

# LabelStar™ Array Handbook

For cDNA labeling and cleanup

December 2002



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## QIAGEN Worldwide

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## Kit Contents

<b>LabelStar™ Array Kit</b>		
<b>Number of reactions*</b>	<b>12</b>	<b>50</b>
<b>Catalog no.</b>	<b>28902</b>	<b>28904</b>
<b>(Box 1 of 2)</b>	<b>Store at -20°C</b>	<b>Store at -20°C</b>
<b>LabelStar Array Kit cDNA Labeling Module</b>		
LabelStar	30 µl	125 µl
Reverse Transcriptase		
Buffer RT, 10x	60 µl	250 µl
dATP, dGTP, dCTP, dTTP; each 20 mM	16 µl each	65 µl each
Oligo-dT Primer, 20 µM	60 µl	250 µl
RNase Inhibitor (40 units/µl)	7 µl	25 µl
Denaturation Solution Plus	100 µl	100 µl
Stop Solution LS	25 µl	100 µl
RNase-free water	1 ml	1 ml
dNTP Mix, empty tube	1	1
<b>(Box 2 of 2)</b>	<b>Store at room temperature (15–25°C)</b>	<b>Store at room temperature (15–25°C)</b>
<b>LabelStar Array Kit Cleanup Module</b>		
MinElute™ Spin Columns	12	50
Buffer PB† (binding buffer)	3.5 ml	30 ml
Buffer PE (wash buffer) concentrate	2.5 ml	2 x 6 ml
Buffer LS (wash buffer)	12 ml	45 ml
Buffer EB (elution buffer)	1 ml	15 ml

\* Number of standard reactions, each using 0.2–50 µg RNA.

† Binding Buffer PB contains chaotropic salts that are irritants. Take appropriate laboratory safety measures and wear gloves when handling.

## Shipping and Storage

LabelStar Array Kits are shipped in two packages. The cDNA Labeling Module (box 1 of 2) is shipped on dry ice and should be stored immediately upon receipt at  $-20^{\circ}\text{C}$  in a constant-temperature freezer.

The Cleanup Module (box 2 of 2) is shipped at room temperature and should be stored at room temperature.

When stored under these conditions and handled correctly, the products can be kept at least until the expiration date (see inside lid of box 1 of 2) without showing any reduction in performance.

## Product Use Limitations

LabelStar Array Kits are developed, designed, and sold for research purposes only. They are not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

## Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN® product does not meet your expectations, simply call your local Technical Service Department. We will credit your account or exchange the product — as you wish.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see inside front cover).

## Quality Control

QIAGEN Quality Control assays the units and carefully checks each lot of Reverse Transcriptase for direct cDNA labeling and functional absence of RNases, exonucleases, endonucleases, and proteases. All buffers and reagents used for cDNA labeling are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination. For more information, please call for a certificate of analysis, or contact one of the QIAGEN Technical Service Departments or local distributors listed (see inside front cover).

### Enzymatic activities of LabelStar Reverse Transcriptase:

- |   |         |
|---|---------|
| • RNA-dependent DNA polymerase (reverse transcriptase): | Yes     |
| • RNA–DNA hybrid-dependent ribonuclease:                | Yes     |
| • DNA-dependent DNA polymerase:                         | Reduced |

## Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN products. If you have any questions or experience any difficulties regarding LabelStar Array Kits or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information please call one of the QIAGEN Technical Service Departments or local distributors listed (see inside front cover).

## Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at [www.qiagen.com/ts/msds.asp](http://www.qiagen.com/ts/msds.asp) where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

**CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.**

The following risk and safety phrases apply to the components of the LabelStar Array Kit. Buffers PB and LS contain guanidine hydrochloride, which can form highly reactive compounds when combined with bleach.

If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

### Buffer PB

Contains guanidine hydrochloride: harmful irritant and flammable. Risk and safety phrases: \* R10-22-36/38 S13-23-26-36/37/39-46

### Buffer LS

Contains guanidine hydrochloride: harmful irritant. Risk and safety phrases: \* R22-36/38 S13-26-36-46

## 24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

*\* R10: Flammable; R22: Harmful if swallowed; R36/38: Irritating to eyes and skin; S13: Keep away from food, drink, and animal feedingstuffs; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36: Wear suitable protective clothing; S36/37/39: Wear suitable protective clothing, gloves and eye/face protection; S46: If swallowed, seek medical advice immediately and show this container or label.*

## Solutions and Reagents to Be Supplied by the User

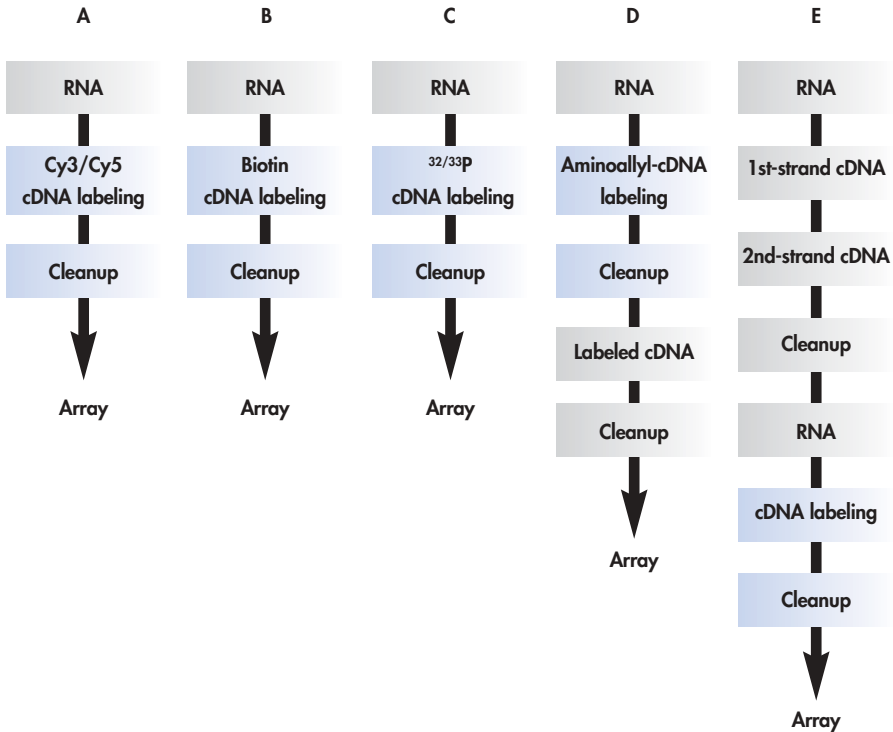
- Ethanol (96–100%)
- Modified nucleotides:  
<sup>32</sup>P-, <sup>33</sup>P-, cyanine 3-, cyanine 5-, biotin-, or aminoallyl-modified dCTP or dUTP are commonly used for labeling cDNA during reverse transcription. A list of modified nucleotides recommended for use with the LabelStar Array Kit appears in Table 1.

**Table 1. Examples of modified nucleotides recommended for use with the LabelStar Array Kit\***

<b>Modified nucleotide</b>	<b>Source</b>	<b>Order no.</b>
Cyanine 3-dCTP	Amersham Biosciences PerkinElmer™ Life Sciences	PA53021 NEL 576
Cyanine 5-dCTP	Amersham Biosciences PerkinElmer Life Sciences	PA55021 NEL 577
Biotin-dCTP	ENZO	42816
Cyanine 3-dUTP	Amersham Biosciences PerkinElmer Life Sciences	PA53022 NEL 578
Cyanine 5-dUTP	Amersham Biosciences PerkinElmer Life Sciences	PA55022 NEL 579
Biotin-dUTP	Roche	1 093 070
5-(3-Aminoallyl)-2'-dUTP	Sigma	A0410
[ $\alpha$ - <sup>32</sup> P]dCTP (3000 Ci/mmol)	Amersham Biosciences	AA0005
[ $\alpha$ - <sup>33</sup> P]dCTP (2500 Ci/mmol)	Amersham Biosciences	AH9905

\* Modified nucleotides are also available from other suppliers.

