GE Healthcare

Amersham CyScribe First-Strand cDNA Labeling Kit

Product Booklet

Codes: RPN6200
RPN6200X
RPN6201
RPN6201X
RPN6202
RPN6202X
27-9606-01
27-9606-02

Page Finder

1. Legal

Previously published 2006

http://www.gelifesciences.com

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2. Handling

2.1. Safety warnings and precautions

Warning: ***For research use only.*** Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.

2.2. Storage

Store CyScribe™ First-Strand cDNA Labeling Kit labeling components at -15°C to -30°C. **Do not store in a frost-free freezer.** Store CyScribe GFX™ Purification Kit at ambient temperature.

2.3. Stability

All components are stable for at least 3 months when stored under recommended conditions.

2.4. Expiry

For expiry details see outer packaging

3. Components

CyScribe First-Strand cDNA Labeling Kit RPN6200

- Anchored oligo(dT), 30 µl
- Random nonamer primers, 30 µl
- 5 x CyScribe buffer, 150 ml, 5 x concentrated buffer pH 8.3
- 0.1 M DTT, 75 µl aqueous solution of Dithiotreitol
- dCTP Nucleotide mix, 30 µl for labeling with CyDye™-dCTP
- dUTP Nucleotide mix, 30 µl for labeling with CyDye-dUTP
- CyScribe reverse transcriptase, 25 µl, in a buffer solution, pH 8.3 containing 50% Glycerol. See Material safety data sheet supplied
- Nuclease free water, 1 µl, water treated with Diethyl Pyrocarbonate (DEPC)
- Control mRNA, 6 µl, 1 mg/ml mixture of synthetic mRNAs of defined sizes (9.2, 7.5, 4.4, 2.4, 1.4 and 0.24 kilobases) in aqueous solution.
- Microarray hybridization buffer, 1 ml

CyScribe First-Strand cDNA Labeling Kit—dUTP RPN6201

- Two of RPN 6200 and in addition
- Cy3-dUTP, 25 nmol, 1.0 mM aqueous solution
- Cy5-dUTP, 25 nmol, 1.0 mM aqueous solution

CyScribe First-Strand cDNA Labeling Kit—dCTP RPN6202

- Two of RPN 6200 and in addition
- Cy3-dCTP, 25 nmol, 1.0 mM aqueous solution.
- Cy5-dCTP, 25 nmol, 1.0 mM aqueous solution

Reagents required for the purification of labeled cDNA are not provided with the kit.

RPN6200X, RPN6201X AND RPN6202X Contain the additional CyScribe GFX Purification Kit components as listed below:

RPN6200X
The additional CyScribe GFX Purification Kit supplied contains enough reagents for 25 purifications, 27-9606-01

- Capture buffer
- Wash buffer*
- Elution buffer
- GFX spin columns
- Collection tubes

*Absolute ethanol provided by the researcher is added immediately prior to use.

RPN6201X and RPN6202X
The additional CyScribe GFX Purification Kit supplied contains enough reagents for 50 purifications, 27-9606-02

- Capture buffer
- Wash buffer*
- Elution buffer
- GFX spin columns
- Collection tubes

*Absolute ethanol provided by the researcher is added immediately prior to use.

4. Other materials required

These materials are not provided with the CyScribe kit.

All reagents should be molecular biology grade and free of contaminating nucleases. Use distilled water for the preparation of solutions unless instructed otherwise.

For synthesis and purification of cDNA

- **Amber microcentrifuge tubes 1.5 ml**
  Eppendorf 0030 120.191
- **2.5 M NaOH**

For	10 ml
NaOH	1 g
Nuclease free water to	10 ml

  Sterilize by filtration with a 0.45 micron filter. Store at room temperature for up to 3 months.
- **2 M HEPES free acid**

For	10 ml
HEPES free acid	4.77 g
Nuclease free water to	10 ml

  HEPES US16926-100 g

  Sterilize by filtration with a 0.45 micron filter. Store at room temperature for up to 3 months.
- **illustra AutoSeq G-50 spin columns**
  GE Healthcare 27-5340-01
- Absolute ethanol

Equipment

- Microcentrafuge capable of generating a g-force of 13 800

For additional protocols

- [α-^{33}P]dATP
  (37–110 TBq/mmol, 1000–3000 Ci/mmol)
- Agarose
  US75817
- Glycerol
  US16374
- Formamide
  US75828
- TEMED
  US76320
- Ammonium Persulfate
  US76322

- ALFexpress™ sizer 50–500
  27-4539-01

The preceding materials are all available from GE Healthcare.

- PEI cellulose chromatography plates with glass support
  Merck 5725
- 1 M KH_2PO_4

For	1 litre
KH_2PO_4	136.09 g

- 5 x TBE buffer pH 8.3

For	1 litre
Tris base	54 g
Boric acid	27.5 g
0.5 M EDTA	20 ml

- 50 x TAE buffer pH 8.5

For	1 litre
Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA	100 ml

- Typhoon™ Variable Mode Imager or equivalent.
- UV visible Spectrophotometer such as Gene Quant 1300 or Ultrospec™ 3100 Pro, or equivalent.
- Microarray slides
- Hybridization chamber
- Microarray scanner
- Oligonucleotide dA_{80}
- Microarray hybridization buffer (1 vial included with the CyScribe kit)
  GE Healthcare RPK0325
- 20 x SSC
  GE Healthcare US75832
- 20% w/v SDS
  GE Healthcare US19629
- Nuclease free water
  GE Healthcare US70783

5. Description

The CyScribe First-Strand cDNA Labeling Kit provides reagents for preparation of Cy3 and Cy5 labeled cDNA probes in first-strand cDNA synthesis reactions. The kit has been designed for use with either Cy3 or Cy5 labeled dCTP and dUTP.

The CyDye-labeled first-strand cDNAs generated with the CyScribe First-Strand cDNA Labeling Kit are intended to provide high signal levels in microarray hybridization applications that can be easily measured on microarray scanners optimized to detect Cy3 and Cy5 fluorescence. For dual color microarray hybridizations, labeling reactions of mRNA are performed separately with Cy3- and Cy5-nucleotides and the two probes are combined in the hybridization solution. This enables simultaneous detection of hybridization signals and comparative analysis of gene expression levels.

