, and is compatible with Cy3™ and Cy5™ dyes from Amersham Biosciences or other monoreactive NHS-ester dyes from a variety of manufacturers.

Advantages of the System

- 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 and higher signal intensity with small amounts of starting material
- Unbiased incorporation of amino-modified dNTPs and the high efficiency of the coupling reaction result in an even distribution of fluorescent signal and high overall levels of fluorescence, increasing the sensitivity and reproducibility of array hybridizations
- System includes all major reagents and materials for preparing fluorescently labeled cDNA, except fluorescent dye

Experimental Outline

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

Isolate total RNA

↓

Perform first-strand cDNA synthesis using SuperScript™ III RT and amino-modified dNTPs.

↓

Purify the amino-modified cDNA using Purification Module (Cat. Nos. L1014-01 and L1014-02) OR Purify the amino-modified cDNA using method of choice (Cat. No L1014-03)

↓

Perform the fluorescent dye coupling reaction.

↓

Purify the labeled cDNA using Purification Module (Cat. Nos. L1014-01 and L1014-02) OR Purify the labeled cDNA using method of choice (Cat. No. L1014-03)

Ready to hybridize

Continued on next page
Overview, continued

Advantages of SuperScript™ III RNase H- 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.

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.

Dye Compatibility

This kit has been developed using Alexa Fluor® fluorescent dyes from Invitrogen and CyDye™ fluorescent dyes from Amersham Biosciences. See page 7 for more information.

Other monofunctional, N-hydroxysuccinimide (NHS)-reactive fluorescent dyes are compatible with this system.

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.

- Monofunctional, NHS-reactive fluorescent dye
- 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 RNA Ladder included in the system to determine the efficiency of the labeling reaction. The section on First-Strand cDNA Synthesis (page 3) describes how to set up the control reaction and page 10 has equations for calculating the efficiency of the labeling procedure.
Detailed 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 14), 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 designed for use with 5–20 µg total RNA or 0.4–2 µg of mRNA. To isolate total RNA, we recommend the Micro-to-Midi Total RNA Purification System or TRIzol® Reagent (Chirgwin et al., 1979; Chomczynski and Sacchi, 1987). To isolate mRNA, we recommend the Micro-FastTrack™ 2.0 or FastTrack® 2.0 mRNA Isolation Kits. Ordering information is provided on page 14.

After you have isolated the RNA, check the quality of your RNA preparation as described on the following 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 3. 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
- 0.5-ml or 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
- 0.24–9.5 Kb Control RNA Ladder, optional
- 3 M Sodium Acetate, pH 5.2

## Control RNA Ladder
A Control RNA Ladder is included in the kit to help you determine the efficiency of the labeling procedure. We strongly recommend that you perform the control reaction if you are a first-time user of the SuperScript™ Indirect cDNA Labeling System.

Instructions are provided on the next page to set up separate cDNA synthesis reactions for your sample and the Control RNA Ladder. Equations for calculating the amount of coupled dye in the control reaction are provided on page 10.

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.

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*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 1 μl of the Control RNA Ladder supplied in the kit (0.5 μg/μl).

1. Mix and briefly centrifuge each component before use.

2. Prepare reaction(s) as follows, using 0.5-ml or 1.5-ml RNase-free tubes:

<table>
<thead>
<tr>
<th>Component</th>
<th>Sample</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>5–20 μg total RNA or 0.4–2 μg mRNA</td>
<td>X μl</td>
<td>—</td>
</tr>
<tr>
<td>Control RNA Ladder</td>
<td>—</td>
<td>1 μl</td>
</tr>
<tr>
<td>Anchored Oligo(dT)20 Primer (2.5 μg/μl)</td>
<td>2 μl</td>
<td>2 μl</td>
</tr>
<tr>
<td>Random hexamers (only if using mRNA)</td>
<td>1 μl *</td>
<td>1 μl *</td>
</tr>
<tr>
<td>DEPC-treated water</td>
<td>to 18 μl</td>
<td>to 18 μl</td>
</tr>
</tbody>
</table>

*For mRNA (including the control ladder), 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 min, and then place on ice for at least 1 min.

4. Add the following to each tube (sample and control) on ice:

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

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, above, 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.