# Labeling Strategies



**Figure 1.** The LabelStar Array Kit is adaptable to several labeling strategies. The steps of labeling and cleanup covered by the LabelStar Array Kit are marked in blue. The LabelStar cDNA labeling and cleanup system is suitable for use in direct labeling (A, B), indirect labeling (D), radioactive labeling (C) and direct labeling after RNA amplification (E).

## Introduction

The LabelStar Array Kit from QIAGEN offers a labeling and purification system for target cDNA used in microarray analysis that gives high signal intensities and low background. The LabelStar Array Kit is adaptable to several labeling reactions such as direct and indirect cDNA labeling (Figure 1, page 9), using a wide range of modified nucleotides and amounts of RNA (0.2–50 µg). The optimized reaction conditions used in labeling and purification ensure reproducibly high sensitivity.

### Procedure

During the LabelStar procedure (see flowchart), isolated RNA is treated with Denaturation Solution Plus to ensure denaturation of the RNA template and neutralization of inhibitors of reverse transcription copurified with the RNA. Incorporation of a modified nucleotide of choice (see Table 1, page 8) is performed during reverse transcription of the denatured RNA using LabelStar Reverse Transcriptase. Remaining RNA is degraded after the labeling reaction by the exonuclease activity of the LabelStar Reverse Transcriptase.

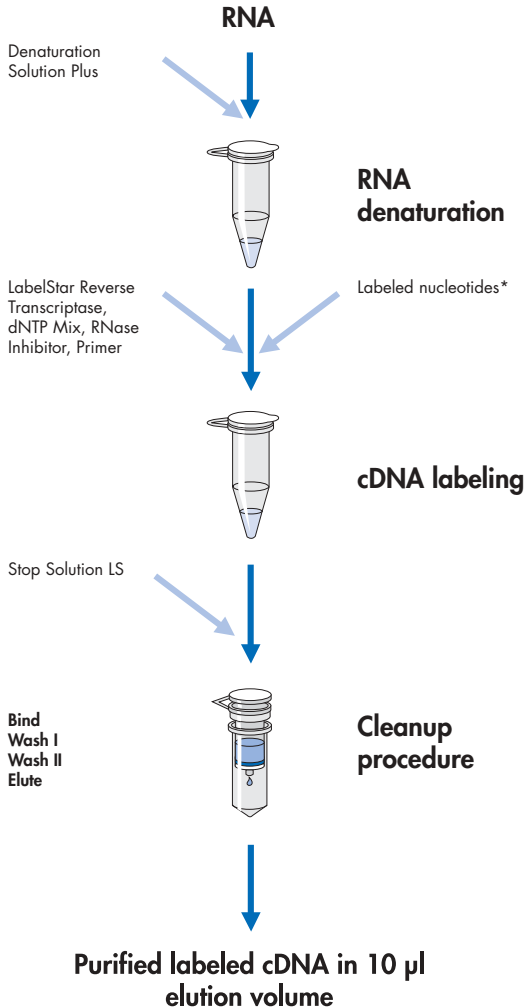
Stop Solution LS stops the labeling reaction and reduces nonspecific binding of labeled reaction components to the array, reducing background signal.

Cleanup of labeled cDNA is performed using MinElute spin columns, and an optimized set of buffers, ensuring high recovery of labeled cDNA in low elution volumes.

cDNA labeled and purified by the LabelStar Array Kit is ready for hybridization to expression arrays, independent of the type of probes (e.g., oligos, PCR fragments) or support material (e.g., glass slides, membrane arrays).

The LabelStar labeling reaction is optimized for a wide range of RNA amounts and for use with a range of different labeled or modified nucleotides, such as fluorophore, haptene, amino-functional, or radioactively modified nucleotides (see Table 1, page 8).

# LabelStar Array Procedure



\* Not included in LabelStar Array Kit

**Table 2. Protocols for the LabelStar Array Kit**

<b>Modified nucleotide</b>	<b>Protocol page</b>
Cyanine 3-dCTP	13
Cyanine 5-dCTP	13
Biotin-dCTP	13
Cyanine 3-dUTP	16
Cyanine 5-dUTP	16
Biotin-dUTP	16
5-(3-Aminoallyl)-2'-dUTP	19
[ $\alpha$ - <sup>32</sup> P]dCTP or [ $\alpha$ - <sup>33</sup> P]dCTP (using mRNA as a template)	22
[ $\alpha$ - <sup>32</sup> P]dCTP or [ $\alpha$ - <sup>33</sup> P]dCTP (using total RNA as a template)	26

## Protocol for Direct Labeling of cDNA with Biotin-dCTP, Cyanine 3-dCTP, or Cyanine 5-dCTP

This is the standard protocol for direct labeling of cDNA with biotin-dCTP, cyanine 3-dCTP, or cyanine 5-dCTP, using 0.2–50 µg RNA. The amount of RNA corresponds to the total amount of RNA present, including any rRNA, mRNA, viral RNA, or carrier RNA present.

### Important notes before starting

- Set up all reactions on ice to avoid premature cDNA synthesis and minimize the risk of RNA degradation.
- Be sure that everything is mixed well by vortexing.
- Ensure that this protocol is suitable for the modified nucleotide that you are using (see Table 2, page 12).
- The protocol is optimized for use with 0.2–50 µg total RNA or 0.2–5 µg mRNA. For amounts >50 µg total RNA, scale up the reaction linearly to the appropriate volume (see Table 4, page 15).
- Optical density measurements of labeled cDNA is not recommended.
- Do not perform an RNA degradation step following the labeling procedure.
- The protocol is optimized using oligo-dT primers. If you intend to use other primers, please refer to Appendix D.

### A) Preparation of dNTP Mix C

**Note:** The total volume of dNTP Mix C (64 µl or 260 µl) is sufficient for 12 or 50 identical labeling reactions respectively (depending on kit size). If using different cDNA labeling protocols with the LabelStar Array Kit ensure that appropriate aliquots of dNTP Mix C are prepared. After use, store the remaining dNTP Mix C in the LabelStar cDNA Labeling Module (box 1 of 2) at –20°C.

1. **Thaw dATP, dCTP, dTTP, and dGTP solution on ice. Mix each solution, for example, by vortexing. Centrifuge briefly and keep on ice.**
2. **Prepare the dNTP Mix C (Table 3) on ice in the tube provided. Mix and keep the tube on ice.**

**Table 3. Preparation of dNTP Mix C using biotin-dCTP, cyanine 3-dCTP, or cyanine 5-dCTP**

Component	Volume (12 reactions)	Volume (50 reactions)	Final concentration
dATP (20 mM)	16.0 $\mu$ l	65 $\mu$ l	5 mM
dGTP (20 mM)	16.0 $\mu$ l	65 $\mu$ l	5 mM
dTTP (20 mM)	16.0 $\mu$ l	65 $\mu$ l	5 mM
dCTP (20 mM)	2.5 $\mu$ l	10 $\mu$ l	0.8 mM
RNase-free water	13.5 $\mu$ l	55 $\mu$ l	–
<b>Total volume*</b>	<b>64.0 <math>\mu</math>l</b>	<b>260 <math>\mu</math>l</b>	

\* Be sure that everything is mixed well by vortexing.

## B) Protocol for cDNA labeling

1. Thaw the template RNA solution, LabelStar Reverse Transcriptase, dNTP Mix C (see Table 3), RNase inhibitor, and labeled dCTP (1 mM) on ice. All other components, oligo-dT primer solution, 10x Buffer RT, Denaturation Solution Plus, and RNase-free water should be thawed at room temperature, mixed, and kept on ice.

**Note:** Biotin-, cyanine 3-, and cyanine 5-labeled dCTP (1 mM) are not provided with this kit. For recommended sources of modified nucleotides see “Solutions and Reagents to Be Supplied by User”, page 8.