Labeling

The labeling reaction, catalyzed by CyScribe reverse transcriptase incorporates Cy3-dCTP, Cy5-dCTP, Cy3-dUTP or Cy5-dUTP into first-strand cDNA. CyScribe is a modified reverse transcriptase that gives excellent yields of first-strand cDNA. Two optimized nucleotide mixes are provided with the kit to be used with CyDye-dCTP or CyDye-dUTP. Equal incoporation of Cy3-dCTP, Cy5-dCTP, Cy3-dUTP and Cy5-dUTP can be achieved with the CyScribe First-Strand cDNA Labeling Kit

Three alternative priming methods are offered in this kit. Priming with anchored oligo(dT) will direct the start of the synthesis of cDNA from the 5′ end of the polyA-tail. This priming method is especially suitable if the hybridization targets on the microarray are derived from the 3′ ends of transcripts. As an alternative, the random nonamers supplied with the kit can be used for priming the synthesis of first-strand cDNA. These random nonamers will anneal to their complementary sequences and direct the synthesis of complementary cDNA

molecules along the length of transcripts. Priming cDNA synthesis with random nonamers will shorten the average length of transcripts, but will not detrimentally affect the use of these cDNA molecules as a hybridization probe. Finally, the standard protocol provided with the CyScribe kit uses both priming methods together to provide uniform coverage of transcripts in the CyDye-labeled cDNA. A schematic presentation of the steps in the standard protocol is illustrated in figure 1. The kit has been developed for use with purified RNA that is free from contaminating DNA, proteins, nucleotides or other materials. Either mRNA or total RNA can be used with the kit. With purified mRNA three priming strategies can be used: oligo(dT) or random primers alone or both together. Protocols for labeling from 50 ng to 1 µg are given in section 6.2., page 15. With eukaryotic total RNA priming with oligo(dT) is the only option. The protocol for labeling from 2.5–25 µg of total RNA is given in section 6.3., page 17. With RNA isolated from species that lack poly A-tails in transcripts, priming with random nonamers can be used, but titration of primer amount to the amount of RNA needs to be carried out to find the optimal ratio.

Purification of cDNA probes

The fluorescent cDNA probes need to be purified from RNA template and unincorporated fluorescent nucleotides in order to maximize hybridization signal and minimize non-specific hybridization background on microarrays. RNA template can be degraded with an alkaline hydrolysis treatment. After neutralization, the labeling reactions are ready for purification. We recommend using CyScribe GFX Purification Kit which has been developed for this purpose. These columns give excellent recovery, typically 50% or higher, of labeled cDNA and are efficient in removing unincorporated nucleotides from the labeled cDNA. Other spin column based purification systems such as illustra AutoSeq™ G-50 (27-5340-01) can be used as an alternative, but recovery of labeled material may be compromised.

Purification of labeled cDNA can be performed in 15 minutes with these spin columns (page 20 describes protocols for the columns). The CyScribe GFX Purification Kit can be purchased with the CyScribe labeling kits.

Monitoring of cDNA yield and incorporation of fluorescent nucleotide

In order to produce high quality results from microarray hybridizations, it is necessary to monitor the success of all the steps involved in the process, including probe labeling, target preparation, slide spotting and hybridization itself. The CyScribe kit provides several protocols for assessing the success of cDNA labeling reactions. These methods give different levels of information and the users can choose those methods that are most suitable for their purposes. The minimum amount of analysis we strongly recommend to be performed after labeling and purification of fluorescently labeled cDNAs is to use UV visible spectrophotometry to determine the amount of Cy3 and Cy5 incorporated into cDNA. This information is required for setting up reproducible and accurate microarray hybridizations. Polyacrylamide gel electrophoresis or agarose gel electrophoresis can also be used to visualize and estimate the incorporation of CyDye fluors into cDNA. If information about the amount of cDNA prepared is required, then spiking with [α-^{33}P]dATP is recommended. Protocols for performing these analyses are given on pages 24–38.

Use of CyDye labeled cDNAs in applications

First-strand cDNA probes prepared using the CyScribe First-Strand cDNA Labeling Kit can be used in many applications where uniformly labeled populations of first-strand cDNAs are used. At the GE Healthcare Laboratories, CyDye-labeled first-strand cDNAs have been successfully used as probes in dual color microarray hybridization assays to detect targets immobilized on microarray

slides using the GE Healthcare microarray system and reagents. Microarray hybridization buffer developed and optimized for giving high signal to noise ratios on aminosilane-treated GE Healthcare microarray slides is included in the CyScribe kit. Although this buffer can be used with other manufacturer's slides, some slide chemistries may not perform well with this buffer. Purified fluorescently labeled cDNA prepared with the CyScribe kit can be used successfully in different types of hybridization buffers.

Use of control reagents

The control mRNA supplied with the labeling kit can be used to monitor the performance of the CyScribe First-Strand cDNA Labeling Kit for troubleshooting purposes, as well as becoming familiarized with the standard protocol. The labeled cDNA prepared from the control mRNA can be analyzed with the additional protocols described on pages 15–23. The quality control test for CyScribe First-Strand cDNA Labeling Kit is based on the use of the control mRNA in the standard labeling protocol. cDNA prepared from the control mRNA cannot be used in a microarray hybridization.

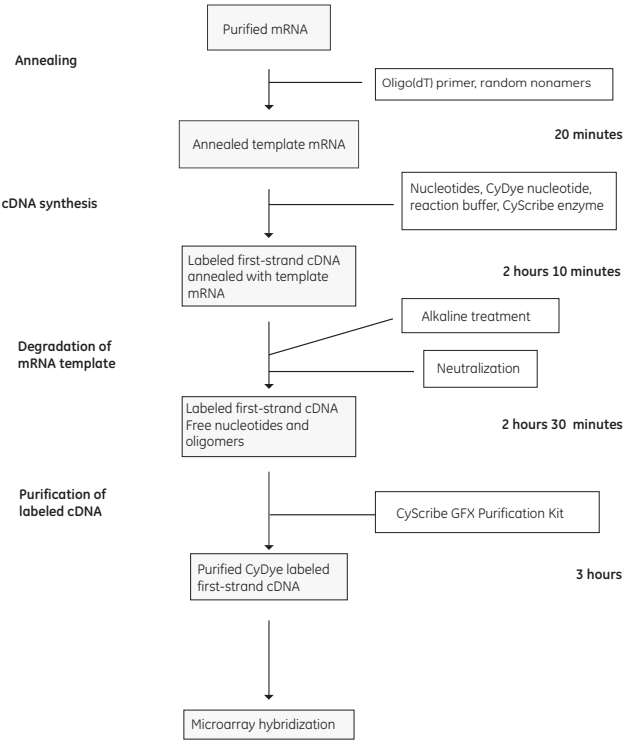

Figure 1. Preparation of CyDye labeled first-strand cDNA with the CyScribe kit

6. Protocol

6.1. Introduction

This protocol provides all the information required to use the CyScribe First-Strand cDNA Labeling Kit to incorporate Cy3-dCTP, Cy5-dCTP, Cy3-dUTP or Cy5-dUTP, into probes for use in dual color microarray hybridizations. It is recommended that the protocol is read thoroughly before using the system and that it is followed precisely.

