GE Healthcare

Amersham
CyScribe Post-Labeling Kit

Product Booklet

Codes: RPN5660
RPN5660X
27-9606-02

Page finder

1. Legal

http://www.gelifesciences.com

GE Healthcare UK Limited.
Amersham Place,
Little Chalfont, Buckinghamshire,
HP7 9NA, UK

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.

Caution: This product may be used with radioactive material. Please follow the manufacturers instruction relating to the handling, use, storage and disposal of such material.

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.

Please note that the protocol may require the use of the following chemicals:
Sodium Hydroxide (Harmful, causes burns); Glycerol (Irritant); Formamide (Harmful, irritant); Ammonium Persulphate (Harmful, causes burns); Ethanol (Flammable); Boric Acid (Harmful, irritant). Please follow the manufacturers safety data sheets relating to the handling and safe use of these materials.

2.2. Storage

Store all components of the CyScribe™ Post-Labeling Kit at -15°C to -30°C. **Do not store in a frost-free freezer.**
Store illustra™ CyScribe GFX™ Purification Kit at ambient temperature

Protect the fluorescent dyes from light. The fluorescent dyes, Cy™3 and Cy5, can be adversely affected by

prolonged exposure to light. Store reagents and any materials prepared using these reagents in the dark. Minimize their exposure to light while performing any of the procedures described in this booklet.

2.3. Storage of labeled probes

In GE Healthcare laboratories, fluorescently 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.

2.4. Expiry

All components are stable for at least 3 months when stored under the recommended conditions. For expiry details see outer packaging.

3. Components

CyScribe Post-Labeling Kit **RPN5660**

- 24 labeling reactions each of 500 ng of mRNA
- Anchored oligo(dT), 80 µl
- Random nonamer primers, 30 µl
- 5 × CyScribe buffer, pH 8.3, 150 µl
- 0.1 M DTT, aqueous solution of Dithiothreitol, 75 µl
- CyScribe Post-Labeling nucleotide mix containing dCTP, dGTP, dATP, dTTP, 30 µl
- CyScribe Post-Labeling Amino Allyl-dUTP, 3 vials each to be reconstituted with 30 µl water
- CyScribe reverse transcriptase, containing 50% Glycerol. pH 8.3, 25 µl
- Nuclease free water, water filtered through 1 µm filter (DEPC), 1 ml
- Control mRNA, 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, 6 µl
- CyScribe Cy3 reactive dye for one labeling reaction, 12 tubes
- CyScribe Cy5 reactive dye for one labeling reaction, 12 tubes
- Microarray hybridization buffer, 1 ml
- Reagents required for the purification of amino allyl-modified cDNA and CyDye labeled cDNA are provided only with RPN5660X.

CyScribe Post-Labeling Kit with
CyScribe GFX Purification Kit **RPN5660X**

- illustra CyScribe Post-Labeling Kit, RPN5660
- illustra CyScribe GFX Purification Kit, 27-9606-02, containing the following reagents:
  - Capture buffer
  - Wash buffer*
  - Elution buffer
  - illustra CyScribe GFX spin columns
  - Collection tubes

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

3.1. Other materials required

These materials are not provided with the CyScribe cDNA Post Labeling Kit.

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

For synthesis and purification of cDNA

- 2.5 M NaOH
  For 10 ml
  NaOH 1 g
  water to 10 ml
  Sterilize by filtration with a 0.45 micron filter.
  Store at ambient for up to 3 months.
- 2 M HEPES free acid
  For 10 ml
  HEPES free acid 4.77 g
  water to 10 ml
  Sterilize by filtration with a 0.45 micron filter.
  Store at ambient for up to 3 months.

- illustra AutoSeq G-50 columns GE Healthcare 27-5340-01

For coupling cDNA to CyDye

- 0.1 M Sodium Bicarbonate pH 9.0
  For 100 ml
  Sodium Bicarbonate 0.84 g
  Sterile water to 100 ml
  Dissolve the salt in 90 ml of water, adjust pH to 9 with 1 M NaOH.
  Add water to 100 ml.
  Note: CyDye coupling will be unsuccessful if pH is not 9.0.
  Sterilize by filtration with a 0.45 micron filter.
  Dispense into aliquots and store at -15°C to -30°C for up to three months.
- 4 M Hydroxylamine Hydrochloride
  For 100 ml
  Hydroxylamine Hydrochloride Sigma H2391 27.8 g
  water to 100 ml
- absolute ethanol

Equipment

- Microcentrifuge capable of generating a g-force of 13 800
- Amber microcentrifuge tubes, 1.5 ml Eppendorf 0030.120.191

For additional products

- [α-^{33}P]dATP,37-110 TBq/mmol, 1000–3000 Ci/mmol)
- Agarose GE Healthcare US75817
- Glycerol GE Healthcare US16374
- Formamide GE Healthcare US75828
- TEMED GE Healthcare US76320
- Ammonium Persulfhate GE Healthcare US76322
- ALFexpress© Sizer 50-500 GE Healthcare 27-4539-01

- PEI cellulose chromatography plates with glass support
  Merc k5725
- 3 M Sodium Acetate pH 5.2
- Absolute Ethanol
- 1 M KH_2PO_4
  For 1 liter
  KH_2PO_4 136.09 g
  Water to 1 l
- 5 × TBE buffer pH 8.3
  For 1 liter
  Tris Base 54 g
  Boric Acid 27.5 g
  0.5 M EDTA 20 ml
  Water to 1 l
- 50 × TAE buffer pH 7.6
  For 1 liter
  Tris base 242 g
  Glacial Acetic Acid 57.1 ml
  0.5M EDTA 100 m
  Water to 1 l
- 70% Ethanol
  For 100 ml
  Absolute Ethanol 70 ml
  Water 30 ml
- Typhoon™ Variable Mode Imager or equivalent.
- UV visible Spectrophotometer such as Gene Quant 1300 or Ultrospec 3100 *Pro*, or equivalent.
- Liquid scintillation counter, glass scintillation vials and scintillation fluid.
- Microarray slides

- Hybridization chamber
- Microarray scanner
- Oligonucleotide dA_{80}
- Microarray hybridization buffer (1 vial included with this CyScribe kit) GE Healthcare RPK0325
- 20 × SSC, GE Healthcare US75832
- 20% (w/v) SDS, GE Healthcare US19629
- Nuclease free water, GE Healthcare US70783

4. Description

The **CyScribe Post-Labeling Kit** provides reagents for preparation of Cy3 and Cy5 labeled cDNA probes.