**Note:** dNTPs and labeled nucleotides are labile reagents. Freeze nucleotides immediately.

2. Adjust the volume of your RNA solution to a total volume of 18  $\mu$ l with RNase-free water.
3. Add 2  $\mu$ l Denaturation Solution Plus to the 18  $\mu$ l RNA solution. Mix and centrifuge briefly.
4. Incubate at 65°C for 5 min and cool down on ice immediately. Centrifuge briefly.
5. Prepare a fresh master mix on ice according to Table 4 (next page). Mix thoroughly and carefully by vortexing. Centrifuge briefly and store on ice.

The master mix contains everything except the denatured template RNA.

**Note:** Be sure to mix the master mix thoroughly before adding the denatured template RNA.

6. Add 20  $\mu$ l of denatured template RNA (step 3 and 4) to the individual tubes containing the master mix. Mix and centrifuge briefly.

**Note:** Be sure that everything is mixed well by vortexing.

7. Incubate for 120 min at 37°C.

**8. Add 2  $\mu$ l of Stop Solution LS to the individual tubes. Mix and centrifuge briefly.**

**Note:** RNA degradation step is not needed.

**9. Proceed to the purification protocol on page 30.**

For optimal sensitivity and background, use the LabelStar Cleanup Module (box 2 of 2). If purification will not be performed immediately, store tubes at  $-20^{\circ}\text{C}$ .

**Table 4. Master mix for cDNA labeling using biotin-, cyanine 3-, or cyanine 5-labeled dCTP**

Component	Volume/reaction	Final concentration
<b>Master mix</b>		
10x Buffer RT	5.0 $\mu$ l	1x
dNTP Mix C (prepared in protocol section A)	5.0 $\mu$ l	0.5 mM for dATP, dTTP, dGTP and 0.08 mM for dCTP*
Biotin-, cyanine 3-, or cyanine 5-labeled dCTP (1 mM) <sup>†</sup>	1.0 $\mu$ l	0.02 mM
Oligo-dT primer (20 $\mu$ M)	5.0 $\mu$ l	2 $\mu$ M
RNase inhibitor (40 units/ $\mu$ l)	0.5 $\mu$ l	20 units (per 50 $\mu$ l reaction)
RNase-free water	11.0 $\mu$ l	–
LabelStar Reverse Transcriptase	2.5 $\mu$ l	
<b>Template RNA</b>		
Denatured template RNA, added in step 6	20.0 $\mu$ l	Up to 50 $\mu$ g <sup>‡</sup> (per 50 $\mu$ l reaction)
<b>Total labeling reaction volume<sup>§</sup></b>	<b>50.0 <math>\mu</math>l</b>	–

\* Optimal labeling of cDNA is obtained by using the recommended ratio of labeled dCTP to unlabeled dCTP.

<sup>†</sup> In general, labeled dNTPs are provided in aliquots of 25  $\mu$ l at a concentration of 1 mM. This corresponds to an amount of 25 nmol of labeled dNTPs.

<sup>‡</sup> This amount corresponds to the total amount of RNA present, including any rRNA, mRNA, viral RNA, and carrier RNA present.

<sup>§</sup> Be sure that everything is mixed well.

# Protocol for Direct Labeling of cDNA with Biotin-dUTP, Cyanine 3-dUTP, or Cyanine 5-dUTP

This is the standard protocol for direct labeling of cDNA with biotin-dUTP, cyanine 3-dUTP, or cyanine 5-dUTP, using 0.2–50 µg RNA. The amount of RNA corresponds to the total amount of RNA present, including any rRNA, mRNA, viral RNA, or carrier RNA present.

## Important notes before starting

- Set up all reactions on ice to avoid premature cDNA synthesis and minimize the risk of RNA degradation.
- Be sure that everything is mixed well by vortexing.
- Ensure that this protocol is suitable for the modified nucleotide that you are using (see Table 2, page 12).
- The protocol is optimized for use with 0.2–50 µg total RNA or 0.2–5 µg mRNA. For amounts >50 µg total RNA, scale up the reaction linearly to the appropriate volume.
- Optical density measurement of labeled cDNA is not recommended.
- Do not perform an RNA degradation step following the labeling procedure.
- The protocol is optimized using oligo-dT primers. If you intend to use other primers, please refer to Appendix D.

## A) Preparation of dNTP Mix U

**Note:** The total volume of dNTP Mix U (64 µl or 260 µl) is sufficient for 12 or 50 labeling reactions respectively (depending on kit size). If using different cDNA labeling protocols with the LabelStar Array Kit ensure that appropriate aliquots of dNTP Mix U are prepared. After use, store the remaining dNTP Mix U in the LabelStar Array Kit cDNA Labeling Module at –20°C.

1. **Thaw dATP, dCTP, dTTP, and dGTP solution on ice. Mix each solution, for example, by vortexing. Centrifuge briefly and keep on ice.**
2. **Prepare the dNTP Mix U (Table 5) on ice (box 1 of 2), in the tube provided. Mix and keep the tube on ice.**

**Table 5. Preparation of dNTP Mix U using biotin-dUTP, cyanine 3-dUTP, or cyanine 5-dUTP**

Component	Volume (12 reactions)	Volume (50 reactions)	Final concentration
dATP (20 mM)	16.0 $\mu$ l	65 $\mu$ l	5 mM
dGTP (20 mM)	16.0 $\mu$ l	65 $\mu$ l	5 mM
dCTP (20 mM)	16.0 $\mu$ l	65 $\mu$ l	5 mM
dTTP (20 mM)	2.5 $\mu$ l	10 $\mu$ l	0.8 mM
RNase-free water	13.5 $\mu$ l	55 $\mu$ l	–
<b>Total volume*</b>	<b>64.0 <math>\mu</math>l</b>	<b>260 <math>\mu</math>l</b>	

\* Be sure that everything is mixed well by vortexing.

## B) Protocol for cDNA labeling

1. Thaw the template RNA solution, LabelStar Reverse Transcriptase, dNTP Mix U (see Table 5), RNase inhibitor, and labeled dUTP (1 mM) on ice. All other components, oligo-dT primer solution, 10x Buffer RT, Denaturation Solution Plus, and RNase-free water should be thawed at room temperature, mixed, and kept on ice.

**Note:** Biotin-, cyanine 3-, and cyanine 5-labeled dUTP (1 mM) are not provided with this kit. For recommended sources of modified nucleotides see “Solutions and Reagents to Be Supplied by User”, page 8.

**Note:** dNTPs and labeled nucleotides are labile reagents. Freeze nucleotides immediately.

2. Adjust the volume of your RNA solution to a total volume of 18  $\mu$ l with RNase-free water.
3. Add 2  $\mu$ l Denaturation Solution Plus to the 18  $\mu$ l RNA solution. Mix and centrifuge briefly.
4. Incubate at 65°C for 5 min and cool down on ice immediately. Centrifuge briefly.
5. For each reaction, prepare on ice a fresh master mix according to Table 6 (next page). Mix thoroughly and carefully by vortexing. Centrifuge briefly and store on ice.

The master mix contains everything except the denatured template RNA.

**Note:** Be sure to mix the master mix thoroughly before adding the denatured template RNA.

6. Add 20  $\mu$ l of denatured template RNA (steps 3 and 4) to the individual tubes containing the master mix. Mix and centrifuge briefly.

**Note:** Be sure that everything is mixed well by vortexing.

7. Incubate for 120 min at 37°C.
8. Add 2  $\mu$ l of Stop Solution LS to the individual tubes. Mix and centrifuge briefly.

**Note:** RNA degradation step is not needed.

## 9. Proceed to the purification protocol on page 30.

For optimal sensitivity and background, use the LabelStar Cleanup Module (box 2 of 2). If purification will not be performed immediately, store tubes at  $-20^{\circ}\text{C}$ .