Both purified mRNA or total RNA can be used with the CyScribe First-Strand cDNA Labeling Kit. The quality of RNA is critical for the success of the labeling reaction. Separate protocols for these starting materials are given in sections 6.2. and 6.3., respectively. In the standard labeling protocols, mRNA or total RNA is first heat denatured to eliminate secondary structure and annealed with primers.The type of the starting material determines the primers strategy. With purified mRNA three priming strategies can be used: oligo(dT) or random priming alone or both together. Highest yield of cDNA is achieved when random nonamers are included in the reaction. With eukaryotic total RNA, priming with oligo(dT) is the only option as the use of random nonamers will result in ribosomal RNA being transcribed predominantly. With RNA isolated from species that lack poly A-tails in transcripts, priming with random nonamers can be used, but titration of primer amount to the amount of RNA needs to be carried out to find optimal ratios. Following the annealing step, other kit components are added. Labeling reactions are incubated at 42ºC for 1.5 hours. **In total this protocol will take less than 2.5 hours to perform.**

When handling mRNA, always avoid contamination with ribonucleases, particularly those present on skin. Wear clean gloves at all times; bake or autoclave all glassware and plasticware and use reagents that have been prepared in nuclease-free water.

The fluorescent properties of Cy3 and Cy5 can be adversely affected by exposure to light. Therefore we recommend that all labeling reactions are performed in amber microcentrifuge tubes and the exposure of Cy3- and Cy5-nucleotides and labeled cDNA to all light sources is kept to a minimum.

6.2. Preparing fluorescently labeled cDNA from mRNA

This protocol can be used to prepare Cy3 or Cy5 labeled cDNA from purified mRNA. In one reaction, 50 ng–1 µg of mRNA can be reverse transcribed into fluorescently labeled cDNA. If more mRNA than 1 µg needs to be labeled, then it is recommended to perform multiple labeling reactions each containing 1 µg or less of mRNA. In the standard protocol, cDNA synthesis is primed with both oligo(dT) and random nonamers, as this combination of primers gives the highest yield of cDNA and uniform representation of mRNA sequences in cDNA.

- Set a water bath at 70°C and another at 42°C.
- Place the required reagents from the kit, excluding the enzyme on ice to thaw. Only place the enzyme on ice immediately prior to use. Make sure that the contents of all tubes are thoroughly thawed and mixed before pipetting solutions from them.

Primer annealing

- To anneal primers to mRNA, add the following labeling reaction components to a 1.5 ml amber microcentrifuge tube on ice:

mRNA, 50 ng–1 µg	X µl
Random nonamers	1 µl
Anchored oligo(dT)	1 µl
Water (supplied)	Y µl
Total	11 µl

The volumes corresponding to x and y should be adjusted so that the total reaction volume is 11 µl. It is possible to use only one type of primer in the reaction. If one of the primers is omitted from the reaction, an extra 1 µl of water should be added to keep the final volume at 11 µl.

- Mix gently by pipetting up and down.
- Incubate the reaction mixture at 70ºC for 5 minutes.
- Let the reaction mixture cool at room temperature for 10 minutes to allow the primers to anneal with the mRNA template.
- Spin down the reaction mixtures for 30 seconds in a microcentrifuge to collect all reaction components at the bottom of the tube.

Extension reaction

- Place the annealed reaction mixture on ice and add the labeling components in the following order, to the existing 11 µl in the tube

5x CyScribe buffer	4 µl
0.1 M DTT	2 µl
dUTP or dCTP nucleotide mix	1 µl
dUTP or dCTP CyDye-labeled nucleotide	1 µl
CyScribe reverse transcriptase	1 µl
Total volume	20 µl

The final reaction volume is 20 µl after addition of all the reaction components. Remove the enzyme from freezer just before removing aliquots into labeling reactions and immediately afterwards return the enzyme to a freezer at -15ºC to -30ºC.

Use the dCTP nucleotide mix in conjunction with either Cy3-dCTP or Cy5-dCTP. Likewise, use dUTP nucleotide mix either with Cy3-dUTP or Cy5-dUTP.

These nucleotide mixtures have been optimized for use with their corresponding CyDye-nucleotide and are not interchangeable.

- Mix the reactions by vortexing and spin them for 30 seconds in a micro centrifuge.
- Incubate the reactions at 42°C for 1.5 hours.
- Store the labeled cDNA on ice for immediate purification or place at -15°C to -30°C for storage. Protect from light and do not store in a frost-free freezer.

6.3. Preparing fluorescently labeled cDNA from total RNA

This protocol can be used to prepare Cy3 or Cy5 labeled cDNA from purified eukaryotic total RNA. In one standard reaction, 2.5–25 µg of total RNA can be reverse transcribed into fluorescently labeled cDNA. If more total RNA than 25 µg needs to be labeled, then it is recommended to perform multiple labeling reactions each containing 25 µg or less of total RNA. In the standard protocol, cDNA synthesis is primed with oligo(dT) alone. This primer anneals to the poly A-tails of transcripts, that only account for 1.5–2% of the total RNA, and selectively primes cDNA synthesis from these RNA molecules. As this method will only create one transcription start site per transcript, the yield of cDNA from total RNA is less than the yield of cDNA prepared from the corresponding amount of mRNA using dual priming.

Note: Total RNA isolated from species, such as bacteria, that have transcripts lacking poly A-tails can only be labeled using random nonamer priming. However, performing this with the CyScribe First-Strand cDNA Labeling Kit requires that an optimal ratio between the amount of random nonamer primers and total RNA is determined empirically for each species.

- Set a water bath at 70ºC and another at 42ºC.

- Place the required reagents from the kit, excluding the enzyme, on ice to thaw. Place the enzyme on ice only immediately prior to use. Make sure that the contents of all tubes are thoroughly thawed and mixed before pipetting solutions from them.

Primer annealing

- To anneal primers to total RNA, add the following labeling reaction components to a 1.5 ml amber microcentrifuge tube on ice:

Total RNA, 2.5–25 µg	X µl
Anchored oligo-dT	1 µl
Water (supplied)	Y µl
Total	11 µl

- The volumes corresponding to x and y should be adjusted so that the total reaction volume is 11 µl.
- Mix gently by pipetting up and down.
- Incubate the reaction mixture at 70°C for 5 minutes.
- Let the reaction mixture cool at room temperature for 10 minutes to allow the primers to anneal with the total RNA template.
- Spin down the reaction mixtures for 30 seconds in a microcentrifuge to collect all reaction components at the bottom of the tube.

Extension reaction

- Place the annealed reaction mixture on ice and add the labeling components in the following order:

5x CyScribe buffer	4 µl
0.1 M DTT	2 µl
dUTP or dCTP nucleotide mix	1 µl
dUTP or dCTP CyDye-labeled nucleotide	1 µl
CyScribe reverse transcriptase	1 µl
Total volume	20 µl

Final reaction volume is 20 µl after addition of all reaction components. Remove the CyScribe enzyme from the freezer just before removing aliquots into labeling reactions and immediately afterwards return the enzyme to a freezer at -15°C to -30°C.

Use the dCTP nucleotide mix in conjunction with either Cy3-dCTP or Cy5-dCTP. Likewise, use dUTP nucleotide mix either with Cy3-dUTP or Cy5-dUTP. These nucleotide mixtures have been optimized for use with their corresponding CyDye-nucleotide and are not interchangeable.

- Mix the reactions by vortexing and spin them for 30 seconds in a microcentrifuge.
- Incubate the reactions at 42°C for 1.5 hours.
- Store the labeled cDNA on ice for immediate purification or place at -15°C to -30°C for storage. Protect from light and do not store in a frost-free freezer.