This protocol has been developed as a two step procedure. The first step involves the incorporation of amino allyl-dUTP (AA-dUTP) during cDNA synthesis using an optimized nucleotide mix. The second step involves chemically labeling the amino allyl modified cDNA using CyDye NHS-esters.

The CyDye cDNA labeled probes generated with the CyScribe Post-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, coupling reactions of amino allyl-modified cDNA are performed separately with Cy3 and Cy5 and the two probes are combined in the hybridization solution. This enables simultaneous detection of hybridization signals and comparative analysis of gene expression levels.

cDNA synthesis

The synthesis reaction, catalyzed by CyScribe reverse transcriptase incorporates AAdUTP into first-strand cDNA. An optimized nucleotide mix is provided with the kit to be used in the synthesis reaction.

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 poly A-tail. This priming method is especially suitable if the hybridization targets on the microarrays 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 from mRNA. These random nonamer oligonucleotides 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 that is provided with the CyScribe Post-Labeling 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 figures 1 and 2 (see pages 16 and 17).

The kit has been developed for use with purified mRNA that is free from contaminating DNA, proteins or nucleotides. 100 ng to 1 mg of mRNA can be used as a template for the synthesis of amino allyl-modified cDNA when using the standard protocol described in this booklet.

Total RNA may be used in a modified cDNA synthesis protocol. The amount of oligo(dT) primers should be increased to 3 µl and the nonamer primers should be omitted from the labeling reaction. Less cDNA will be synthesized from total RNA than from the corresponding amount of mRNA.

Purification of amino allyl-modified cDNA

The amino allyl modified cDNA needs to be purified from RNA template, unincorporated nucleotides and any compounds containing amino groups in order to achieve optimum conditions for CyDye coupling. The RNA template can be degraded by alkaline hydrolysis treatment. After neutralization, the synthesis reactions are ready for purification. We recommend the use of illustra CyScribe GFX Purification Kit, which is supplied with CyScribe Post Labeling Kit RPN5660X, for the removal of free nucleotides and other reaction components from amine modified cDNA. Purification of cDNA can be performed in less than 30 minutes with these spin columns. Alternatively, the amine-modified cDNA can be purified by Ethanol precipitation.

Coupling reaction
The purified cDNA from each synthesis reaction is coupled with an excess of reactive CyDye to give a labeled cDNA probe. The amount of reactive CyDye has been optimized and pre-dispensed to enable one aliquot of CyDye to be coupled with cDNA produced from one synthesis reaction. The coupling reaction is terminated by the addition of Hydroxylamine. These steps can be completed in less than two hours.

Purification of CyDye labeled cDNA
The fluorescent cDNA probe needs to be purified from unreacted CyDye, in order to maximize hybridization signal and minimize non-specific background. We recommend illustra CyScribe GFX Purification Kit (27-9606-01) for the removal of free CyDye from fluorescently labeled cDNA. These columns give excellent recovery, typically 50% or higher, of labeled cDNA and are efficient in removing unincorporated nucleotides from the labeled cDNA. As an alternative purification system illustra AutoSeq G-50 (27-5340-01) can be used, although the recovery of labeled cDNA may be more variable. It is important that the amount of CyDye labeled material recovered from the labeling step is quantified before using it in a microarray hybridization. Do not purify more than one sample with one column. The reagents for removal of RNA template are not included in the CyScribe Post Labeling Kit.

Monitoring of cDNA yield and incorporation of AA-dUTP
High quality results from microarray hybridizations are usually achieved using the CyScribe Post-Labeling Kit. However, it is recommended to monitor the performance of the steps involved in probe production. The CyScribe Post-Labeling Kit provides several protocols for monitoring the stages involved in the production of CyDye labeled cDNA probes. These methods give different levels of information and the user may choose those methods that are

most suitable for their purposes. Spiking with [α-^{33}P]dATP provides quantitative information about cDNA yield and recovery after purification. UV visible spectrometry alone, or in conjunction with [α-^{33}P]dATP spiking, gives quantitative information about the amount of CyDye incorporated into the cDNA product. Finally, polyacrylamide or Agarose gel electrophoresis can be used to visualize the CyDye labeled probes. Protocols for performing these analyses are given on pages 26–38.

Use of CyDye labeled cDNA probes in microarray applications

cDNA probes prepared using the CyScribe Post-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 cDNA probes have been successfully used in dual color microarray hybridization assays to detect targets immobilized on microarray slides. Microarray hybridization buffer developed and optimized for giving high signal to noise ratios on aminosilane treated microarray slides from GE Healthcare is included in this 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 this CyScribe kit can be used successfully with different types of hybridization buffers.

Use of control reagents

The control mRNA supplied can be used to monitor the performance of the CyScribe Post-Labeling Kit for troubleshooting purposes, as well as for familiarization with the standard protocol. cDNA preparation and labeling using the control mRNA can be analyzed using the additional protocols described on pages 24–34. The quality control test for the CyScribe cDNA Post-Labeling Kit is based on the use of the control mRNA in the standard labeling protocol.