Purifying First-Strand cDNA

Introduction

After you have generated cDNA with amino-modified nucleotides, you need to purify the cDNA to remove unincorporated dNTPs and hydrolyzed RNA.

Catalog Numbers L1014-01 and L1014-02 include a Purification Module developed for use with the system. Use the S.N.A.P.™ Column Purification procedure on the next page to purify your amino-modified cDNA using this Purification Module.

Catalog Number L1014-03 does not include a Purification Module. Use your preferred method of purification instead of the S.N.A.P.™ Column Purification procedure, and proceed to the Ethanol Precipitation procedure on the next page.

Important

You must perform the Ethanol Precipitation step on page 6 even if you are using your own purification procedure.

Before Starting

The following materials are supplied by the user:

- Microcentrifuge
- 1.5-ml microcentrifuge tube
- 100% Ethanol
- 75% Ethanol

The following materials are supplied in the Core Module:

- 2X Coupling Buffer
- 3 M Sodium Acetate, pH 5.2
- Glycogen (20 mg/ml)

The following materials are supplied in the Purification Module (Catalog Numbers L1014-01 and L1014-02):

- DEPC-treated water
- S.N.A.P.™ column(s) and clear collection tube(s)
- Loading Buffer plus isopropanol (see page v for preparation)
- Wash Buffer plus ethanol (see page v for preparation)

Important

The pellet should be completely dry at the end of the purification procedure to ensure complete removal of the ethanol. The presence of ethanol can inhibit the labeling reaction.

Continued on next page
Purifying First-Strand cDNA, continued

S.N.A.P.™ Column Purification

Use the following procedure to purify the cDNA using the components of the Purification Module (Cat. nos. L1014-01 and L1014-02).

If you are using Cat. No. L1014-03, purify using your method of choice and then proceed to Ethanol Precipitation below.

Note: Before starting the procedure, be sure to add isopropanol to the Loading Buffer and ethanol to the Wash Buffer as described on page v.

1. Add 500 µl of Loading Buffer prepared as directed on page v to the neutralized cDNA (from Alkaline Hydrolysis and Neutralization, Step 4, previous page). Mix well by vortexing.
2. Place a S.N.A.P.™ Column on a collection tube and load your sample on the S.N.A.P.™ Column.
3. Centrifuge at 14,000 × g at room temperature for 60 seconds. Remove the collection tube and discard the flow-through.
4. Place the S.N.A.P.™ Column onto the same collection tube and add 700 µl of Wash Buffer prepared as directed on page v.
5. Centrifuge at 14,000 × g at room temperature for 60 seconds. Remove the collection tube and discard the flow-through.
6. Repeat Steps 4–5 above.
7. Centrifuge one more time at 14,000 × g at room temperature for 60 seconds. Remove the collection tube and discard the flow-through.
8. Place the S.N.A.P.™ Column onto a new 1.5-ml microcentrifuge tube.
9. Add 50 µl of DEPC-treated water to the S.N.A.P.™ Column and incubate at room temperature for 1 minute. Centrifuge at 14,000 × g at room temperature for 1 minute.
10. Repeat Step 9, using the same microcentrifuge tube. Proceed directly to Ethanol Precipitation on the next page.

Ethanol Precipitation

In the second part of the purification procedure, perform an ethanol precipitation of the cDNA:

1. Add 10 µl of 3 M Sodium Acetate, pH 5.2, to the tube from Step 10, previous page.
2. Add 2 µl of 20 mg/ml glycogen to the tube and mix.
3. Add 300 µl of ice-cold 100% ethanol, and incubate the tube at -20°C for at least 30 minutes.

Note: You can incubate the tube overnight if desired.

4. Spin the tube at 14,000 × g at 4°C for 10–20 minutes. Carefully remove and discard the supernatant.

Note: You can spin at room temperature if a refrigerated centrifuge is unavailable; however, the yield may be slightly less.

5. Add 250 µl of ice-cold 75% ethanol and spin the tube at 14,000 × g for 2 minutes. Carefully remove and discard the supernatant.
6. Air dry the sample for 10 minutes.
7. Resuspend the sample in 5 µl of 2X Coupling Buffer.
Labeling with Fluorescent Dye

Introduction

After cDNA synthesis and purification, you are ready to label the amino-modified cDNA with fluorescent dye.