6.4. Purification of labeled cDNA

It is necessary to remove the RNA template, whether mRNA or total RNA, from the single-stranded cDNA to promote hybridization of the cDNA probe to immobilized microarray targets and to minimize hybridization with complementary RNA in solution. Removal of unincorporated CyDye-nucleotides is also necessary for minimizing hybridization background and for improving the sensitivity of detection of low abundance targets. The removal of RNA can be achieved in two steps. First RNA is degraded into short oligomers with Alkaline treatment. Then these short oligomers, as well as unincorporated nucleotides, are removed with spin column chromatography. We recommend using CyScribe GFX Purification kit for removing unincorporated nucleotides from labeled cDNA. This kit has been developed specially for this purpose and it efficiently removes shorter than 50 nucleotides. A high recovery of labeled

cDNA, typically over 50%, is achieved with this kit. CyScribe GFX Purification kit is supplied as a part of some CyScribe kits (ordering codes with the suffix X). As an alternative purification system illustra AutoSeq G-50 (27-5340-01) can be used, although the recovery of labeled cDNA may be variable. It is important that the amount of CyeDye labeled material recovered from the labeling step is quantified before using it in a microarray hybridization, see protocol on page 24 for performing UV visible spectrophotometry analysis. Do not purify more than one sample with one column. The reagents for removal of RNA template are not included in the CyScribe First-Strand cDNA Labeling Kit .

Degradation of RNA

- Adjust a waterbath to 37°C.
- Add 2 µl of 2.5 M NaOH into each microcentrifuge tube containing labeling reactions.
- Mix the reaction mixtures by vortexing and spin them for 30 seconds in a microcentrifuge.
- Incubate the samples at 37°C for 15 minutes.
- Add 10 µl of 2 M HEPES free acid to each reaction tube.
- Mix the reaction mixtures by vortexing to ensure that all of the contents are neutralized and spin them for 30 seconds in a microcentrifuge.
- The labeling reactions are now ready for purification or can be stored at -15°C to -30°C.

Purification of labeled cDNA with CyScribe GFX Purification kit
Preparation of wash buffer

For details on how to purify CyDye labeled cDNA using the CyScribe GFX Purification Kit, please refer to the pack leaflet that is supplied with the kit.

Removal of nucleotides and short oligomers with AutoSeq G-50 columns

AutoSeq G-50 columns are not supplied with the CyScribe kits, see page 7 for details.

AutoSeq G-50 columns contain Sephadex™ G-50 DNA grade F and are supplied pre-equilibrated in water containing 0.05% Kathon™ cG/ICP Biocide as preservative.

Note: At this stage in the protocol the sample labeling reaction has a volume of 32 µl. AutoSeq G-50 columns have a loading volume range of 12 to 25 µl. Therefore, two AutoSeq G-50 columns are needed to purify one labeling reaction. Alternately, the labeling reaction may be dried to a volume of 20 µl and applied to a single AutoSeq G-50 column for purification.

Prepare the columns for nucleotide removal as follows:

- Resuspend the resin in the column by vortexing gently.
- Loosen the cap a quarter of a turn and snap off the bottom closure.
- Place the column in a 1.5 ml screw-cap microcentrifuge tube for support. Alternatively, remove the cap from a standard microcentrifuge tube and use this tube for support. If using a 1.5 ml microcentrifuge tube, 10–20 µl of fluid will remain in the tip of the column after spinning. Blot this fluid from the column using a clean paper towel before applying sample into the column.
- Spin the column for 1 minute at 2000 x g. Start the timer and the microcentrifuge simultaneously. Use the column immediately after preparation to avoid drying of the matrix.

Purify the labeled cDNA as follows:

- Place the column in a new 1.5 ml tube and slowly apply the sample to the centre of the angled surface of the compacted resin bed, being careful not to disturb the resin. Do not allow any of the liquid to flow around the sides of the bed.

- Spin the column for 1 minute at 2000 x g. Start the timer and the microcentrifuge simultaneously. The purified sample is collected at the bottom of the tube. Discard the column.
- The purified cDNA is ready for use in microarray hybridizations or can be stored at -15°C to -30°C protected from light. Guidelines for performing microarray hybridizations with CyDye-labeled cDNAs are given on page 35 in this booklet

7. Additional information

7.1. Monitoring the success of labeling reactions

Monitoring the incorporation of CyDye into labeled cDNA probe is one of the most critical success factors for obtaining high quality microarray data. The yield of fluorescent probe is determined not only by the success of the labeling step and by the amount of template RNA used in the reaction, but more importantly, by the recovery of labeled material from the purification system. Reproducible microarray experiments require the use of balanced and optimal amounts of fluorescently labeled samples in hybridizations. This can only be achieved if the amounts of Cy3 and Cy5 labeled samples are quantified before setting up hybridization reactions. In this section we provide several protocols for assessing the performance of CyDye incorporation into first-strand cDNA. These protocols include:

- using UV visible spectroscopy to determine the amount of CyDye incorporated into purified cDNA
- agarose or denaturing polyacrylamide gel electrophoresis to determine the size and fluorescence of labeled cDNA.
- spiking with [α-^{33}P]dATP to determine the yield of cDNA and to monitor the loss of cDNA in purification steps

It is recommended as a minimum that UV visible spectroscopy is used to determine the amount of CyDye in each sample before microarray hybridization.

7.2. Determination of the incorporation of Cy3- and Cy5-labeled nucleotides into cDNA.

The incorporation of Cy3- and Cy5-nucleotides into first-strand cDNA can be quantified with UV visible spectrophotometry. Cy3 and Cy5

have absorption maximum at 550 and 650 nm, respectively. Using their extinction coefficients the total amount of CyDye molecules incorporated into cDNA can be calculated. Purification of the labeled cDNA is essential as residual Cy3- or Cy5-nucleotides will interfere with the measurements. The Ultrospec range of UV/Visible spectrophotometers from GE Healthcare, most notably the Ultrospec 3100 Pro, is particularly suitable for this application. The Ultrospec 3100 Pro has an embedded cDNA application software that displays a UV/visible scan of the sample and automatically calculates yield and CyDye incorporation data (see GE Healthcare catalogue or web page www.gelifesciences.com for further details).

Alternatively, agarose gel electrophoresis or denaturing polyacrylamide gel electrophoresis can be used to visualize the fluorescence of Cy3- and Cy5-labeled cDNA. Denaturing polyacrylamide gel electrophoresis will also provide information about the size of labeled cDNAs. Fluorescent cDNA separated in both types of gels can be analyzed with Typhoon variable mode scanner which has been developed for the detection of Cy3 and Cy5 fluorescence (see the GE Healthcare catalogue or web page http://www.gelifesciences.com for further details).