**Table 6. Master mix for cDNA labeling using biotin-, cyanine 3-, or cyanine 5-labeled dUTP**

Component	Volume/reaction	Final concentration
<b>Master mix</b>		
10x Buffer RT	5.0 $\mu\text{l}$	1x
dNTP Mix U (prepared in protocol section A)	5.0 $\mu\text{l}$	0.5 mM for dATP, dCTP, dGTP and 0.08 mM for dTTP*
Biotin-, cyanine 3-, or cyanine 5-labeled dUTP (1 mM) <sup>†</sup>	1.0 $\mu\text{l}$	0.02 mM
Oligo-dT primer (20 $\mu\text{M}$ )	5.0 $\mu\text{l}$	2 $\mu\text{M}$
RNase inhibitor (40 units/ $\mu\text{l}$ )	0.5 $\mu\text{l}$	20 units (per 50 $\mu\text{l}$ reaction)
RNase-free water	11.0 $\mu\text{l}$	–
LabelStar Reverse Transcriptase	2.5 $\mu\text{l}$	–
<b>Template RNA</b>		
Denatured template RNA, added in step 6	20.0 $\mu\text{l}$	Up to 50 $\mu\text{g}$ <sup>‡</sup> (per 50 $\mu\text{l}$ reaction)
<b>Total labeling reaction volume<sup>§</sup></b>	<b>50.0 <math>\mu\text{l}</math></b>	<b>–</b>

\* Optimal labeling of cDNA is obtained by using the recommended ratio of labeled dUTP to unlabeled dTTP.

<sup>†</sup> In general, labeled dNTPs are provided in aliquots of 25  $\mu\text{l}$  at a concentration of 1 mM. This corresponds to an amount of 25 nmol of labeled dNTPs.

<sup>‡</sup> This amount corresponds to the total amount of RNA present, including any rRNA, mRNA, viral RNA, and carrier RNA present.

<sup>§</sup> Be sure that everything is mixed well by vortexing.

## Protocol for Indirect Labeling of cDNA with Aminoallyl-dUTP

This is the standard protocol for indirect labeling of cDNA with aminoallyl-dUTP, using 0.2–50 µg RNA. The amount of RNA corresponds to the total amount of RNA present, including any rRNA, mRNA, viral RNA, or carrier RNA present.

### Important notes before starting

- **Do not use the Denaturation Solution Plus provided with the kit. Denature RNA in steps 2 and 3 using RNase-free water.**
- Set up all reactions on ice to avoid premature cDNA synthesis and minimize the risk of RNA degradation.
- Be sure that everything is mixed well by vortexing.
- Ensure that this protocol is suitable for the modified nucleotide that you are using (see Table 2, page 12).
- The protocol is optimized for use with 0.2–50 µg total RNA or 0.2–5 µg mRNA. For amounts >50 µg total RNA, scale up the reaction linearly to the appropriate volume.
- Optical density measurements of labeled cDNA is not recommended.
- Do not perform an RNA degradation step following the labeling procedure.
- The protocol is optimized using oligo-dT primers. If you intend to use other primers, please refer to Appendix D.

### A) Preparation of dNTP Mix aa

**Note:** The total volume of dNTP Mix aa (64 µl or 260 µl) is sufficient for 12 or 50 labeling reactions respectively (depending on kit size). If using different cDNA labeling protocols with the LabelStar Array Kit ensure that appropriate aliquots of dNTP Mix aa are prepared. After use, store the remaining dNTP Mix aa in the LabelStar Array Kit cDNA Labeling Module at –20°C.

1. **Thaw dATP, dCTP, and dGTP solution on ice. Mix each solution, for example, by vortexing, centrifuge briefly, and keep them on ice.**
2. **Prepare a 20 mM aminoallyl-dUTP solution by dissolving 5 mg (3-aminoallyl)-2'-dUTP in 478 µl RNase-free 10 mM Tris-Cl, pH 8.5.**
3. **Prepare the dNTP Mix aa (Table 7) on ice, in the tube provided. Mix and keep the tube on ice.**

**Table 7. Preparation of dNTP Mix aa using aminoallyl-dUTP**

<b>Component</b>	<b>Volume (12 reactions)</b>	<b>Volume (50 reactions)</b>	<b>Final concentration</b>
dATP (20 mM)	16 µl	65 µl	5 mM
dGTP (20 mM)	16 µl	65 µl	5 mM
dCTP (20 mM)	16 µl	65 µl	5 mM
Aminoallyl-dUTP (20 mM)	16 µl	65 µl	5 mM
<b>Total volume*</b>	<b>64 µl</b>	<b>260 µl</b>	

The protocol uses a high concentration of aminoallyl-dUTP, the amount of which is optimized for the LabelStar procedure.

\* Be sure that everything is mixed well by vortexing.

## B) Protocol for cDNA labeling

1. Thaw the template RNA solution, LabelStar Reverse Transcriptase, dNTP Mix aa (see Table 7), and RNase inhibitor on ice. All other components, oligo-dT primer solution, 10x Buffer RT, and RNase free water should be thawed at room temperature, mixed, and kept on ice.

**Note:** Aminoallyl-dUTP is not provided with this kit. For recommended sources of modified nucleotides see “Solutions and Reagents to Be Supplied by User”, page 8.

**Note:** dNTPs and modified nucleotides are labile reagents. Freeze nucleotides immediately.

2. Adjust the volume of your RNA solution to a total volume of 20 µl with RNase-free water.
3. Incubate at 65°C for 5 min and cool down on ice immediately. Centrifuge briefly.
4. For each reaction, prepare on ice a fresh master mix according to Table 8. Mix thoroughly and carefully by vortexing. Centrifuge briefly and store on ice.

The master mix contains everything except the denatured template RNA.

**Note:** Be sure to mix the master mix thoroughly before adding the denatured template RNA.

5. Add 20 µl of denatured template RNA (step 3) to the individual tubes containing the master mix. Mix and centrifuge briefly.
 

**Note:** Be sure that everything is mixed well by vortexing.
6. Incubate for 120 min at 37°C.
7. Add 2 µl of Stop Solution LS to the individual tubes. Mix and centrifuge briefly.
 

**Note:** RNA degradation step is not needed.

**8. Proceed to the purification protocol on page 30.**

For optimal sensitivity and background, use the LabelStar Cleanup Module (box 2 of 2). If purification will not be performed immediately, store tubes at  $-20^{\circ}\text{C}$ .

**Note:** After purification of aminoallyl-cDNA and before hybridization carry out a procedure for aminoallyl coupling with monofunctional NHS-ester dyes according to the manufacturer's protocol.

**Table 8. Master mix for cDNA labeling using aminoallyl-dUTP**

<b>Component</b>	<b>Volume/reaction</b>	<b>Final concentration</b>
<b>Master mix</b>		
10x Buffer RT	5.0 $\mu\text{l}$	1x
dNTP Mix aa (prepared in protocol section A)	5.0 $\mu\text{l}$	0.5 mM for dATP, dCTP, dGTP, and Aminoallyl-dUTP
Oligo-dT primer (20 $\mu\text{M}$ )	5.0 $\mu\text{l}$	2 $\mu\text{M}$
RNase inhibitor (40 units/ $\mu\text{l}$ )	0.5 $\mu\text{l}$	20 units (per 50 $\mu\text{l}$ reaction)
RNase-free water	12.0 $\mu\text{l}$	–
LabelStar Reverse Transcriptase	2.5 $\mu\text{l}$	
<b>Template RNA</b>		
Denatured template RNA, added in step 5	20.0 $\mu\text{l}$	Up to 50 $\mu\text{g}^*$ (per 50 $\mu\text{l}$ reaction)
<b>Total labeling reaction volume<sup>†</sup></b>	<b>50.0 <math>\mu\text{l}</math></b>	–

\* This amount corresponds to the total amount of RNA present, including any rRNA, mRNA, viral RNA, and carrier RNA present.

† Be sure to mix everything well by vortexing.

# Protocol for Direct Labeling of cDNA with <sup>32</sup>P- or <sup>33</sup>P-Labeled dCTP Using mRNA as a Template

This is the standard protocol for direct labeling of cDNA with <sup>32</sup>P- or <sup>33</sup>P-labeled dCTP, using 0.2–5 µg mRNA.