Dye Information

This kit has been validated with the following dyes and dye packs:

- Alexa Fluor® 555 Reactive Dye Decapack (10 vials) (A-32756)
- Alexa Fluor® 647 Reactive Dye Decapack (10 vials) (A-32757)
- Alexa Fluor® 555 and Alexa Fluor® 647 Reactive Dye Decapacks (10 vials each dye) (Cat. no. A-32755)
- CyDye Post-Labeling Reactive Dye Pack (12 vials each Cy3™ and Cy5™) (Amersham Biosciences, #RPN 5661)
- Cy3™ Mono-Reactive Dye Pack (Amersham Biosciences, #PA 23001)
- Cy5™ Mono-Reactive Dye Pack (Amersham Biosciences, #PA 25001)

This kit is also compatible with other monofunctional, NHS-reactive fluorescent dyes.

Before Starting

The following items will be used in the following procedure:

- DMSO (supplied in the kit)
- DEPC-treated water (if using Alexa Fluor® dyes)
- Fluorescent dye(s)

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.

CAUTION

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.

Labeling Reaction

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

1. **Alexa Fluor® dye** vials — Add 2 μl of DMSO directly to each dye vial.
   
   **Cy3™ or Cy5™ dye** vials —
   
   Individual reaction size (RPN 5661): Add 5 μl DMSO directly to each dye vial.
   
   Large size (PA 23001 and PA 25001): Add 45 μl DMSO directly to each dye vial.
   
   Use 5 μl of this DMSO/dye solution in the next step.
   
   Dye from another manufacturer: Prepare as directed.

2. Add the DMSO/dye solution to the tube from Ethanol Precipitation, Step 7, previous page. If you are using Alexa Fluor® dyes, add 3 μl of DEPC-treated water to the tube to bring the total volume to 10 μl.

3. Mix well and incubate the tube at room temperature in the dark for 1 hour. Reaction can be stored overnight if necessary. Store any unused dye solution according to manufacturer’s directions.
Purifying Labeled cDNA

Introduction

In this step, you purify the labeled cDNA to remove any unreacted dye. Cat nos. L1014-01 and L1014-02 include a Purification Module developed for use with the system. Follow the procedure below to purify your labeled cDNA.

Cat no. L1014-03 does not include a Purification Module. Use your preferred method of labeled cDNA purification, and then continue to hybridization.

Before Starting

The following items are supplied by the user:

- Microcentrifuge

The following items are supplied in the Purification Module (Cat nos. L1014-01 and L1014-02):

- 3 M Sodium Acetate, pH 5.2
- DEPC-treated water (supplied in the kit)
- S.N.A.P.™ column(s) and collection tube
- Amber collection tubes
- Loading Buffer plus isopropanol (see page v for preparation)
- Wash Buffer plus ethanol (see page v for preparation)

S.N.A.P.™ Column Purification

Use the following procedure to purify the cDNA:

2. Add 500 µl of Loading Buffer plus isopropanol to the cDNA solution. Mix well by vortexing.
3. Place a S.N.A.P.™ Column onto a clear collection tube and load the cDNA/buffer solution onto the S.N.A.P.™ Column.
4. Centrifuge at 14,000 × g at room temperature for 60 seconds. Remove the collection tube and discard the flow-through.
5. Place the S.N.A.P.™ Column on the same collection tube and add 700 µl of Wash Buffer plus ethanol to the column.
6. Centrifuge at 14,000 × g at room temperature for 60 seconds. Remove the collection tube and discard the flow-through.
7. Repeat Steps 5–6 above, using the same collection tube.
8. Centrifuge one more time at 14,000 × g at room temperature for 60 seconds. Remove the collection tube and discard the flow-through.
9. Place the S.N.A.P.™ Column onto a new amber collection tube.
10. Add 50 µl of DEPC-treated water to the S.N.A.P.™ Column and incubate at room temperature for 1 minute.
11. Centrifuge at 14,000 × g at room temperature for 1 minute and collect the flow-through. The flow-through contains your purified dye-coupled 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 on page 10.
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 the following procedure and formulas to measure the amount of Alexa Fluor®-labeled or CyDye™-labeled cDNA and determine the efficiency of the reaction. The expected amounts of labeled cDNA using the Control RNA Ladder are noted below.