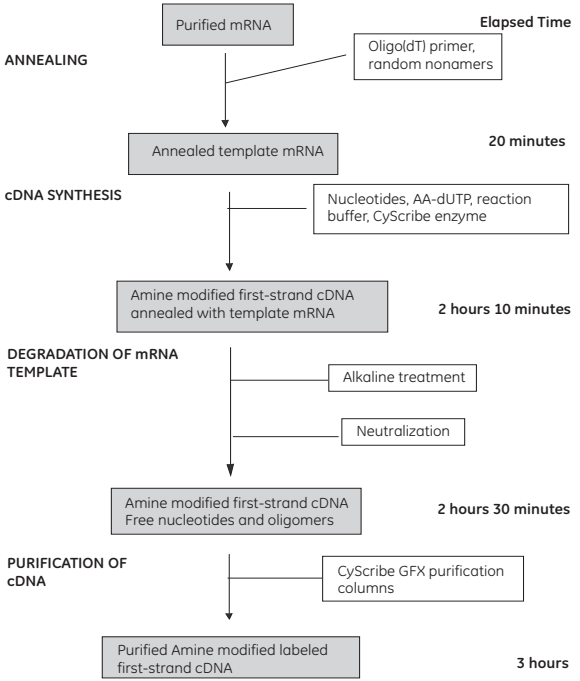

Figure 1. Preparation of amino allyl modified first-strand cDNA with CyScribe Post-Labeling Kit

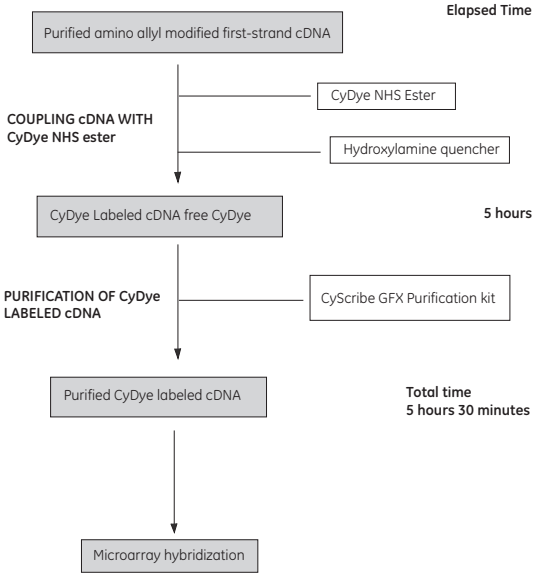

Figure 2. Preparation of CyDye labeled cDNA with the CyScribe Post-Labeling Kit

5. Protocol

5.1. Introduction

This protocol provides all the information required to use the CyScribe Post-Labeling Kit to produce Cy3 and Cy5 labeled cDNA 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.

5.2. Labeling first-strand cDNA with amino allyl-dUTP

In the standard labeling protocol, mRNA is first heat denatured to eliminate secondary structure and annealed with a mixture of anchored oligo(dT) and random nonamer primers. Alternatively, anchored oligo(dT) and random nonamers can be used separately. However, this will reduce cDNA yields. The standard protocol has been developed for the preparation of amino allyl-modified cDNA from 500 ng mRNA. 100–1000 ng of mRNA can be used as template in one synthesis reaction. Following the annealing step, reaction buffer, DTT, nucleotide mix, AA-dUTP and enzyme are added. Synthesis reactions are incubated at 42°C for 1.5 hours.

Total RNA can be used as a template for cDNA synthesis. It is important to omit the random nonamers when total eukaryotic RNA is being copied to cDNA in order to avoid copying ribosomal RNA. The amount of anchored oligo(dT) primer can be increased to 3 µl to increase the yield of cDNA from total RNA.

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

1. Set a water bath at 70°C and another at 42°C.

   Alternatively, primer annealing and extension incubation steps can be done with use of PCR tubes (200 µl) and a thermocycler,

2. Place the required reagents from the kit, excluding the enzyme, on ice to thaw. Place the enzyme on ice only **immediately prior to use.** Ensure that the contents of all tubes are thoroughly thawed and mixed.

3. Take one aliquot of lyophilized AA-dUTP and spin for 15 seconds in a microcentrifuge to collect all the AA-dUTP at the bottom of the tube. The lyophilized AA-dUTP may not be visible.

4. Resuspend the AA-dUTP in 30 µl nuclease free water. Ensure all contents have been completely resuspended. Record the reconstitution date. Place on ice.
   Note: After use, any remaining AA-dUTP solution should be stored at -15°C to -30°C and discarded after one month and a fresh aliquot prepared.

Primer annealing

1. Add the following components to a 1.5 ml microcentrifuge tube on ice:

mRNA, 500 ng	X µl
Random nonamers	1 µl
Anchored oligo(dT)	1 µl
Water (supplied)	Y µl
Total volume	11 µl

   The volumes corresponding to X and Y should be adjusted so that the total reaction volume is 11 µl. **If using total RNA omit the random nonamers and use 3 µl of anchored oligo(dT).**

2. Mix gently by pipetting up and down.

3. Incubate reactions at 70°C for 5 minutes.

4. Cool reactions at room temperature for 10 minutes to allow the primers and the mRNA template to anneal.

5. Spin down reactions for 15 seconds in a microcentrifuge.

Extension reaction

1. Place reactions on ice and add the following components to each, adding the enzyme last:

5 × CyScribe buffer	4 µl
0.1 M DTT	2 µl
Nucleotide mix	1 µl
AA-dUTP	1 µl
CyScribe reverse transcriptase	1 µl
Total volume	20 µl

Remove the CyScribe enzyme from freezer just before addition to reactions. Return the enzyme to a freezer at -15°C to -30°C immediately after use.