## Important notes before starting

- The final concentration of dCTP in the dNTP Mix P1 depends on the amount of mRNA used for the labeling reaction. See Table 9 for the amount of 1:10 diluted dCTP (20 mM) to add to dNTP Mix P.
- Set up all reactions on ice to avoid premature cDNA synthesis and minimize the risk of RNA degradation.
- Be sure that everything is mixed well by vortexing.
- Ensure that this protocol is suitable for the modified nucleotide that you are using (see Table 2, page 12).
- The protocol is optimized for use with 0.2–5 µg mRNA. For amounts >5 µg total RNA, scale up the reaction linearly to the appropriate volume.
- Take appropriate safety measures when working with radioactive isotopes.
- Do not perform an RNA degradation step following the labeling procedure.
- The protocol is optimized using oligo-dT primers. If you intend to use other primers, please refer to Appendix D.

## A) Preparation of dNTP Mix P1

**Note:** The total volume of dNTP Mix P1 (64 µl or 260 µl) is sufficient for 12 or 50 labeling reactions respectively (depending on kit size). If using different cDNA labeling protocols with the LabelStar Array Kit ensure that appropriate aliquots of dNTP Mix P1 are prepared. After use, store the remaining dNTP Mix P1 in the LabelStar Array Kit cDNA Labeling Module at –20°C.

1. **Thaw dATP, dCTP, dTTP, and dGTP solution on ice. Mix each solution, for example, by vortexing. Centrifuge briefly, and keep on ice.**
2. **Prepare a 1:10 dilution of dCTP (20 mM) (i.e., dilute 3 µl dCTP [20 mM] with 27 µl RNase-free water) to give a 2 mM solution.**
3. **Prepare the dNTP Mix P1 (Table 9) on ice in the tube provided. Add diluted dCTP to dNTP Mix P1 according to the volumes given in Table 10. Mix and keep the tube on ice.**

**Table 9. Preparation of dNTP Mix P1 using mRNA as a template**

<b>Component</b>	<b>Volume (12 reactions)</b>	<b>Volume (50 reactions)</b>	<b>Final concentration</b>
dATP (20 mM)	16 µl	65 µl	5 mM
dGTP (20 mM)	16 µl	65 µl	5 mM
dTTP (20 mM)	16 µl	65 µl	5 mM
dCTP*	Varies	Varies	Varies
RNase-free water	Make volume up to 64 µl	Make volume up to 260 µl	
<b>Total volume†</b>	<b>64 µl</b>	<b>260 µl</b>	

\* Concentration and volume of dCTP added varies. See Table 10.

† Be sure that everything is mixed well by vortexing.

**Table 10. Addition of diluted dCTP (2 mM) to dNTP Mix P1 if reverse transcribing mRNA**

<b>mRNA (µg)</b>	<b>Volume of dCTP (2 mM) added to dNTP Mix P1 (12 reactions)</b>	<b>Volume of dCTP (2 mM) added to dNTP Mix P1 (50 reactions)</b>
5	5.0 µl	21.0 µl
4	4.0 µl	16.5 µl
3	3.0 µl	12.5 µl
2	2.0 µl	8.5 µl
1	1.0 µl	4.0 µl
0.5	0.5 µl	2.0 µl
0.2	0.2 µl	1.0 µl

## B) Protocol for cDNA labeling

1. Thaw the template RNA solution, LabelStar Reverse Transcriptase, dNTP Mix P1 (see Table 9), and RNase inhibitor on ice. All other components, oligo-dT primer solution, 10x Buffer RT, Denaturation Solution Plus, <sup>32</sup>P- or <sup>33</sup>P-labeled dCTP (2500–3000 Ci/mmol), and RNase-free water should be thawed at room temperature, mixed, and kept on ice.

**Note:** <sup>32</sup>P- or <sup>33</sup>P-labeled dCTP (2500–3000 Ci/mmol) is not provided with this kit. For recommended sources of modified nucleotides see “Solutions and Reagents to Be Supplied by User”, page 8.

**Note:** dNTPs and modified nucleotides are labile reagents. Freeze nucleotides immediately.

2. Adjust the volume of your mRNA solution to a total volume of 18 µl using RNase-free water.
3. Add 2 µl Denaturation Solution Plus to the 18 µl mRNA solution, mix, and centrifuge briefly.
4. Incubate at 65°C for 5 min and cool down on ice immediately. Centrifuge briefly.
5. For each reaction, prepare on ice a fresh master mix according to Table 11. Mix thoroughly and carefully by vortexing. Centrifuge briefly and store on ice.

The master mix contains everything except the denatured template RNA.

**Note:** Be sure to mix the master mix thoroughly before adding the denatured template RNA.

6. Add 20 µl of denatured template mRNA (steps 3 and 4) to the individual tubes containing the master mix, and centrifuge briefly.

**Note:** Be sure that everything is mixed well by vortexing

7. Incubate for 120 min at 37°C.
8. Add 2 µl of Stop Solution LS to the individual tubes. Mix and centrifuge briefly.

**Note:** RNA degradation step is not needed.

9. Proceed to the purification protocol on page 30.

For optimal sensitivity and background, use the LabelStar Cleanup Module (box 2 of 2). If purification will not be performed immediately, store tubes at –20°C.

**Table 11. Master mix for cDNA labeling using <sup>32</sup>P- or <sup>33</sup>P-labeled dCTP**

<b>Component</b>	<b>Volume/reaction</b>	<b>Final concentration</b>
<b>Master mix</b>		
10x Buffer RT	5.0 µl	1x
dNTP Mix P1 (prepared in protocol section A)	5.0 µl	0.5 mM for dATP, dTTP, dGTP and variable for dCTP*
<sup>32</sup> P- or <sup>33</sup> P-labeled dCTP (2500–3000 Ci/mmol)	5.0 µl	50 µCi (per 50 µl reaction)
Oligo-dT primer (20 µM)	5.0 µl	2 µM
RNase inhibitor (40 units/µl)	0.5 µl	20 units (per 50 µl reaction)
RNase-free water	7.0 µl	–
LabelStar Reverse Transcriptase	2.5 µl	
<b>Template RNA</b>		
Denatured template RNA, added in step 6	20.0 µl	Up to 5 µg (per 50 µl reaction)
<b>Total labeling reaction volume<sup>†</sup></b>	<b>50.0 µl</b>	<b>–</b>

\* Optimal labeling of cDNA is obtained by using the recommended ratio of radioactively labeled dCTP to unlabeled dCTP.

<sup>†</sup> Be sure to mix everything well by vortexing.

# Protocol for Direct Labeling of cDNA with <sup>32</sup>P- or <sup>33</sup>P-Labeled dCTP Using Total RNA as a Template

This is the standard protocol for direct labeling of cDNA with <sup>32</sup>P- or <sup>33</sup>P-labeled dCTP, using 0.2–50 µg total RNA. Total RNA corresponds to the total amount of RNA present, including any rRNA, mRNA, viral RNA, or carrier RNA present.

## Important notes before starting

- The final concentration of dCTP in the dNTP Mix P2 depends on the amount of RNA used for the labeling reaction. See Table 12 for the amount of 1:100 diluted dCTP (20 mM) to add to dNTP Mix P2.
- Set up all reactions on ice to avoid premature cDNA synthesis and minimize the risk of RNA degradation.
- Be sure that everything is mixed well by vortexing.
- Ensure that this protocol is suitable for the modified nucleotide that you are using (see Table 2, page 12).
- The protocol is optimized for use with 0.2–50 µg total RNA. For amounts >50 µg total RNA, scale up the reaction linearly to the appropriate volume.
- Take appropriate safety measures when working with radioactive isotopes.
- Do not perform an RNA degradation step following the labeling procedure.
- The protocol is optimized using oligo-dT primers. If you intend to use other primers, please refer to Appendix D.

## A) Preparation of dNTP Mix P2

**Note:** The total volume of dNTP Mix P2 (64 µl or 260 µl) is sufficient for 12 or 50 labeling reactions respectively (depending on kit size). If using different cDNA labeling protocols with the LabelStar Array Kit ensure that appropriate aliquots of dNTP Mix P2 are prepared. After use, store the remaining dNTP Mix P2 in the LabelStar Array Kit cDNA Labeling Module at –20°C.