UV visible spectrophotometry

Before performing this protocol, purify labeled cDNA according to the protocol on page 14. Any residual unincorporated CyDye-labeled nucleotides will interfere with the detection of CyDye-labeled cDNA. The amount of CyDye incorporated into purified cDNA can be used as a guide to optimize the amount of label in the hybridization probe, and to adjust the relative amounts of Cy3 and Cy5 to account for any imbalances in the detection of these dyes with scanning instruments. For most accurate results, follow the protocol for incorporation of [α-^{33}P]dATP into cDNA. This enables the determination of incorporation of CyDye per microgram of cDNA. Without this data, however, the amount of CyDye in total sample can still be estimated.

- For spectrophotometry, dilute an aliquot of purified cDNA with nuclease free water.
- Use the smallest cuvettes available, preferably with volumes less than 100 µl.
- Measure absorbance of the dilution against blank at 550 nm for Cy3 and at 650 nm for Cy5 using cuvettes with known path length. The diluted cDNA sample can be recovered from the measuring cell, dried down and used in microarray hybridization.

The amounts of Cy3 and Cy5 incorporated into cDNA can be calculated from their respective extinction coefficients, (which are 150 000 l mol^{-1} cm^{-1} at 550 nm for Cy3 and 250 000 l mol^{-1} cm^{-1} at 650 nm for Cy5) as follows:

pmoles Cy3 or Cy5 in sample = (A/E) x (1/W) x (Z) x df x 10^6

Where:

A = absorbance Cy3 at 550 nm or Cy5 at 650 nm

E = the extinction coefficient for Cy3 or Cy5

Z = original volume expressed in microliters

W = optical path of cuvette expressed in centimeters

df = dilution factor

Incorporation of CyDye = $\frac{\textbf{pmol of CyDye in sample}}{\textbf{µg of nucleic acid in sample}}$

For example

If 20 µl of Cy3-labeled cDNA at nucleic acid concentration of 0.025 µg/µl that has been diluted 5-fold gives absorbance of 0.06 at 550 nm and half a cm light path, then...

pmoles of Cy3 in sample = (0.06/150000) x (1/0.5) x (20) x 5 x 10^6 = 80 pmoles

$$\text{Incorporation of Cy3} = \frac{\text{80 pmoles of Cy3}}{\text{0.5 µg nucleic acid}} = \text{160 pmoles Cy3 per µg of nucleic acid}$$

Note: the factor of 10^6 is derived to express the answer in terms of pmoles.

Typical yield of labeled cDNA with the recommended purification systems varies from 40–100 pmol of Cy3 or Cy5 incorporated into probe using 0.5 µg control mRNA as template.

Agarose gel electrophoresis

Agarose gel electrophoresis provides a relatively easy way of monitoring the incorporation of CyDye into cDNA without purifying the cDNA prior to analysis. Non-denaturing agarose gel electrophoresis will not reflect accurately the size distribution of the CyDye-labeled single-stranded cDNA molecules.

- Prepare 1% (w/v) agarose gel in 1x TBE. Alternatively 1x TAE buffer can be used.
- Dilute an aliquot of labeling reaction to 10 µl with water. 0.1–1 µl of labeling reaction should be enough for detecting fluorescent cDNA. Add 2 µl of 50% (v/v) glycerol. Do not use normal loading buffer which contain dyes such as bromophenol blue or xylene cyanol, as these will interfere with the detection of fluorescence.
- Dilute 4 µl of fluorescent markers (ALFexpress sizer 50–500; 27-4539-01) with 6 µl of water and add 2 µl of 50% (v/v) glycerol.
- Separate the diluted samples on the agarose gel by performing electrophoresis at 7.5 V/cm in 1x TBE. Protect the samples from light during the electrophoresis. In order to help monitor the progress of electrophoresis, load an aliquot of loading buffer containing bromophenol blue into a side well on the gel, well separated from labeled cDNA samples. Let electrophoresis proceed until the bromophenol marked dye has reached the last third of the length of the gel.
- Scan the gel with Typhoon Variable Mode Imager. Detect Cy3 by excitation with 532 nm laser and using emission filter 555BP20.

Detect Cy5 by excitation with 633 nm laser and using emission filter 670BP30. Set PMT to 800 V, focal plane to +3 mm and use normal sensitivity.

- If necessary, the gel can be returned to the gel tank and electrophoresis continued until samples have separated adequately from free CyDye-nucleotides. CyDye-nucleotides do not separate true to their size under these conditions.

This method can be used to estimate the success of the purification step, if samples taken before and after purification of the labeled cDNA are separated on the same gel.

Polyacrylamide gel electrophoresis

If the size range of the first-strand cDNA needs to be determined, then denaturing polyacrylamide gel electrophoresis using a standard mini gel can be performed.

The incorporation of CyDye into cDNA can be determined from these gels by scanning the fluorescence associated with the cDNAs with a Typhoon Variable Mode Imager.

- Prepare a 6% (w/v) polyacrylamide gel. Cast a gel according to the instructions provided with your electrophoresis equipment. This method can be used to estimate the success of the purification step, if samples taken before and after purification of the labeled cDNA are separated on the same gel.
- Dilute 1 µl of the labeling reaction with 9 µl of sterile water. 0.1–1 µl of labeling reaction should be enough for detection of CyDye-labeled cDNA.
- Further dilute 1, 2.5 and 5 µl aliquots of this 1:10 dilution to 5 µl with water. Add 2 µl of 100% (v/v) formamide to each sample. Do not use normal loading/denaturation buffers which contain dyes such as bromophenol blue or xylene cyanol as these will interfere with the detection of fluorescence.

- Dilute 4 µl of fluorescent markers (ALFexpress sizer 50–500; 27-4539-01) with 1 µl of water and add 2 µl of 100% (v/v) formamide. The use of the ALFexpress sizer 50–500 will allow determination of the size range of the fluorescent cDNAs.
- Denature these samples by boiling for 2 minutes at 95°C. Snap cool on ice before loading on to the gel.
- Load samples to the gel and perform electrophoresis according to the instructions provided with your equipment. Use 1 x TBE as buffer. Protect the samples from light during the electrophoresis.
- In order to help monitor the progress of electrophoresis, an aliquot of loading buffer containing bromophenol blue can be loaded into a side well on the gel well separated from the labeled cDNAs. Stop the electrophoresis when the bromophenol dye is near the bottom of the gel.
- Remove one of the gel plates before scanning and make sure that the back of the remaining plate is clean. Do not let the gel dry before scanning.
- Scan the gel with a Typhoon Variable Mode Imager. Detect Cy3 by excitation with 532 nm laser and using emission filter 555BP20. Detect Cy5 by excitation with 633 nm laser and using emission filter 670BP30. Set PMT to 800 V, focal plane to +3 mm and use normal sensitivity.

Fluorescent cDNA should be visible as a smear when less than 1 µl of labeling reaction is analyzed. A high proportion of cDNA molecules should be longer than 300 nucleotides.