2. Mix by very gently pipetting or by stirring with a pipette tip and spin for 15 seconds in a microcentrifuge.
Note: Vigorous pipetting will denature the enzyme.

3. Incubate the reactions at 42°C for 1.5 hours.

4. Store the amino allyl modified cDNA on ice for immediate purification or place at -15°C to -30°C for storage. Do not store in a frost-free freezer.

5.3. Purification of amino allyl modified cDNA

It is necessary to remove mRNA from the single-stranded cDNA to promote hybridization of the cDNA probe to immobilized microarray targets and to minimize hybridization with complementary mRNA in solution. The removal of mRNA can be achieved in two steps: First mRNA is degraded into short oligomers with alkaline treatment, then these short oligomers, as well as unincorporated nucleotides,

are removed by spin column chromatography. We recommend the use of illustra CyScribe GFX Purification Kit for this purpose as these columns give consistent recoveries of amine modified cDNA. As an alternative, illustra GFX gel and PCR Purification Kit or ethanol precipitation can be used (see additional information section). Some loss of material will occur during the purification procedure. illustra CyScribe GFX Purification kit can be purchased with CyScribe Post Labeling Kit (RPN5660X).

Degradation of mRNA

1. Adjust a waterbath to 37°C.

2. Add 2 µl 2.5 M NaOH to each cDNA reaction.

3. Mix reactions by vortexing and spin for 15 seconds in a microcentrifuge.

4. Incubate reactions at 37°C for 15 minutes.

5. Add 10 µl 2 M HEPES free acid to each reaction.

6. Mix by vortexing and spin for 15 seconds in a microcentrifuge.

7. The cDNA reactions are now ready for purification or can be stored at -15°C to -30°C.

Purification of cDNA with illustra CyScribe GFX Purification kit

Solutions containing Tris can react with CyDye NHS esters, reducing the yield of CyDye labeling during the coupling step. Therefore, 80% ethanol (v/v, in water) is used as the wash buffer and 0.1 M Sodium bicarbonate is used as the elution buffer.

GFX purification of aminoallyl-labeled cDNA

Note: Sodium bicarbonate (0.1 M, pH 9.0) must be prepared by the researcher before proceeding with GFX purification. If the pH falls below 8.5, prepare fresh Sodium bicarbonate. (see page 9).

1. For every 20–100 µl of cDNA labeling reaction to be purified, place one GFX column into a clean collection tube. Add 500 µl of capture buffer to each GFX column.
2. Transfer the unpurified aminoallyl-labeled cDNA products into each GFX column; mix the cDNA by gently pipetting up and down 5 times.
3. Centrifuge each column immediately in a microcentrifuge at 13 800 × *g* for 30 seconds (equivalent to 13 000 rpm in an Eppendorf model 5415C microcentrifuge with an 18 position fixed-angle rotor).
4. Remove the GFX column and discard the liquid at the bottom of each collection tube. Return each GFX column into the used collection tube.
5. Add 600 µl of 80% ethanol to each column and centrifuge at 13 800 × *g* for 30 seconds.
6. Remove the GFX column and discard the collected liquid. Repeat step 5 twice for a total of 3 washes. After the final wash, discard the liquid and place each column back in the used collection tube.
7. Centrifuge each column at 13 800 × *g* for an additional 10 seconds to remove all traces of 80% ethanol in the tip of the column. Discard the collection tube.
8. Transfer each GFX column to a fresh 1.5 ml microcentrifuge tube and add 60 µl of 0.1 M sodium bicarbonate directly to the top of the glass fiber matrix in each GFX column. It is crucial that this buffer completely covers the membrane.
9. Incubate the GFX column at room temperature for 1–5 minutes. Centrifuge at 13 800 × *g* for 1 minute to collect the purified aminoallyl-labeled cDNA.

Note: Repeating step 8 might increase the yield of labeled cDNA yield by approximately 10%.

10. Proceed immediately to the coupling reaction procedure.

5.4. Labeling of amino allyl-modified cDNA with CyDye

Labeling of the cDNA is achieved when CyDye NHS–esters react with the amino allyl groups incorporated into the cDNA during first-strand synthesis.

The CyDye supplied with the CyScribe Post-Labeling Kit is derivatized to carry only one reactive group on each dye molecule. This allows for accurate labeling of amino allyl groups found in amino allyl modified cDNA.

The CyDye is supplied in optimized aliquots. Each aliquot contains the correct amount of CyDye for optimal labeling of purified amino allyl modified cDNA, produced from one synthesis reaction. The aliquots are supplied in individually sealed foil packs to protect the reactive dye from atmospheric moisture, which can degrade the reactive groups.

Addition of 4 M Hydroxylamine after CyDye labeling will inactivate any unreacted CyDye NHS-ester molecules.

Do not attempt to use one CyDye aliquot to label more than one complete cDNA reaction. The CyScribe Post-Labeling Kit has been developed to use the purified cDNA from one synthesis reaction with one CyDye aliquot.

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 and CyDye labeled cDNA to all light sources is kept to a minimum. Atmospheric moisture can degrade the reactive CyDye, so do not open the foil-packs containing CyDye vials until ready to use them in labeling reactions.

Post-labeling coupling reaction

1. Add the purified aminoallyl cDNA (in 0.1 M sodium bicarbonate) directly into one aliquot of CyDye NHS ester in a 1.5 ml tube. Resuspend the NHS ester completely by pipetting several times.

   Centrifuge at 13 800 × *g* for 1 minute to collect the liquid at the bottom of the tube.

2. Incubate at room temperature in the dark for 60–90 minutes.
3. Add 15 µl of 4 M hydroxylamine to each coupling reaction.
4. Pipette up and down several times to mix the components, and then incubate at room temperature in the dark for 15 minutes.
5. Purify the CyDye-labeled cDNA immediately according to the following protocol.

