invitrogen™
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SuperScript® Indirect cDNA Labeling System

For generating fluorescently labeled cDNA to use in microarray screening

Catalog numbers 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.

Cat no.	Number of Labeling Reactions	Modules
L1014-01	10	Core and Purification
L1014-02	30	Core and Purification
L1014-03	30	Core only

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 –20ºC and store the components of the Purification Module at room temperature.

Core Module

The components of the Core Module should be stored at –20ºC.

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 $MgCl_2$	1020 µl	1020 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 at optimal concentrations in DEPC-treated water	20 µl	60 µl
2X Coupling Buffer	—	100 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
Glycogen	20 mg/ml	20 µl	60 µl
DMSO	—	200 µl	750 µl
RNaseOUT™	40 U/µl	10 µl	30 µl
DEPC-treated Water	—	2 ml	6 ml
3 M Sodium Acetate	pH 5.2	1 ml	2 ml
Control HeLa RNA	1 µg/µl	20 µl	20 µl

Continued on next page

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.

Item	Kit Size	
	10 Rxns	30 Rxns
Loading Buffer (you must add 100% isopropanol to create the final buffer; see below)	4.3 ml	13 ml
Wash Buffer (you must add 100% ethanol to create the final buffer; see below)	8.33 ml	25 ml
S.N.A.P.™ Columns	22 columns	62 columns
Clear Collection Tubes	22 tubes	62 tubes
Amber Collection Tubes	11 tubes	31 tubes

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.

	10-Rxn Kit	30-Rxn Kit
Loading Buffer	4.3 ml (entire bottle)	13 ml (entire bottle)
100% Isopropanol	10.0 ml	30 ml
Total Volume	14.3 ml	43 ml

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.

	10-Rxn Kit	30-Rxn Kit
Wash Buffer	8.33 ml (entire bottle)	25 ml (entire bottle)
100% Ethanol	25.00 ml	75 ml
Total Volume	33.33 ml	100 ml

Product Use

For research use only. Not intended for any human or animal diagnostic or therapeutic uses.

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.
Alexa Fluor® 555 and Alexa Fluor® 647 Reactive Dye Decapacks	2 × 10 vials	A-32755
Alexa Fluor® 555 Reactive Dye Decapack	10 vials	A-32756
Alexa Fluor® 647 Reactive Dye Decapack	10 vials	A-32757
RNase *AWAY*™ Reagent	250 ml	10328-011
Yeast tRNA	25 mg 50 mg	15401-011 15401-029
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 200 ml	15596-026 15596-018
FastTrack® 2.0 mRNA Isolation Kit	6 reactions 18 reactions	K1593-02 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
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™ 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® Indirect cDNA Labeling System is a highly efficient system for generating fluorescently labeled cDNA for use on microarrays in gene expression studies (De Risi *et al.*, 1996; Eisen & Brown, 1999). 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.

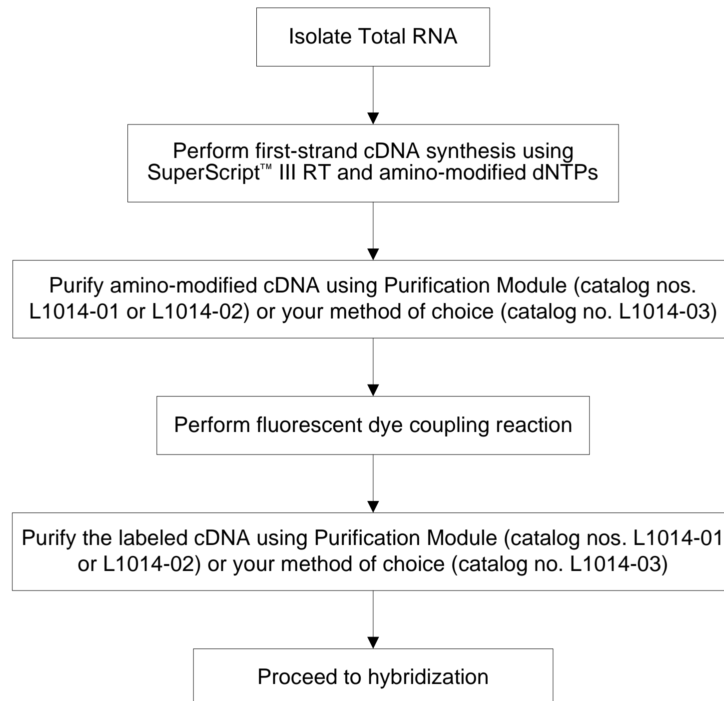
This system uses 5–20 µg of total RNA or 0.4–2 µg of mRNA as starting material, and is compatible with Alexa Fluor® 555 and Alexa Fluor® 647 fluorescent dyes from Invitrogen, Cy3™ and Cy5™ dyes from Amersham Biosciences, or other monoreactive NHS-ester dyes from a variety of manufacturers.