Estimation of the yield of CyDye labeled cDNA with UV spectrophotometry

The amount of CyDye labeled cDNA that has been purified with CyScribe GFX Purification kit can be estimated with UV spectrophotometry at 260 nm. This method only provides an estimation of the cDNA amount as the CyDye incorporated into the

cDNA contributes slightly to the absorbance values measured at 260 nm (typically, less than 1% contribution at the labeling densities achieved with CyScribe First-Strand cDNA Labeling kit). Labeled cDNA purified with other methods may give even less accurate results because of the presence of varying amounts of impurities absorbing at 260 nm, derived from purification columns.

7.3. Determination of the yield of cDNA with [α-33P]dATP spiking

Spiking a radioactive nucleotide into the labeling reaction provides an alternative method for determining the yield of labeled cDNA. This method can be used regardless of the RNA template being used. [α-^{33}P]dATP is suitable for use as a spike with either CyDye-labeled dCTP or dUTP. [α-^{33}P]dATP will not interfere with the incorporation of these CyDye into cDNA nor with the subsequent use of the cDNA as a fluorescent hybridization probe. After labeling, the incorporation of [α-^{33}P]dATP into cDNA can be determined with thin layer chromatography on PEI-cellulose matrix. The yield of cDNA is directly proportional to the percentage of the radionuclide incorporated into cDNA.

Spiking labeling reaction with [α-^{33}P]dATP

- Start setting up a labeling reaction according to the standard protocol for labeling cDNA on page 15.
- In the annealing step, reduce the total volume to 9 µl by reducing the amount of water added to the reaction. Pipette the following into an amber 1.5 ml conical polypropylene tube:

mRNA, 1 µg	X µl
Random nonamers	2 µl
Anchored oligo(dT)	1 µl
Water (supplied)	Y µl
Total volume	9 µl

- Mix gently by pipetting up and down.
- Incubate the reaction mixture at 70°C for 5 minutes.
- Let the reaction mixture to cool at room temperature for 10 minutes to allow the primers to anneal with the mRNA template.
- Spin down the reaction mixtures for 30 seconds in a microcentrifuge to collect all reaction components at the bottom of the tube.
- Place the annealed reaction mixture on ice and add the labeling components in the following order, to the existing 9 µl in the tube:

5 x CyScribe buffer	4 µl
0.1 M DTT	2 µl
dUTP or dCTP nucleotide mix	1 µl
1:10 diluted [α-^{33}P]dATP	2 µl
CyDye-nucleotide	1 µl
CyScribe reverse transcriptase	1 µl
Total volume	9 µl

The final reaction volume is 20 µl after addition of all the reaction components.

Dilute [α-^{33}P]dATP, (37–110 TBq/mmol, 1000–3000 Ci/mmol, in nuclease free water. 2 µl of this dilution will contain 74 kBq (2 µCi) [α-^{33}P]dATP, which has been found to be sufficient for monitoring the incorporation of this nucleotide into cDNA as well as for assessing the recovery of cDNA from the purification step. Do not use ^{33}P-labeled nucleotides dissolved in colored or fluorescent buffers, as these will interfere with the detection of the CyDye.

- Perform the rest of the standard labeling protocol as described on pages 17–19.

- Perform the protocol for degradation of mRNA as described on page 20.

Quantification of the incorporation of [α-^{33}P]dATP with thin layer chromatography

The incoproration of [α-^{33}P]dATP into cDNA is proportional to the yield of first-strand cDNA. The incorporated [α–^{33}P]dATP can easily be separated from unincorporated nucleotides with thin layer chromatography on PEI Cellulose chromatography plates. Quantification of the amount of incorporated [α-^{33}P]dATP as a proportion of the total [α-^{33}P]dATP in the reaction will enable the calculation of cDNA yield. Perform this analysis before proceeding with the purification of cDNA.

- Prepare 20 x 20 cm PEI Cellulose chromatography plate for use by cutting a thin linear groove into the nitrocellulose layer at 1 cm distance from the top edge of the plate. Mark sample positions along a line that is 3 cm from the bottom edge of the plate.
- Pipette duplicate 0.5 µl samples of labeling reactions along the marked line. The sample spots should be about 1 cm apart. Do not damage the PEI-cellulose layer with the tip.
- Place the plate in a rectangular chromatography tank so that the bottom of the plate is immersed 2 cm deep in 1 M KH_2PO_4. Cover the tank and let sample separation take place. Make sure that the level of the buffer is below the level of the marked sample line. [α-^{33}P]dATP that has been incorporated into cDNA will not move far from the sample line. Free [α-^{33}P]dATP will move progressively towards the top of the plate.
- When the buffer has reached the top groove, i.e when the samples have migrated the full available length of the 20 cm plate, remove the plate from the tank and let it air-dry.

- Wrap the plate in cling-film and expose it to a phosphoscreen for 1–6 hours. Take care not to overexpose the phosphoscreen as it will saturate the signal.
- Scan the phosphoscreen with a Typhoon scanner or equivalent with recommended settings. As an alternative to the use of phosphoscreens, any instrument that can measure quantitatively the radioactivity associated with different spots on the chromatography plate can be used.
- Use ImageQuant™ software to calculate the proportion of [α-^{33}P]dATP that is associated with cDNA. Consult the manual provided with the software for details about performing quantification.

Calculation of cDNA yield

Percentage of labeled [α-^{33}P]dATP incorporated	=Y%
Amount of unlabeled dATP in reaction	= 2 nmol
Therefore assume amount of unlabeled dATP incorporated	= Y% x 2 nmol = Y/50 nmol
Therefore amount of total dNTPs incorporated	= 4 x Y/50 nmol = Y/12.5 nmol
Assume residue molecular weight of dNMP (1 mole)	= 330 g
Therefore weight of cDNA synthesized	= 330 x Y/12.5 ng = 26.4Y ng

7.4. Monitoring the purification of labeled cDNA

The success of the purification of labeled cDNA can be monitored by spiking the labeling reactions with [α-^{33}P]dATP as described on page

29. Recovery of material can be determined with liquid scintillation counting.

- Remove 1 µl aliquots from spiked labeling solutions before and after purification with AutoSeq G-50 columns.
- Add 5 ml of aqueous scintillation liquid to these samples
- Count the samples with a liquid scintillation counter with settings suitable for the detection of ^{33}P.
- Assess the purity of recovered material with thin layer chromatography on PEI-cellulose, as described on page 32. The purity of the cDNA can be calculated as the percentage of [α-^{33}P]dATP incorporated into cDNA as a proportion of total counts after purification.
- Calculate the recovery of cDNA:

$$\text{recovery \%} = \frac{\text{(cpm after) x (purity \%/100) x volume recovered}}{\text{(cpm before) x (incorporation \%/100) x volume purified}} \times 100\%$$

Determination of labeling density

In order to determine the labeling density of the sample, the amount of CyDye in the sample needs to be related to the amount of cDNA present in the sample. This calculation requires that the yield of cDNA, and the recovery of material from the purification step have been determined using thin layer chromatography and liquid scintillation counting, as described above.