5.5. Purification of CyDye-labeled cDNA

Removal of unincorporated CyDye molecules is necessary for minimizing hybridization background and for improving the sensitivity of detection of low abundance targets. We recommend the use of illustra CySribe GFX Purification kit for this purpose as these columns give good recoveries of CyDye labeled cDNA and efficiently remove fragments shorter than 50 nucleotides. Some loss of material will occur during the purification procedure, typically 50% or more of labeled cDNA is recovered. Alternatively, illustra AutoSeq G-50 columns that contain Sephadex™ G-50 DNA grade can be used, although the recovery of labeled cDNA may be variable.

Purification of CyDye labeled cDNA with illustra CyScribe GFX Purification kit

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

Purification of labeled cDNA with illustra AutoSeq G-50 columns
Briefly dry the labeling reaction volume to 45 µl in a speed-vac. The final volume of the labeling reaction before purification should be 45 µl therefore **two AutoSeq columns are needed to purify one labeling reaction.**

Prepare the columns as follows:

1. Resuspend the resin in each column by vortexing gently.

2. Loosen the caps a quarter of a turn and snap off the bottom closures.

3. Place the columns into 2.0 ml screw-cap microcentrifuge tubes for support. Alternatively, remove the cap from standard microcentrifuge tubes and use this tube for support. If using a 1.5 ml microcentrifuge tube, 10–20 ml 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.

4. Spin the columns for 1 minute at 2000 × *g*.
It is very important that the right rpm corresponding to 2000 × *g* is used. The speed can be calculated as follows:

revolutions per minute $= (1000) \times (1786/r)^{1/2}$, where r is the radius of the rotor in mm. Measure the radius from the centre of the spindle to the bottom of the rotor bucket.

5. Start the timer and the microcentrifuge simultaneously. Use the columns immediately after preparation to avoid drying of the matrix.

6. Place the columns into new 1.5 ml amber microcentrifuge tubes (without cap, as above) and slowly apply 22.5 µl of the sample to the centre of each column. The sample should be applied to the angled surfaces of the compacted resin beds, being careful not to disturb the resin. Do not allow any of the liquid to flow around the sides of the resin.

7. Spin the columns for 1 minute at 2000 × *g*. Start the timer and the microcentrifuge simultaneously. The purified samples are collected in the microcentrifuge tubes. Discard the columns.
8. Pool the recovered material from the same labeling reactions and measure the total volume.

The purified CyDye labeled 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 38 in this booklet. It is recommended that, as a minimum, UV visible spectrophotometry be used to determine the amount of CyDye in each probe before microarray hybridizations (see page 33).

6. Additional information

6.1. Purification of amino allyl modified cDNA by Ethanol precipitation

The following protocol has been used successfully to purify Amino allyl modified cDNA.

1. Add 3 µl of 3 M Sodium Acetate to each cDNA sample.

2. Add 105 µl of absolute Ethanol.

3. Vortex to mix and spin in a microcentrifuge for 15 seconds.

4. Incubate on dry ice for 30 minutes or at -70°C for 1 hour.

5. Microcentrifuge at 13 000 rpm for 30 minutes, at ambient.

6. Remove the supernatant with a pipette, taking care not to disturb the pellet.

7. Add 1 ml of 70% (v/v) Ethanol to each sample.

8. Microcentrifuge at 13 000 rpm for 15 minutes, at ambient.

9. Remove the supernatant with a pipette, taking care not to disturb pellet.

10. Air dry the pellet and fully resuspend the cDNA in 40 µl of 0.1 M Sodium bicarbonate buffer.

11. Proceed with step 1 of Post-labeling coupling reaction as outlined on page 24. The purified cDNA is now ready for coupling with CyDye or can be stored at -15°C to -3°C.

6.2. Monitoring the performance of the CyScribe Post-Labeling Kit

In this section, protocols are provided for determining the initial yield of amino allyl modified cDNA and for determining the recovery of cDNA after purification. These protocols involve spiking the reactions with [α-^{33}P]dATP.

Protocols are also supplied for determining the amount of CyDye present in the labeled cDNA probe. Agarose or denaturing polyacrylamide gel electrophoresis may be used to determine the size and fluorescence of labeled cDNA.

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

Estimation of the yield of CyDye labeled cDNA with UV spectrophotometry

The amount of CyDye labeled cDNA that has been purified with illustra 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 Post-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.

6.3 Determination of the initial yield of cDNA with [α-^{33}P]dATP spiking

The yield of cDNA can be determined by incorporating a radioactive nucleotide into the synthesis reaction. [α-^{33}P]dATP is suitable for use as a spike as it does not interfere with the incorporation of AA-dUTP into cDNA nor with the subsequent use of the cDNA as a fluorescent hybridization probe. After synthesis, 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 the cDNA.

We recommend using [α-^{33}P]dATP, diluted in nuclease free water. 2 µl of a 1:10 dilution will contain 74 kBq, 2 µCi of [α-^{33}P]dATP. This 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 steps. Do not use ^{33}P-labeled nucleotides dissolved in colored or fluorescent buffers as these may interfere with CyDye detection.

6.4. Spiking synthesis reaction with [α-^{33}P]dATP

Set up a synthesis reaction according to the following protocol:

1. Set a water bath at 70°C and another at 42°C.

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

3. Prepare a fresh AA-dUTP solution only if required, as described on page 19.

4. In the annealing step, adjust the total volume to 9 µl by altering the amount of water added to the reaction. Pipette the following into a microcentrifuge tube:

mRNA, 500 ng	X µl
Random nonamers	1 µl
Anchored oligo(dT)	1 µl
Water (supplied)	Y µl
Total volume	9 µl

If using total RNA, omit the random nonamers and use 3 µl of anchored oligo(dT) primers.