1. **Thaw dATP, dCTP, dTTP, and dGTP solution on ice. Mix each solution, for example, by vortexing, centrifuge briefly, and keep on ice.**
2. **Prepare a 1:100 dilution of dCTP (20 mM) (i.e., dilute 2 µl dCTP [20 mM] with 198 µl RNase-free water) to give a 0.2 mM solution**
3. **Prepare the dNTP Mix P2 (Table 12) on ice in the tube provided. Mix and keep the tube on ice. Add diluted dCTP to dNTP Mix P2 according to the volumes given in Table 13.**

**Note:** If using <5 µg total RNA for the reverse transcription no unlabeled dCTP is added to dNTP Mix P2.

**Table 12. Preparation of dNTP Mix P using <sup>32</sup>P- or <sup>33</sup>P-labeled dCTP using total RNA as a template**

Component	Volume (12 reactions)	Volume (50 reactions)	Final concentration
dATP (20 mM)	16 µl	65 µl	5 mM
dGTP (20 mM)	16 µl	65 µl	5 mM
dTTP (20 mM)	16 µl	65 µl	5 mM
dCTP*	Varies	Varies	Varies
RNase-free water	Make volume up to 64 µl	Make volume up to 260 µl	
<b>Total volume†</b>	<b>64 µl</b>	<b>260 µl</b>	

\* Concentration and volume of dCTP added varies. See Table 12.

† Be sure that everything is mixed well by vortexing.

**Table 13. Addition of diluted dCTP (0.2 mM) to dNTP Mix P2 if reverse transcribing total RNA**

Total RNA (µg)	Volume of diluted dCTP (0.2 mM) added to dNTP Mix P2 (12 reactions)	Volume of diluted dCTP (0.2 mM) added to dNTP Mix P2 (50 reactions)
50	12 µl	50.0 µl
25	6 µl	25.0 µl
20	5 µl	21.0 µl
15	4 µl	16.5 µl
10	2 µl	8.0 µl
5	1 µl	4.0 µl
<5	0 µl	0 µl

## B) Protocol for cDNA labeling

1. Thaw the template RNA solution, LabelStar Reverse Transcriptase, dNTP Mix P2 (see Table 12, page 27), and RNase inhibitor on ice. All other components, oligo-dT primer solution, 10x Buffer RT, Denaturation Solution Plus, <sup>32</sup>P- or <sup>33</sup>P-labeled dCTP (2500–3000 Ci/mmol), and RNase-free water, should be thawed at room temperature, mixed, and kept on ice.

**Note:** <sup>32</sup>P- or <sup>33</sup>P-labeled dCTP (2500–3000 Ci/mmol) is not provided with this kit. For recommended sources of modified nucleotides by see “Solutions and Reagents to Be Supplied by User”, page 8.

**Note:** dNTPs and modified nucleotides are labile reagents. Freeze nucleotides immediately.

2. Adjust the volume of your RNA solution to a total volume of 18 µl using RNase-free water.
3. Add 2 µl Denaturation Solution Plus to the 18 µl RNA solution. Mix and centrifuge briefly.
4. Incubate at 65°C for 5 min and cool down on ice immediately. Centrifuge briefly.
5. For each reaction, prepare on ice a fresh master mix according to Table 14. Mix thoroughly and carefully by vortexing. Centrifuge briefly and store on ice.

The master mix contains everything except the denatured template RNA.

**Note:** Be sure to mix the master mix thoroughly before adding the denatured template RNA.

6. Add 20 µl of denatured template RNA (step 3 and 4) to the individual tubes containing the master mix, and centrifuge briefly.

**Note:** Be sure that everything is mixed well by vortexing.

7. Incubate for 120 min at 37°C.
8. Add 2 µl of Stop Solution LS to the individual tubes. Mix and centrifuge briefly.

**Note:** RNA degradation step is not needed.

9. Proceed to the purification protocol on page 30.

For optimal sensitivity and background, use the LabelStar Cleanup Module (box 2 of 2). If purification will not be performed immediately, store tubes at –20°C.

**Table 14. Master mix for cDNA labeling using <sup>32</sup>P- or <sup>33</sup>P-labeled dCTP and total RNA**

<b>Component</b>	<b>Volume/reaction</b>	<b>Final concentration</b>
<b>Master mix</b>		
10x Buffer RT	5.0 µl	1x
dNTP Mix P2 (prepared in protocol section A)	5.0 µl	0.5 mM for dATP, dTTP, dGTP and variable for dCTP*
<sup>32</sup> P- or <sup>33</sup> P-labeled dCTP (2500–3000 Ci/mmol)	5.0 µl	50 µCi (per 50 µl reaction)
Oligo-dT primer (20 µM)	5.0 µl	2 µM
RNase inhibitor (40 units/µl)	0.5 µl	20 units (per 50 µl reaction)
RNase-free water	7.0 µl	–
LabelStar Reverse Transcriptase	2.5 µl	
<b>Template RNA</b>		
Denatured template RNA, added in step 6	20.0 µl	Up to 50 µg <sup>†</sup> (per 50 µl reaction)
<b>Total labeling reaction volume<sup>‡</sup></b>	<b>50.0 µl</b>	<b>–</b>

\* Optimal labeling of cDNA is obtained by using the recommended ratio of radioactively labeled dCTP to unlabeled dCTP.

<sup>†</sup> This amount corresponds to the total amount of RNA present, including any rRNA, mRNA, viral RNA, and carrier RNA present.

<sup>‡</sup> Be sure that everything is mixed well by vortexing.

# Protocol for Purification of Labeled cDNA

## Using a Microcentrifuge

### Important notes before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at  $>10,000 \times g$  (13,000 rpm) in a conventional tabletop centrifuge.
- Ensure that reactions are mixed well by vortexing.
- For users performing dual-color analysis: pooling of dual-color labeled cDNA prior to purification is possible if the amount of RNA used per individual reaction was less than 25  $\mu\text{g}$ . If  $>25 \mu\text{g}$  RNA is used per reaction, purify dual-color labeled cDNAs separately using two MinElute spin columns.
- Take appropriate safety measures when working with radioactive isotopes.

1. **Add 260  $\mu\text{l}$  Buffer PB to the total cDNA labeling reaction and mix by gentle vortexing.**  
**Note:** Add 520  $\mu\text{l}$  if two reactions are pooled for purification.
2. **Place a MinElute spin column in a 2 ml collection tube.**
3. **To bind cDNA, apply the sample to the MinElute spin column and centrifuge for 1 min.**
4. **Discard the flow-through fraction and replace the MinElute spin column in the same 2 ml collection tube.**

**IMPORTANT:** If using radioactively labeled modified nucleotides, use a new tube for each step and dispose of radioactive waste according to your institution's safety regulations.

5. **To wash, add 0.75 ml Buffer LS to the MinElute spin column and centrifuge for 1 min.**  
**Note:** If cleaning up radioactively labeled cDNA, use 0.7 ml Buffer LS.
6. **Discard the flow-through and place the MinElute spin column back in the same 2 ml collection tube.**
7. **To wash, add 0.75 ml Buffer PE to the MinElute spin column and centrifuge for 1 min.**  
**Note:** If cleaning up radioactively labeled cDNA, use 0.7 ml Buffer PE.  
**Note:** Buffer PE is supplied as a concentrate. Ensure that ethanol is added before use (see "Important notes before starting").
8. **Discard the flow-through. Place the MinElute spin column back in the same 2 ml collection tube and centrifuge for an additional 1 min at maximum speed.**
9. **Place the MinElute spin column in a clean 1.5 ml microcentrifuge tube.**

10. To elute the cDNA, add 10  $\mu$ l Buffer EB (10 mM Tris-Cl, pH 8.5) to the center of the MinElute spin column membrane. Wait for 1 min, and centrifuge the column for 1 min.

**Note:** Ensure that the elution buffer is dispensed directly onto the MinElute spin column membrane for complete elution of bound cDNA. The average eluate volume is 9  $\mu$ l from 10  $\mu$ l elution buffer.

**IMPORTANT:** Buffer EB should be used for elution in all cases, including those where dye-coupling to aminoallyl-cDNA will be performed after purification.