Absorption Wavelengths and Baselines

The following table shows the absorbance and baseline wavelengths for CyDyes™ and Alexa Fluor® dyes:

<table>
<thead>
<tr>
<th>Label</th>
<th>Absorbance Wavelength</th>
<th>Baseline Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor® 555 or Cy3™</td>
<td>550 nm</td>
<td>650 nm</td>
</tr>
<tr>
<td>Alexa Fluor® 647 or Cy5™</td>
<td>650 nm</td>
<td>750 nm</td>
</tr>
</tbody>
</table>

Calculating the Amount of Coupled Dye

To calculate the amount of coupled dye:

1. Transfer the undiluted sample from Step 11, page 8, into a clean cuvette, and scan at 240–800 nm using a UV/visible spectrophotometer. If you are using a 100-µl cuvette, transfer the entire sample; for smaller cuvettes, transfer an appropriate amount of sample.

   Note: The labeled cDNA must be purified as described on page 8 before scanning, as any unreacted dye will interfere with the detection of labeled cDNA.

2. Calculate the amount of labeled cDNA using the formula below:

   Labeled cDNA (pmoles) = (A_{260}–A_{320}) \times 40 \times 50 \text{ (elution volume)}/0.304

   The amount of cDNA generated from the Control RNA Ladder should be > 700 pmoles. If it is < 700 pmoles, see Troubleshooting on page 12.

3. Calculate the amount of fluorescently labeled dye using a formula below:

   - Alexa Fluor® 555 (pmole) = (A_{550}–A_{650})/0.15 \times 50 \text{ (elution volume)}
   - Alexa Fluor® 647 (pmole) = (A_{650}–A_{750})/0.24 \times 50 \text{ (elution volume)}
   - Cy3™ (pmole) = (A_{550}–A_{650})/0.15 \times 50 \text{ (elution volume)}
   - Cy5™ (pmole) = (A_{650}–A_{750})/0.25 \times 50 \text{ (elution volume)}
Determining cDNA Yield Using TCA Precipitation

Introduction

Instructions are provided below to calculate the yield of your first-strand synthesis reaction using TCA precipitation.

Before Starting

Have the following items on hand before starting:

- [α-32P]dCTP
- Yeast tRNA (see page 14 for ordering information)
- 20 mM EDTA
- Glass fiber filters (Fisher Catalog no. 1822-914)
- Heat lamp
- 5% trichloroacetic acid (TCA)
- 10% TCA containing 1% sodium pyrophosphate
- Scintillation counter
- Sterile microcentrifuge tube

Procedure

1. Prepare a first-strand synthesis reaction as described on page 4. Add 1 µl of [α-32P]dCTP (10 mCi/ml, 3,000 mCi/mmol) to the listed components in Step 4.
2. Add 2 µl of the radio-labeled first-strand reaction mix from Step 5, page 4, to a sterile microcentrifuge tube containing 43 µl of 20 mM EDTA (pH 7.5) and 5 µl of yeast tRNA (5 µg). Mix well by vortexing.
3. Spot two 10-µl aliquots from the tube onto separate glass fiber filters.
4. Dry the filters under a heat lamp.
5. Set one filter aside. This will be used to determine the specific activity of dCTP in the reaction.
6. Wash the second filter once in ice-cold 10% TCA containing 1% sodium pyrophosphate for 10 minutes at room temperature on a rotary shaker.
7. Wash the filter twice in 5% TCA for 10 minutes.
8. Wash the filter with 95% ethanol for 10 minutes at room temperature. This filter will be used to determine the yield of the first-strand cDNA.
9. Count both filters using a standard scintillation counter.

Calculating the Yield

Calculate first-strand synthesis yield as follows:

Specific activity (cpm/pmol dCTP) = \( \frac{\text{cpm of unwashed filter}}{200 \text{ pmol dCTP}} \)

Amount of cDNA (µg) = \( \frac{\text{cpm of washed filter} \times 5 \times 15 \times 4 \text{ pmole dNTP/pmol dCTP}}{\text{Specific activity} \times 3,030 \text{ pmole dNTP/µg cDNA}} \)

Yield = \( \frac{\text{Amount of cDNA (µg)} \times 100}{\text{Amount of mRNA used (µg)*}} \)

If the yield is low, see Troubleshooting on page 12.