5. Mix gently by pipetting up and down.

6. Incubate reactions at 70°C for 5 minutes.

7. Cool reactions at ambient for 10 minutes to allow the primers and the mRNA template to anneal.

8. Spin down the reactions for 15 seconds in a microcentrifuge.

9. Place the reactions on ice and add the following components to each, adding the enzyme last:

5 × CyScribe buffer	4 µl
0.1 M DTT	2 µl
Nucleotide mix	1 µl
AA-dUTP	1 µl
[α-^{33}P]dATP diluted 1:10	2 µl
CyScribe reverse transcriptase	1 µl
Total volume	20 µl

Remove the CyScribe enzyme from freezer just before addition to reactions. Return enzyme to freezer at -15°C to -30°C immediately after use.

10. Mix by very gentle pipetting or by stirring with a pipette tip and spin for 15 seconds in a microcentrifuge.

Note: Vigorous pipetting will denature the enzyme.

11. Incubate the reactions at 42°C for 1.5 hours.

12. Store the amino Allyl modified cDNA on ice for degradation of mRNA or place at -15°C to -30°C for storage. Do not store in a frost-free freezer.

13. Perform the protocol for degradation of mRNA as described on page 21.

14. Retain a minimum of 4 µl of the unpurified spiked reaction at 2–8°C for monitoring the purification of the amino Allyl modified cDNA.

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

Perform this analysis before proceeding with the purification of cDNA.

The incorporation of [α-^{33}P]dATP into cDNA is proportional to the yield of first-strand cDNA. The incorporated [α-^{33}P]dATP can be easily separated from unincorporated nucleotides using 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.

1. Prepare 20 × 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.
2. Pipette duplicate 1 µl samples of synthesis reactions along the marked line. The sample spots should be about 1 cm apart. Do not damage the PEI-cellulose layer with the tip.
3. Place the plate in a rectangular chromatography tank so that the bottom of the plate is immersed 2 cm deep in 1 M K_2HPO_4. Cover the tank and let sample separation take place. Ensure 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.
4. When the buffer front has reached the top groove, remove the plate from the tank and allow to air-dry.
5. Wrap the plate in plastic-wrap and expose to a Phosphor Screen for 1–6 hours. Care should be taken not to over-expose the Phosphor Screen as this will saturate the signal.

6. Scan the Phosphor Screen using a Typhoon scanner or equivalent with recommended settings. As an alternative to the use of Phosphor Screens, any instrument that can measure quantitatively the radioactivity associated with different spots on the chromatography plate can be used.
7. Use appropriate software, such as ImageQuant™, to calculate the proportion of [α-^{33}P]dATP associated with cDNA. Consult the manual provided with the software for details about performing the quantification.

6.6. Calculation of cDNA yield

Percentage of [α-^{33}P]dATP incorporated	= Y%
Amount of unlabeled dATP in reaction	= 4 nmol
∴ assume amount of unlabeled dATP incorporated	= Y% × 4 nmol
	= Y/25 nmol
∴ amount of total dNTPs incorporated	= 4 × Y/25 nmol
	= Y/6.25 nmol
Assume residue molecular weight of dNTP (1 mole)	= 330 g
∴ weight of cDNA synthesized	= 330 × Y/6.25 ng
	= 52.8 Y ng

6.7. Monitoring the purification of amino allyl modified cDNA

The success of the purification of amino allyl-modified cDNA can be monitored by spiking the synthesis reactions with [α-^{33}P]dATP as described on page 28. Recovery of material can be determined with liquid scintillation counting:

1. Remove 1 µl aliquots from spiked synthesis reactions, before and after purification.

2. Add 5 ml of aqueous scintillation liquid to each sample.
3. Count the samples with a liquid scintillation counter, with settings suitable for the detection of ^{33}P.
4. Assess the purity of recovered material with thin layer chromatography on PEI-cellulose, as described on page 29. 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.
5. Calculate the recovery of cDNA using the following equation:

$$\text{Recovery \%} = \frac{(\text{cpm after}) \times (\text{purity \%}/100) \times (\text{volume recovered from CyScribe GFX})}{(\text{cpm before}) \times (\text{incorporation\%}/100) \times (\text{volume purified})} \times 100$$

'cpm before and after' data is generated from liquid scintillation before and after purification.

'incorporation' data is generated from thin layer chromatography before purification.

'purity' data is generated from thin layer chromatography after purification.

6.8. Monitoring the purification of CyDye labeled cDNA

The success of the purification of CyDye labeled cDNA can be monitored by spiking the synthesis reactions with [α-^{33}P]dATP, as described on page 29. Recovery of material can be determined with liquid scintillation counting:

1. Remove 1 µl aliquots from spiked CyDye coupling reactions, before purification and after purification.
2. Add 5 ml of aqueous scintillation liquid to each sample.
3. Count the samples with a liquid scintillation counter, with settings suitable for the detection of ^{33}P.

4. Calculate the recovery of cDNA:

$$\text{Recovery \%} = \frac{(\text{cpm after}) \times (\text{volume recovered from purification})}{(\text{cpm before}) \times (\text{volume purified})} \times 100$$

Where volume purified = 45 µl

'cpm before' is generated from liquid scintillation data obtained before labeling but after purification.

'cpm after' is generated from liquid scintillation data after purification.

6.9. Determination of the incorporation of Cy3 and Cy5 into cDNA

The incorporation of Cy3 and Cy5 into amino allyl modified cDNA can be quantified with UV visible spectroscopy. Cy3 and Cy5 have absorption maxima 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 free CyDye 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 (see the GE Healthcare catalogue or web page www.gehealthcare.com/lifesciences for further details).