If w µl of synthesized cDNA (total volume was 32 µl) was applied to the purification column, then

amount of cDNA in purified sample =

cDNA yield x recovery %/100 x (w/32)

incorporation of CyDye = $\frac{\text{pmol of CyDye in sample}}{\text{µg of cDNA in sample}}$

Typical values achieved with the CyScribe First-Strand cDNA Labeling Kit are 70–120 pmol of Cy3 or Cy5 incorporated per µg of cDNA synthesized using human mRNA as template.

7.5. Use of control reagents

The CyScribe First-Strand cDNA Labeling Kit contains synthetic mRNA for control labeling reactions. This mRNA is a mixture of mRNAs of defined sizes; 0.24, 1.4, 2.4, 4.4 , 7.5 and 9.2 kilobases. Enough of this reagent is provided for 6 control reactions of 1 µg of mRNA per reaction. This mRNA can be used as a template for cDNA synthesis in labeling reactions using the standard protocol on page 15. The quantity of labeled cDNA can be determined by spiking the labeling reaction with [α-^{33}P]dATP as has been detailed on page 32. Typical values for the incorporation of [α-^{33}P]dATP into CyDye-labeled cDNA synthesized from control mRNA, as determined with PEI-cellulose thin layer chromatography, exceed 18%. The incorporation of the CyDye-labeled nucleotides can be determined with any of the three methods described on pages 25–30.

7.6. Guidelines for microarray hybridization

CyDye are a range of fluorescent dyes based on the benefits of Cyanine fluors. Cy3 and Cy5 offer bright and intense colors with narrow emission spectra that make them ideal for multicolor detection in microarray and other fluorescence based genome analysis techniques. Key benefits from the use of Cy3- and Cy5-labeled cDNA are their high sensitivity, relatively high photostability, insensitivity to pH and high water solubility.

We recommend that the users of the CyScribe First-Strand cDNA Labeling Kit follow the instructions provided with their microarray slides and analysis systems for specific information about using those reagents and equipment in microarray analysis. Labeled and purified fluorescent cDNA prepared with the CyScribe First-Strand

cDNA Labeling Kit can be successfully used with different types of microarray slides and equipment. However, as these systems use different attachment chemistries they may require the use of special reagents for pretreatment and hybridization buffers for achieving optimal results. Although the CyScribe First-Strand cDNA Labeling Kit is supplied with a microarray hybridization buffer, this buffer may not be compatible with all available microarray slide types. Therefore we recommend testing this buffer alongside the recommended buffer for the slides being used.

70–150 pmol of CyDye is typically incorporated in a standard labeling reaction with 1 µg of mRNA. Some loss of labeled cDNA will occur during purification, so the actual amount of labeled cDNA will vary from sample to sample. CyDye-labeled cDNAs prepared with the CyScribe First-Strand cDNA Labeling Kit have been successfully used in hybridizations on different types of microarray glass slides. For achieving optimal results we recommend that the amount of both Cy3 and Cy5 labeled material is quantified independently before setting up hybridization reactions. This allows for a balanced amount of each of the labeled samples for use in hybridization. Higher or lower amounts of labeled samples, or unequal amounts of Cy3 and Cy5 will lead to loss of signal intensity, decrease in sensitivity of detection and poor data quality.

Microarray hybridization protocol

The CyScribe First-Strand cDNA Labeling Kit is supplied with a formamide-based microarray hybridization buffer that has been developed and optimized for use with aminosilane-treated microarray slides from GE Healthcare. The following protocol gives general instructions for using this buffer in a 30 µl hybridization reaction with coverslips measuring 64 x 33 mm.

NOTE: This protocol has been optimized for GE Healthcare aminosilane-slides from GE Healthcare. Other manufacturers'

microarray slides may not give optimal results with this protocol. If using microarray slides other than those from GE Healthcare, please refer to the instructions provided with the slides you are going to use. Some optimization of slide treatments, hybridization and wash conditions, may be required for achieving best results with different microarray systems and with different immobilized microarray targets. Labeled and purified cDNA prepared with CyScribe First-Strand cDNA Labeling Kit can be used with different hybridization buffers and with different microarray protocols.

- Pretreat microarray slides that contain immobilized nucleic acid targets according to the instructions provided with your microarray system/slide manufacturer.
- For dual color hybridization, combine Cy3- and Cy5-labeled cDNAs into one tube. Dry down the cDNA solution in a SpeedVac™ concentrator in an amber microcentrifuge tube. Protect the solutions from light.
- Dissolve the cDNA in 6 µl of nuclease free water.
- Denature the cDNA by heating at 95°C for 2 minutes.
- Cool the cDNA solution on ice for 30 seconds.
- Add 1.5 µl of oligonucleotide dA_{80} (1 mg/ml) and mix well. This step is optional and is designed to prevent the interaction of the poly-T tails on cDNAs with A-rich regions on the immobilized targets.
- Incubate the mixture at 75ºC for 45 minutes.
- Add 7.5 µl of microarray hybridization buffer (supplied in the CyScribe kit) and 15 µl of 100% (v/v) Formamide. Mix well and spin for 30 sec in a microcentrifuge to collect all reaction components at the bottom of the tube.
- Pipette hybridization mixture on to a microarray slide on an area that does not contain targets and cover with a cover slip. It is important that no air bubbles are trapped beneath the coverslip

and that all of the slide surface containing immobilized targets is covered with hybridization buffer.

- Hybridize 14–18 hours at 42°C in a humid hybridization chamber. Protect the slides from light during this and subsequent steps.
- Prepare wash buffer in distilled water using stock solution of 20 x SSC, 20% SDS. Pre-warm wash buffers at 55°C and keep at 42°C until ready to use
- Wash hybridized slides with 1x SSC, 0.2% (w/v) SDS for 10 minutes at room temperature using a rotary shaker. Remove cover slips only after the slides have been immersed in the washing buffer. Make sure that the slides are fully covered with washing buffer. Wash twice with 0.1x SSC, 0.2% (w/v) SDS for 10 minutes at room temperature. Finally, dip the slides into distilled water for 10 seconds. The stringency of washes can be controlled by altering the wash temperature: increasing the temperature will result in higher stringency and conversely lowering the wash temperature to 37°C will result in lower stringency. Suitable wash parameters may need to be determined empirically.
- Dry the slides immediately and thoroughly with a gentle air stream and store desiccated in the dark.
- Scan the slides with a microarray scanner that is suitable for detecting Cy3 and Cy5 Fluorescence according to the instructions provided with the scanner.

8. Troubleshooting

All batches of CyScribe First-Strand cDNA Labeling Kit are the subject of careful quality control before despatch to ensure efficient performance. Should poor results be obtained we recommend the following points for consideration.