*If you are using total RNA as your starting material, the mRNA will be 1–2% of total RNA.
## Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>28S and 18S bands are not observed after isolation of total RNA and agarose gel electrophoresis</td>
<td>Too little RNA loaded on the gel</td>
<td>Be sure to load at least 250 ng of RNA for analysis.</td>
</tr>
<tr>
<td></td>
<td>RNA is degraded due to RNase activity</td>
<td>Follow the guidelines on page 1 to avoid RNase contamination. Use a fresh sample for RNA isolation.</td>
</tr>
<tr>
<td>28S and 18S bands are not observed after isolation of total RNA and agarose gel electrophoresis</td>
<td>RNA is degraded due to RNase activity</td>
<td>Follow the guidelines on page 1 to avoid RNase contamination. Use a fresh sample for RNA isolation.</td>
</tr>
<tr>
<td></td>
<td>Temperature too high during cDNA synthesis</td>
<td>Perform the cDNA synthesis at 46°C.</td>
</tr>
<tr>
<td></td>
<td>Incorrect reaction conditions used</td>
<td>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.</td>
</tr>
<tr>
<td></td>
<td>Concentration of template RNA is too low</td>
<td>Increase the concentration of template RNA. Use at least 5 µg of total RNA or 0.4 µg of mRNA.</td>
</tr>
<tr>
<td></td>
<td>Poor quality RNA used or RNA is degraded</td>
<td>Check the quality of your RNA preparation (see page 2). If RNA is degraded, use fresh RNA.</td>
</tr>
<tr>
<td></td>
<td>RNase contamination</td>
<td>Use the RNaseOUT™ included in the kit to prevent RNA degradation.</td>
</tr>
<tr>
<td></td>
<td>RT inhibitors are present in your RNA sample</td>
<td>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.</td>
</tr>
<tr>
<td></td>
<td>Improper storage of SuperScript™ III RT</td>
<td>Store the enzyme at -20°C.</td>
</tr>
<tr>
<td>28S and 18S bands are not observed after isolation of total RNA and agarose gel electrophoresis</td>
<td>Improper storage of SuperScript™ III RT</td>
<td>Store the enzyme at -20°C.</td>
</tr>
<tr>
<td></td>
<td>Yield of cDNA from the first-strand synthesis reaction is low</td>
<td>Perform the cDNA synthesis at 46°C.</td>
</tr>
<tr>
<td></td>
<td>Incorrect reaction conditions used</td>
<td>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.</td>
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<td></td>
<td>Improper storage of SuperScript™ III RT</td>
<td>Store the enzyme at -20°C.</td>
</tr>
<tr>
<td>28S and 18S bands are not observed after isolation of total RNA and agarose gel electrophoresis</td>
<td>Improper storage of SuperScript™ III RT</td>
<td>Store the enzyme at -20°C.</td>
</tr>
<tr>
<td></td>
<td>Yield of labeled cDNA from the control reaction is low</td>
<td>Follow the S.N.A.P™ Column purification procedure and ethanol precipitation procedure without modifications. Overnight ethanol precipitation may result in higher yields.</td>
</tr>
<tr>
<td></td>
<td>cDNA has been lost in the purification step following cDNA synthesis</td>
<td>Follow the S.N.A.P™ Column purification procedure and ethanol precipitation procedure without modifications. Overnight ethanol precipitation may result in higher yields.</td>
</tr>
<tr>
<td></td>
<td>cDNA has been lost in the purification step after labeling</td>
<td>Measure the amount of labeled cDNA in the control reaction before and after purification. Follow the purification procedure without modifications.</td>
</tr>
</tbody>
</table>