Denaturing polyacrylamide gel electrophoresis can be used to visualize the fluorescence of Cy3- and Cy5-labeled cDNA. This will also provide information about the size and purity of labeled cDNAs. Alternatively, Agarose gel electrophoresis can be used. Fluorescent cDNA separated in either type of gel can be analyzed with Typhoon variable mode Imager. This scanner has been developed for the detection of Cy3 and Cy5 fluorescence (see the GE Healthcare catalogue or web page www.gehealthcare.com/lifesciences for further details).

6.10. UV visible spectrophotometry for measuring CyDye incorporation

Before performing this protocol, purify labeled cDNA using illustra CyScribe GFX Purification Kit or illustra AutoSeq G-50 columns, according to the protocols on pages 21–24. Any residual unincorporated CyDye will interfere with the detection of Cy-labeled cDNA. The amount of CyDye in total cDNA can be used as a guide to optimizing the amount of label required as a hybridization probe. Additionally, the relative amount of Cy3 and Cy5 in dual color hybridizations can be adjusted to account for any imbalances in the detection of these dyes with scanning instruments. In order to relate the amount of CyDye to the amount of cDNA present in the measured dilution, the yield of cDNA, recovery of cDNA after purification and the purity of the cDNA needs to be determined as well. These can be determined with thin layer chromatography and liquid scintillation counting as has been described on pages 30–33. If using illustra CyScribe GFX Purification Kit the yield of cDNA can be estimated with spectrophotometry at 260 nm

1. For spectrophotometry, dilute an aliquot of CyDye labeled cDNA with nuclease free water. Depending on the volume of the measuring cells available, up to 20 µl of purified cDNA may be required. Typically, 20 µl of CyDye labeled cDNA diluted 1:5 will give absorbencies in the range of 0.01–0.1 units.
2. Measure the absorbance of the dilution against a blank at 550 nm for Cy3 and at 650 nm for Cy5 using cuvettes with a 1 cm path length. Ensure that the cuvettes are thoroughly clean before applying cDNA samples to them.
3. The cDNA can be recovered from the measuring cuvette, dried down and used for microarray hybridization.

The amounts of Cy3 and Cy5 incorporated into cDNA can be calculated from their respective extinction coefficients,

(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 = $\underline{(A/E)} \times (1/W) \times (Z) \times df \times 10^6$

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

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

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 = $(0.06/150000) \times (1/0.5) \times (20) \times 5 \times 10^6 = 80$ pmoles

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

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.

6.11. Polyacrylamide gel electrophoresis (PAGE)

For qualitative analysis of CyDye incorporation, denaturing polyacrylamide gel electrophoresis using standard sequencing gel apparatus, can be performed. The fluorescence of CyDye labeled

cDNA can be determined from these gels by scanning with a Typhoon Variable Mode Imager.

The success of purification in removal of free CyDye from the labeled cDNA can also be monitored from the gel. Successful purification should result in most fluorescence being associated with the cDNA.

The size range of the CyDye labeled cDNA can also be determined if an appropriate size marker is included on the gel during electrophoresis.

Agarose gels may be used in a similar manner. However, non-denaturing Agarose gel electrophoresis will not accurately reflect the size distribution of the CyDye labeled cDNA.

6.12. Preparation of polyacrylamide gels

1. Prepare a 6% (w/v) sequencing gel. Cast the gel according to the instructions provided with the PAGE equipment.
2. 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.
3. Further dilute 1, 2.5 and 5 µl aliquots of this 1:10 dilution to 5 µl with water. Add 2 µl of Formamide to each sample. Do not use normal loading/denaturation buffers that contain dyes such as Bromophenol Blue or Xylene Cyanol as these will interfere with the detection of fluorescence.
4. Dilute 4 µl of fluorescent markers (ALFexpress sizer, GE Healthcare) with 1 µl of water and add 2 µl of 50% (v/v) Formamide. The use of the ALFexpress sizer 50–500 will allow determination of the size range of the fluorescent cDNAs.
5. Denature these samples by boiling for 2 minutes at 95°C. Snap-cool on ice before loading on to the gel.

6. Load samples onto the gel and perform electrophoresis according to instructions provided with the equipment. Use 1 × TBE as a running buffer. Protect the samples from light during electrophoresis.
7. In order to help monitor the progress of electrophoresis, an aliquot of loading buffer containing Bromophenol Blue may be loaded into a side well on the gel, fully separated from the labeled cDNAs. Stop the electrophoresis when the Bromophenol Blue dye has reached the bottom of the gel.
8. Remove one of the gel plates before scanning and ensure that the back of the remaining plate is clean. Do not let the gel dry before scanning.
9. 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.

6.13. Use of control reagents

The CyScribe Post-Labeling Kit contains synthetic mRNA for control labeling reactions. This is a mixture of mRNAs of defined sizes: 0.24, 1.4, 2.4, 4.4, 7.5 and 9.2 kilobases. Enough reagent is provided for 12 control reactions of 500 ng mRNA per reaction. This mRNA can be used as template for cDNA synthesis in labeling reactions using the standard protocol on page 18. The yield of labeled cDNA can be determined by spiking the labeling reaction with [α-^{33}P]dATP as has been detailed on page 30. 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 20%.

The incorporation of the CyDye can be determined with either of the methods described on page 35–36. The incorporation of Cy3 and Cy5 into cDNA is expected to be equal when the control mRNA is used.

6.14. Guidelines for microarray hybridization

The guidance provided here is based on our experience with the GE Healthcare microarray system (www.gehealthcare.com/lifesciences) for further information) which includes microarray slides that have been chemically modified and optimized for use with immobilized cDNA 'target' sequences. We recommend that you refer to the instructions provided with your microarray analysis system, before setting up hybridizations, and make appropriate modifications to the protocol.