11. **Optional: Repeat the elution with a further 10  $\mu$ l elution buffer. If labeling was performed using >25  $\mu$ g RNA, this second elution step is recommended for increased recovery.**
12. **Proceed with hybridization of labeled cDNA to the array or with dye-coupling using NHS-esters (if aminoallyl-dUTP was used).**

**Note:** Purified labeled cDNA is ready for hybridization to arrays. Proceed with a typical hybridization protocol. If necessary, pool cDNAs that were labeled with cyanine-3 and cyanine-5. Precipitation or drying of labeled cDNA may be necessary to obtain a smaller volume prior to hybridization. Mix labeled cDNA with hybridization solution and follow your standard procedure.

# Troubleshooting Guide

## Comments and suggestions

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### Little or no signal

#### I) Chip scanning

- |    |   |  |
|----|---|--|
| a) | Incorrect channel for excitation and emission | Chip scanning of dye-labeled cDNA should be performed at an excitation wavelength and emission wavelength suitable for the dye.  |
| b) | Incorrect excitation time                     | Check the correct excitation time before scanning the chip. Increase excitation time.  |
| c) | Incorrect location of the array on the slide  | Check the location of the array on the slide prior to scanning.  |
| d) | Slide not in focus                            | Check the focus of your scanner or reader. Be sure to scan the (bottom or top) side in focus. Change side of slide for scanning. |

#### II) Chip hybridization and washing

- |    |   |   |
|----|---|---|
| a) | cDNA is lost                              | Often cDNA and competitor DNA are coprecipitated prior to hybridization. If you carry out such a coprecipitation, ensure that the pellet is not lost during decanting of the ethanol solution.                |
| b) | Insufficient denaturation of labeled cDNA | Insufficient denaturation of labeled cDNA may reduce signal intensity. Increase denaturation temperature and/or denaturation time. We have found that denaturation for 10 min at 95°C gives the best results. |
| c) | Temperature too high                      | Check temperature during chip hybridization and washing with a calibrated thermometer. Decrease temperature for chip hybridization and/or washing.  |
| d) | Inappropriate salt conditions             | Be sure to set up hybridization and washing solutions using the correct salt concentrations. Increase salt concentration for chip hybridization and/or washing.   |

**III) cDNA-labeling and purification**

- |  |  |
|--|--|
| a) Reaction setup                                      | Be sure to set up the reaction on ice. Be sure that everything is mixed well by vortexing.   |
| b) Temperature of reaction                             | Reverse transcription should be carried out at 37°C. Check the temperature of your heating block or water bath. In rare cases, when analyzing RNAs with a very high degree of secondary structure, it may be advantageous to increase the temperature to 42°C.   |
| c) Pipetting error or missing reagent                  | Calibrate the pipets used for experimental setup. Mix all reagents well after thawing, store on ice immediately after thawing, and repeat reverse transcription reaction.  |
| d) Nucleotide concentration                            | Be sure that the concentration of labeled and non-labeled nucleotides are as recommended. After preparing the dNTP mix, mix well by vortexing, and centrifuge briefly.   |
| e) Poor quality or wrong quantity of starting template | Check the concentration, integrity, and purity of starting template (see "Appendix B: Storage, Quantification, and Determination of Quality of RNA", page 38). Mix well after thawing the RNA template, and use RNase inhibitor at a final concentration of 0.4 U/µl in the assay. Even minute amounts of RNases can affect the length of cDNA-synthesis products, especially when using small amounts of RNA.<br><br>Denature RNA using Denaturation Solution Plus at 65°C for 5 min to get rid of inhibitors, except if cDNA labeling is performed with aminoallyl-dUTP. |
| f) RNA concentration too high or too low               | The LabelStar Array Kit is designed for use with 0.2–50 µg total RNA. This amount corresponds to the total amount of RNA present, including any rRNA, mRNA, viral RNA, and carrier RNA present. When using >50 µg RNA, scale up the reaction linearly to the appropriate volume.   |

## Comments and suggestions

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- g) Incorrect primers Use the oligo-dT primer provided in the kit at the recommended concentration. If using primers other than the oligo-dT primer provided, please refer to Appendix D.
- h) Incorrect nucleotide concentration/degradation Use the dNTP solutions provided in the kit. Different nucleotide concentrations can reduce the amount of cDNA product. Storage of nucleotides at room temperature will cause degradation of the nucleotides.
- i) Incorrect denaturation conditions Denature the RNA–primer mix using Denaturation Solution Plus provided with the kit, except if cDNA labeling is performed with aminoallyl-dUTP. In this case, denature RNA in RNase-free water provided with the kit. High denaturation temperatures (>65°C) or prolonged denaturation time (>5 min) can affect the integrity of RNA, causing shortened cDNA products.
- j) Two phases are present in the reaction In rare cases two phases are observable after adding denatured RNA to the master mix. Ensure that the reaction is mixed well by vortexing prior to incubation of the reaction mix.
- k) Incubation time too short The standard labeling reaction requires a 120 minute incubation. Shorter incubation times may reduce the yield of labeled cDNA and sensitivity of analysis.
- l) Buffer PE did not contain ethanol Ethanol must be added to Buffer PE (concentrate) before use. Repeat procedure with correctly prepared Buffer PE.
- m) Inappropriate elution buffer DNA will only be eluted efficiently in the presence of low-salt buffer (e.g., Buffer EB: 10 mM Tris·Cl, pH 8.5) or water.
- n) Elution buffer incorrectly dispensed Add elution buffer to the center of the MinElute membrane to ensure that the buffer completely covers the membrane.
- o) Salt concentration in eluate too high Modify the wash step by incubating the column for 5 min at room temperature after adding 750 µl of Buffer PE, then centrifuge.

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## Comments and suggestions

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- p) Eluate contains residual ethanol

Ensure that the wash flow-through is drained from the collection tube and that the MinElute column is then centrifuged at  $10,000 \times g$  (~13,000 rpm) for an additional 1 min.

### Unspecific signal and/or background signal

#### I) Chip scanning

- Incorrect excitation time

Check the correct excitation time before scanning chip. Increase excitation time.

#### II) Chip hybridization and washing

- a) Competitor DNA absent

In some systems competitor DNA is necessary. Ensure that you have added competitor DNA before hybridization if required.

- b) Temperature too low

Check temperature during chip hybridization and washing with a calibrated thermometer. Increase temperature used for chip hybridization and/or washing.

- c) Inappropriate salt conditions

Be sure to set up hybridization and washing solutions using the correct salt concentrations. Increase salt concentration for chip hybridization and/or washing.

#### III) cDNA labeling

- a) Stopping the labeling reaction

Be sure that Stop Solution LS has been used to stop the labeling reaction.

- b) Salt concentration in eluate too high

Modify the wash step by incubating the column for 5 min at room temperature after adding 750  $\mu$ l of Buffer PE, then centrifuge.

- c) Eluate contains residual ethanol

Ensure that the wash flow-through is drained from the collection tube and that the MinElute column is then centrifuged at  $10,000 \times g$  (~13,000 rpm) for an additional 1 min.

# Appendix A: General Remarks on Handling RNA

## Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to degrade RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the isolation procedure. In order to create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and non-disposable vessels and solutions while working with RNA.

## General handling

Proper microbiological, aseptic techniques should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible.

## Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

## Non-disposable plasticware

Non-disposable plasticware should be treated before use to ensure that it is RNase-free. Plasticware should be thoroughly rinsed with 0.1 M NaOH, 1 mM EDTA followed by RNase-free water (see "Solutions", next page). Alternatively, chloroform-resistant plasticware can be rinsed with chloroform to inactivate RNases.

## Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent, thoroughly rinsed, and oven baked at 240°C for four or more hours (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC\* (diethyl pyrocarbonate). Fill glassware with 0.1% DEPC (0.1% in water), allow to stand overnight (12 hours) at 37°C, and then autoclave or heat to 100°C for 15 minutes to eliminate residual DEPC.