*Continued on next page*
## Troubleshooting, continued

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of coupled dye in the control reaction is low (≤40 pmoles) and/or fluorescence of labeled cDNA is low</td>
<td>Control reaction was set up using only anchored oligo(dT)$_{20}$</td>
<td>Prepare the control reaction as specified on page 4, using both anchored oligo(dT)$_{20}$ and random hexamers.</td>
</tr>
<tr>
<td></td>
<td>Reaction tubes have been exposed to light</td>
<td>Avoid direct exposure of the labeling reaction to light. Use an amber tube for collection of the final product.</td>
</tr>
<tr>
<td></td>
<td>Dye solution has been exposed to light</td>
<td>Repeat labeling reaction with fresh mixture of dye, being careful to avoid direct exposure to light.</td>
</tr>
<tr>
<td></td>
<td>DMSO used to prepare dye mixture was contaminated with water.</td>
<td>Prepare a new mixture of dye using fresh DMSO. Carefully follow the instructions for storing and handling DMSO in the Caution on page 7.</td>
</tr>
<tr>
<td></td>
<td>Inefficient labeling due to improper purification</td>
<td>Follow all purification steps carefully and without modification.</td>
</tr>
<tr>
<td></td>
<td>2X Coupling Buffer was not stored properly</td>
<td>Store 2X Coupling Buffer at -20°C.</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Product</th>
<th>Quantity</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor® 555 and Alexa Fluor® 647 Reactive Dye Decapacks</td>
<td>2 × 10 vials</td>
<td>A-32755</td>
</tr>
<tr>
<td>Alexa Fluor® 555 Reactive Dye Decapack</td>
<td>10 vials</td>
<td>A-32756</td>
</tr>
<tr>
<td>Alexa Fluor® 647 Reactive Dye Decapack</td>
<td>10 vials</td>
<td>A-32757</td>
</tr>
<tr>
<td>RNase AWAY™ Reagent</td>
<td>250 ml</td>
<td>10328-011</td>
</tr>
<tr>
<td>Yeast tRNA</td>
<td>25 mg</td>
<td>15401-011</td>
</tr>
<tr>
<td></td>
<td>50 mg</td>
<td>15401-029</td>
</tr>
<tr>
<td>Micro-FastTrack™ 2.0 mRNA Isolation Kit</td>
<td>20 reactions</td>
<td>K1520-02</td>
</tr>
<tr>
<td>FastTrack® 2.0 mRNA Isolation Kit</td>
<td>6 reactions</td>
<td>K1593-02</td>
</tr>
<tr>
<td></td>
<td>18 reactions</td>
<td>K1593-03</td>
</tr>
<tr>
<td>Micro-to-Midi Total RNA Purification System</td>
<td>50 reactions</td>
<td>12183-018</td>
</tr>
<tr>
<td>TRIZol® Reagent</td>
<td>100 ml</td>
<td>15596-026</td>
</tr>
<tr>
<td></td>
<td>200 ml</td>
<td>15596-018</td>
</tr>
<tr>
<td>RNaseOUT™ Recombinant Ribonuclease Inhibitor</td>
<td>5000 units</td>
<td>10777-019</td>
</tr>
<tr>
<td>Human Cot-1 DNA®</td>
<td>500 µg</td>
<td>15279-011</td>
</tr>
<tr>
<td>Mouse Cot-1 DNA®</td>
<td>500 µg</td>
<td>18440-016</td>
</tr>
<tr>
<td>Random primers</td>
<td>9 A260 units</td>
<td>48190-011</td>
</tr>
<tr>
<td>UltraPure™ DEPC-treated water</td>
<td>4 × 1.25 ml</td>
<td>10813-012</td>
</tr>
<tr>
<td>UltraPure™ 10% SDS solution</td>
<td>4 × 100 ml</td>
<td>15553-027</td>
</tr>
<tr>
<td>UltraPure™ 20X SSC</td>
<td>1 L</td>
<td>15557-044</td>
</tr>
<tr>
<td>UltraPure™ 20X SSPE</td>
<td>1 L</td>
<td>15591-043</td>
</tr>
</tbody>
</table>
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• Explore our catalog with full color graphics
• Obtain citations for Invitrogen products
• Request catalog and product literature

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http://www.invitrogen.com

...and the program will connect directly. Click on underlined text or outlined graphics to explore. Don’t forget to put a bookmark at our site for easy reference!

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our Web page (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_service@invitrogen.com

Japanese Headquarters:
Invitrogen Japan K.K.
Nihonbashi Hama-Cho Park Bldg. 4F
2-35-4, Hama-Cho, Nihonbashi
Tel: 81 3 3663 7972
Fax: 81 3 3663 8242
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

MSDS Requests


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