- Check that the procedures have been followed as specified in the standard protocol.
- Perform labeling reactions with the control mRNA provided in the kit according to the protocol detailed in the section: Use of control reagents, page 35. Monitor the yield of the cDNA and the incorporation of CyDye according to the protocols detailed in sections: Determination of the yield of cDNA with [α-^{33}P]dATP spiking and Determination of the incorporation of Cy3- and Cy5-labeled nucleotides into cDNA, pages 24–35.
- Check the purity and concentration of RNA. For efficient cDNA synthesis it is important that the mRNA template should be free of contaminating proteins and DNA. It is also important that the RNA is accurately quantified before use in labeling reactions. See Ausubel *et al.* (2000) for additional information on purification, analysis and handling of RNA and mRNA. Always handle RNA carefully to avoid ribonuclease contamination.
- If poor results are obtained in a microarray application using cDNAs prepared with CyScribe First-Strand cDNA Labeling Kit, consider each of the steps of the whole microarray procedure in troubleshooting. The microarray signal observed from the microarray slide reflects the success of all the preceding steps, and failure in any of these steps can cause poor results. It is recommended to always check the success of the labeling step and to determine the amounts of purified cDNAs before setting up microarray hybridization reactions.

Problem: Little or no cDNA synthesized

Possible causes	Remedy
1. One or more of the reaction components is missing.	**1.** Repeat labeling reaction with all reagents.
2. RNA is contaminated with ribonuclease.	**2.** Check the quality of RNA with gel electrophoresis/Northern blotting.
3. Wrong nucleotide mix was used. **dUTP and dCTP nucleotide mixes are not interchangeable.**	**3.** Repeat reactions with the correct nucleotide mix for the CyDye-nucleotide that is being used as the label.
4. RNA is contaminated with compounds that inhibit CyScribe reverse transcriptase.	**4.** Re-purify RNA.
5. The amounts of CyDye nucleotide and nucleotide mix were not pipetted accurately.	**5.** Check the accuracy of pipettes and repeat labeling reaction with correct amounts.
6. The amount of RNA template was less than recommended.	**6.** Re-analyze the concentration of RNA template, or use more RNA in the labeling reaction.

Problem: No labeled cDNA longer than 300 bp present

Possible causes	Remedy
1. RNA is contaminated with ribonuclease.	**1.** Check the quality of RNA with gel electrophoresis/Northern blotting.
2. Too little of nucleotide mix included in reaction.	**2.** Repeat labeling reaction with the correct amount.
3. Wrong nucleotide mix was used in the reaction.	**3.** Repeat labeling reaction with the correct nucleotide mix according to the CyDye-nucleotide being used as the label.
4. Random primers were used with total RNA	**4.** Repeat labeling using oligo (dT) primers only.

Problem: cDNA is not fluorescent

Possible causes	Remedy
1. Reaction tubes have been exposed to light.	**1.** Take care to avoid exposure of labeling reactions to daylight. Always keep the reactions in amber microcentrifuge tubes.
2. CyDye-nucleotides have been exposed to light.	**2.** Repeat labeling reactions with fresh CyDye-nucleotide.
3. Samples have been exposed to light during analysis of incorporation of CyDye.	**3.** Repeat analysis and minimize exposure of samples to light at all times.
4. cDNA has been lost in purification steps.	**4.** Monitor the amount of cDNA before and after purification.

Problem: Low recovery of labeled cDNA

Possible causes	Remedy
1. The Ethanol concentration in the wash buffer was less than 76%.	**1.** Add absolute Ethanol to wash buffer prior to use and cap bottle tightly for storage.
2. Poor elution of fragments from GFX column	**2.** Elution buffer must cover all of the capture membrane.

9. Storage of labeled probes

In GE Healthcare laboratories, CyDye-labeled cDNAs are routinely stored at -15°C to -30°C in a freezer. Do not store in a frost free freezer. Store protected from light.

10. References

1. Ausubel, F .M. *et al., Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Interscience, New York, 2000

11. Related products

CyScribe cDNA Post Labeling kit	RPN5660
CyScribe Direct mRNA Labeling kit	RPN5665
Cy3-dCTP 25 nmol	PA53021
Cy5-dCTP 25 nmol	PA55021
Cy3-dUTP 25 nmol	PA53022
Cy5-dUTP 25 nmol	PA55022
illustra AutoSeq G-50 Dye Terminator Removal Kit 27-5340-01	
illustra AutoSeq 96 G-50 Dye Terminator Removal Kit	28903427
illustra GFX PCR DNA and Gel Band Purification Kit	28-9034-70
ALFexpress sizer 50–500 50 loadings	27-4539-01
Microarray hybridization buffer	RPK0325
CyScribe First-strand cDNA Labeling kit	
25 labeling reactions each of up to 1 µg of mRNA	RPN6200
25 labeling reactions each of up to 1 µg of mRNA plus CyScribe GFX Purification kit for 25 reactions	RPN6200X
CyScribe First-strand cDNA Labeling system	
50 labeling reactions each of up to 1 µg of mRNA plus 25 nmol Cy3-dUTPand 25 nmol Cy5-dUTP	RPN6201
50 labeling reactions each of up to 1 µg of mRNA plus 25 nmol Cy3-dUTPand 25 nmol Cy5-dUTP plus CyScribe GFX Purification kit for 50 reactions	RPN6201X
50 labeling reactions each of up to 1 µg of mRNA plus 25 nmol Cy3-dCTPm and 25 nmol Cy5-dCTP	RPN6202
50 labeling reactions each of up to 1 µg of mRNA plus 25 nmol Cy3-dCTP and 25 nmol Cy5-dCTP plus CyScribe GFX Purification kit for 50 reactions	RPN6202X

CyScribe GFX Purification Kit for 25 reactions	27-9606-01
CyScribe GFX Purification Kit for 50 reactions	27-9606-02

Extraction of RNA and mRNA see section for DNA/RNA Synthesis and Purification of the GE Healthcare BioDirectory™.

See the current GE Healthcare catalogue or web site http://www.gelifesciences.com for further details.

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RPN6200PL Rev J 06/2008

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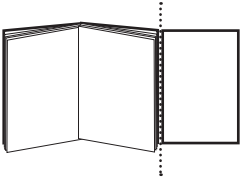

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Amersham
CyScribe First-Strand cDNA Labeling Kit
Product protocol card

RPN6200/6201/6202/
RPN6200X/6201X/6202X

Using the CyScribe™ First-Strand cDNA Labeling Kit

Denaturing and annealing

- **Mix the following in an amber 1.5 ml microcentrifuge tube:**
  - up to 1 µg of mRNA
  - 1 µl of oligo(dT) primer
  - 1 µl random nonamers
  - y µl water to 11 µl
- **Heat the mix at 70°C for 5 minutes**
- **Let the mix cool at room temperature for 10 minutes**

Labeling reaction

- **Add the following:**
  - 4 µl 5x CyScribe™ buffer
  - 2 µl 0.1 M DTT
  - 1 µl of dUTP or dCTP nucleotide mix
  - 1 µl of CyDye™-dUTP or CyDye- dCTP
  - z µl water to 20 µl (total volume)
  - 1 µl CyScribe reverse transcriptase
- **Mix well and incubate at 42°C for 1.5 hours**

Degradation of mRNA

- Add 2 µl of 2.5 M NaOH
- Incubate at 37°C for 15 minutes
- Add 10 µl of 2 M Hepes free acid
- Mix well

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