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:



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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 55ºC, 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 9 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 HeLa RNA included in the system to determine the efficiency of the labeling reaction. The section on **First-Strand cDNA Synthesis** (page 5) describes how to set up the control reaction and page 12 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 vi), to remove RNase contamination from surfaces. For further information on controlling RNase contamination, see Ausubel, et al. and Sambrook, et al (Ausubel *et al.*, 1994; 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 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 vi.

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

Continued on next page

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 13.

Storing RNA

After preparing the RNA, we recommend that you proceed directly to **First-Strand cDNA Synthesis** on page 5. Otherwise, store the RNA at –80ºC.

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 46ºC and 70ºC
- 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
- Control HeLa RNA, optional
- 3 M Sodium Acetate, pH 5.2

Control HeLa RNA

Control HeLa RNA 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 HeLa RNA. Equations for calculating the amount of coupled dye in the control reaction are provided on page 12.

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 (1 µg/µl) supplied in the kit.

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:

Component	Sample	Control
5–20 µg total RNA or 0.4–2 µg mRNA	X µl	—
Control HeLa RNA (1 µg/µl)	—	10 µl
Anchored Oligo$(dT)_{20}$ Primer (2.5 µg/µl)	2 µl	2 µl
Random hexamers (only if using mRNA)	1 µl *	—
DEPC-treated water	to 18 µl	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 70ºC for 5 minutes, and then place on ice for at least 1 minute.
4. Add the following to each tube (sample and control) on ice:

Component	Volume
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)	2 µl
Total 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.

Alkaline 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 70º C for 10 minutes.
3. Add 15 µl of 1 N HCl to neutralize the pH and mix gently.
4. Add 20 µl 3 M Sodium Acetate, pH 5.2, 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 included 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 8 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 –20ºC for at least 30 minutes.

   Note: You can incubate the tube overnight if desired.
4. Spin the tube at 14,000 × *g* at 4ºC 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.

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 –20ºC, 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 (RPN5661): Add 5 µl DMSO directly to each dye vial. Large size (PA23001 and PA25001): 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:

1. Add 20 µl of 3 M Sodium Acetate, pH 5.2, to the dye-coupled cDNA solution from Step 3, page 9.
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 of this procedure, 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 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 12.

Continued on next page

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 amount of labeled cDNA using the Control HeLa RNA is noted below.

Absorption Wavelengths and Baselines

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

Label	Absorbance Wavelength	Baseline Wavelength
Alexa Fluor® 555 or Cy3™	550 nm	650 nm
Alexa Fluor® 647 or Cy5™	650 nm	750 nm

Calculating the Amount of Coupled Dye

To calculate the amount of coupled dye:

1. Transfer the **undiluted** sample from Step 11, page 10, 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 10 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:

   cDNA (ng) = $(A_{260}–A_{320}) \times 37$ ng/µl × Volume in µl

The amount of cDNA generated from 10 µg of Control HeLa RNA should be >250 ng. If it is <250 ng, see **Troubleshooting** on page 13.

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

   Alexa Fluor® 555 (pmole) = $(A_{550}–A_{650})/0.15 \times 50$ (elution volume)

   Alexa Fluor® 647 (pmole) = $(A_{650}–A_{750})/0.24 \times 50$ (elution volume)

   Cy3™ (pmole) = $(A_{550}–A_{650})/0.15 \times 50$ (elution volume)

   Cy5™ (pmole) = $(A_{650}–A_{750})/0.25 \times 50$ (elution volume)

Troubleshooting

Observation	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 3 to avoid RNase contamination. Use a fresh sample for RNA isolation.
Yield of cDNA from the first-strand synthesis reaction is low	Temperature too high during cDNA synthesis	Perform the cDNA synthesis at 46ºC.
	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 4). 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 –20ºC.
Yield of labeled cDNA from the control reaction is low	cDNA has been lost in the purification step following cDNA synthesis	Follow the S.N.A.P.™ Column purification procedure and ethanol precipitation procedure without modifications. Overnight ethanol precipitation may result in higher yields.
	cDNA has been lost in the purification step after labeling	Measure the amount of labeled cDNA in the control reaction before and after purification. Follow the purification procedure without modifications.

Continued on next page

Troubleshooting, continued

Observation	Cause	Solution
Amount of coupled dye in the control reaction is low (<40 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 an amber tube for collection of the final product.
	Dye solution has been exposed to light	Repeat labeling reaction with 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 9.
	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 –20ºC.

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