## Electrophoresis tanks

Electrophoresis tanks should be cleaned with detergent solution (e.g., 0.5% SDS), thoroughly rinsed with RNase-free water, and then rinsed with ethanol† and allowed to dry.

## Solutions

Solutions (water and other solutions) should be treated with 0.1% DEPC.\* DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO<sub>2</sub>. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carboxymethylation. Carboxymethylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

**Note:** QIAGEN LabelStar buffers and RNase-free water are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

\* DEPC is a suspected carcinogen and should be handled with great care. Wear gloves and use a fume hood when using this chemical.

† Plastics used for some electrophoresis tanks are not resistant to ethanol. Take proper care and check the supplier's instructions.

# Appendix B: Storage, Quantification, and Determination of Quality of RNA

## Storage of RNA

Purified RNA can be stored at  $-20^{\circ}\text{C}$  or  $-70^{\circ}\text{C}$  in water.

## Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm ( $A_{260}$ ) in a spectrophotometer. To ensure significance, readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 40  $\mu\text{g}$  of RNA per ml ( $A_{260}=1 \rightarrow 40 \mu\text{g/ml}$ ). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer at a neutral pH. As discussed below (see "Purity of RNA"), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1 M NaOH, 1 mM EDTA followed by washing with RNase-free water (see "Solutions", page 37). Use the buffer in which the RNA is diluted to zero the spectrophotometer.

An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100  $\mu\text{l}$

Dilution = 20  $\mu\text{l}$  of RNA sample + 180  $\mu\text{l}$  distilled water (1/10 dilution).

Measure absorbance of diluted sample in a 0.2 ml cuvette (RNase-free).

$A_{260} = 0.2$

Concentration of RNA sample =  $40 \mu\text{g/ml} \times A_{260} \times \text{dilution factor}$

=  $40 \mu\text{g/ml} \times 0.2 \times 10$

= 80  $\mu\text{g/ml}$

Total amount = concentration  $\times$  volume of sample in ml

=  $80 \mu\text{g/ml} \times 0.1 \text{ ml}$

= 8  $\mu\text{g}$  of RNA

## Purity of RNA

The ratio of the readings at 260 nm and 280 nm ( $A_{260}/A_{280}$ ) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. However, the  $A_{260}/A_{280}$  ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting  $A_{260}/A_{280}$  ratio can vary greatly. Lower pH results in a lower  $A_{260}/A_{280}$  ratio and reduced sensitivity to protein contamination.\* For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5. Pure RNA has an  $A_{260}/A_{280}$  ratio of 1.9–2.1† in 10 mM Tris·Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution.

For determination of RNA concentration, however, we still recommend dilution of the sample in a buffer at a neutral pH since the relationship between absorbance and concentration ( $A_{260}$  reading of 1 = 40 µg/ml RNA) is based on an extinction coefficient calculated for RNA in water (see “Quantification of RNA”).

## Integrity of RNA

The integrity and size distribution of total RNA can be checked by denaturing agarose gel electrophoresis and ethidium bromide staining (see “Appendix C: Protocol for Formaldehyde Agarose Gel Electrophoresis”, page 40). The respective ribosomal bands (Table 15) should appear as sharp bands on the stained gel. Ribosomal RNA bands of 28S should be present at approximately twice the amounts of the 18S RNA band. If the ribosomal bands in a given lane are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the RNA sample suffered major degradation during preparation.

**Table 15. Size of ribosomal RNAs from various sources**

Source	rRNA	Size (kb)
Mouse	18S	1.9
	28S	4.7
Human	18S	1.9
	28S	5.0

\* Wilfinger, W.W., Mackey, M., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *BioTechniques* **22**, 474.

† Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris·Cl, pH 7.5) with some spectrophotometers.

## Appendix C: Protocol for RNA Formaldehyde Agarose Gel Electrophoresis

The following protocol for formaldehyde agarose (FA) gel electrophoresis is routinely used at QIAGEN and gives enhanced sensitivity for gel and subsequent analysis (e.g., northern blotting). A key feature is the concentrated RNA loading buffer that allows a larger volume of RNA sample to be loaded onto the gel than conventional protocols (e.g., Sambrook et al., eds. (1989) *Molecular cloning — a laboratory manual*, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

### FA gel preparation

To prepare FA gel (1.2% agarose) of size 10 x 14 x 0.7 cm, mix:

1.2 g            agarose  
10 ml            10x FA gel buffer (see composition below)  
add RNase-free water to 100 ml

If smaller or larger gels are needed, adjust the quantities of components proportionately.

Heat the mixture to melt agarose. Cool to 65°C in a water bath. Add 1.8 ml of 37% (12.3 M) formaldehyde\* and 1 µl of a 10 mg/ml ethidium bromide\* stock solution. Mix thoroughly and pour onto gel support. Prior to running the gel, equilibrate in 1x FA gel running buffer (see composition below) for at least 30 min.

### RNA sample preparation for FA gel electrophoresis

Add 1 volume of 5x RNA loading buffer (see composition below) to 4 volumes of RNA sample (for example 10 µl of loading buffer and 40 µl of RNA) and mix.

Incubate for 3–5 min at 65°C, chill on ice, and load onto the equilibrated FA gel.

\* Toxic and/or mutagenic. Take appropriate safety measures.

## Gel running conditions

Run gel at 5–7 V/cm in 1x FA gel running buffer.

Composition of FA gel buffers

10x FA gel buffer

200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (free acid)

50 mM sodium acetate

10 mM EDTA

pH to 7.0 with NaOH

1x FA gel running buffer

100 ml 10x FA gel buffer

20 ml 37% (12.3 M) formaldehyde\*

880 ml RNase-free water

5x RNA loading buffer

16  $\mu$ l saturated aqueous bromophenol blue solution<sup>†</sup>

80  $\mu$ l 500 mM EDTA, pH 8.0

720  $\mu$ l 37% (12.3 M) formaldehyde\*

2 ml 100% glycerol

3.084 ml formamide

4 ml 10x FA gel buffer

RNase-free water to 10 ml

Stability: approximately 3 months at 4°C

\* Toxic and/or mutagenic. Take appropriate safety measures.

<sup>†</sup> To make a saturated solution, add solid bromophenol blue to distilled water. Mix and continue to add more bromophenol blue until no more will dissolve. Centrifuge to pellet the undissolved powder, and carefully pipet the saturated supernatant.

## Appendix D: Priming of RNA

The priming strategy has a strong impact on the cDNA labeling result and subsequent array analysis. The LabelStar Array Kit contains an oligo-dT primer. Oligo-dT primers select the fraction to be labeled as well as the 3' poly-A tail as a start point which is common to all primed mRNAs. There are three labeling strategies however which require specific or random primers.

- cDNA labeling of bacterial RNA — bacterial mRNA does not contain polyA tails therefore random or specific priming is needed during the labeling reaction.
- cDNA labeling of amplified RNA — depending on the RNA amplification scheme and RNA orientation, polyA tails are missing the amplified RNA, therefore specific or random primers are needed for the LabelStar procedure.
- Whole transcriptome labeling — for some applications it is necessary to label the complete transcriptome using random primers.

Using random primers, random octamer primers are recommended at a final concentration of 10  $\mu$ M in the reaction mixture. We do not recommend using random hexamers, because they lead to reduced cDNA synthesis. When using random octamers, use the setup and reaction temperatures given in the protocol.

Using specific primers, the final concentration needs to be optimized according to the length of the primer and target RNA.

## Ordering Information

Product	Contents	Cat. No.
LabelStar Array Kit (12)	For 12 labeling reactions: LabelStar Reverse Transcriptase, dNTPs, * RNase Inhibitor, Oligo-dT Primer, 12 MinElute Spin Columns, RNase-free Reagents, Buffers	28902
LabelStar Array Kit (50)	For 50 labeling reactions: LabelStar Reverse Transcriptase, dNTPs, * RNase Inhibitor, Oligo-dT Primer, 50 MinElute Spin Columns, RNase-free Reagents, Buffers	28904

\* 20 mM solutions each; labeled nucleotides to be supplied by user.

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## Notes

## Notes

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