CyDye is 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 photo-stability, insensitivity to pH and high solubility in water.

100–170 pmol of CyDye can be incorporated into cDNA in a standard labeling reaction with the CyScribe Post-Labeling Kit, using 0.5 µg of mRNA. Not all of this cDNA will be recovered as some loss of labeled cDNA will occur during purification. Typical yield of probe varies from 40–100 pmol of CyDye incorporated into purified cDNA.

Labeled and purified fluorescent cDNA prepared with the CyScribe Post-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 of slides prior to hybridization and hybridization buffers for achieving optimal results. Although the CyScribe Post-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. At GE Healthcare, we recommend that 15 pmol of each CyDye is used per slide in a hybridization volume of 30 µl in Formamide-based hybridization buffer. This volume is suitable for use with a cover slip measuring 64 × 22 mm. Lower amounts of CyDye labeled cDNAs can be used successfully, but some loss of signal intensity and sensitivity of detection will occur.

6.15. Suggested microarray hybridization protocol

1. Pre-treat microarray slides that contain immobilized nucleic acid targets according to the instructions provided with your microarray system/slide manufacturer. Perform prehybridization according to instructions provided with your slides.
2. For dual color hybridization, combine Cy3- and Cy5-labeled cDNAs into one tube. Dry down the cDNA solution in a rotary evaporator, in an amber microcentrifuge tube. Protect the solutions from light.
3. Dissolve the cDNA in 6 µl of nuclease free water.
4. Denature the cDNA by heating at 95°C for 2 minutes.
5. Cool the cDNA solution on ice for 30 seconds.
6. Add 1.5 µl oligonucleotide dA_{80} (1 mg/ml) and mix well. The purpose of this step is to prevent the poly-T tail on the cDNAs interacting non-specifically with A-rich regions on the immobilized templates.

7. Incubate the mixture at 75°C for 45 minutes.
8. Add 7.5 µl of microarray hybridization buffer (supplied in the CyScribe Post-Labeling Kit) and 15 µl of 100% Formamide. Mix well and spin for 15 seconds in a microcentrifuge to collect all reaction components at the bottom of the tube.
9. Pipette the hybridization mixture onto a microarray slide on an area that does not contain targets and cover with a coverslip. 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 hybrization buffer.
10. Hybridize at 42°C for 14–18 hours in a humid hybridization chamber. Protect the slides from light during this and subsequent steps.
11. Wash hybridized slides with 1 × SSC, 0.2% (w/v) SDS pre-warmed to 55°C, for 10 minutes, at ambient 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. Follow with 2 × 10 minute room temperature washes with 0.1 × SSC, 0.2% (w/v) SDS pre-warmed to 55°C. Wash the slides for 2 × 1 minute in room temperature 0.1 × SSC. Prepare the wash buffers from stock solutions of 20 × x SSC and 20% (w/v) SDS using distilled water. Finally, dip the slides into distilled water for a maximum of 10 seconds. Immediately dry the slides with a gentle air stream. 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.

12. Scan the slides with a microarray scanner suitable for detecting Cy3 and Cy5 fluorescence according to the instructions provided with the scanner.

7. Troubleshooting guide

All batches of CyScribe Post-Labeling Kit are subjected to 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 synthesis and CyDye labeling reactions with the control mRNA provided in the kit according to the protocol detailed in the section: Use of control reagents, page 40. Monitor the yield of the cDNA and the incorporation of CyDye according to the protocols detailed in sections: Determination of the initial yield of cDNA with [α-^{33}P]dATP spiking and Determination of the incorporation of Cy3 and Cy5 cDNA, pages 29-35.
- Check the purity and concentration of mRNA. For efficient cDNA synthesis, it is important that the mRNA template should be free of contaminating ribosomal RNA, proteins and DNA. It is also important that the mRNA is accurately quantified before use in labeling reactions. See Ausubel *et al.* (2000) for additional information on purification, analysis and handling of mRNA. Always handle mRNA carefully to avoid ribonuclease contamination.
- Refer to the instructions provided by the supplier of your microarray system.

Problem	Possible cause and remedy
Little or no cDNA synthesized	1. **Possible cause:** One or more of the reaction components is missing. **Remedy:** Repeat synthesis reaction with all reagents 2. **Possible cause:** mRNA is contaminated with ribonuclease. **Remedy:** Check the quality of mRNA with gel electrophoresis/Northern blotting. 3. **Possible cause:** mRNA is contaminated with compounds that inhibit CyScribe reverse transcriptase. **Remedy:** Re-purify mRNA. 4. **Possible cause:** Lyophilized AA-dUTP has not been completely resuspended. **Remedy:** Reconstitute a fresh tube of lyophilized AA-dUTP making sure all contents are collected at the bottom of the tube before addition of water. 5. **Possible cause:** AA-dUTP has been reconstituted for longer than four weeks.

Problem	Possible cause and remedy
Little or no cDNA synthesized *continued.*	**Remedy:** Prepare a fresh AA-dUTP aliquot. 6. **Possible cause:** cDNA lost during purification method. **Remedy:** Use illustra CyScribe GFX Purification Kit (27-9606-01) to purify aminoallyl labeled cDNA.
No labeled cDNA longer than 300 bp present	1. **Possible cause:** mRNA is contaminated with ribonuclease. **Remedy:** Check the quality of mRNA with gel electrophoresis/Northern blotting. 2. **Possible cause:** Random nonamer primers were used in synthesis reactions with total RNA. **Remedy:** Use oligo(dT) only at optimized concentrations.
cDNA is not fluorescent	1. **Possible cause:** Reaction tubes have been exposed to light **Remedy:** Take care to avoid exposure of labeling reactions to daylight. Keep the reactions always in amber microcentrifuge tubes.

Problem	Possible cause and remedy
cDNA is not fluorescent *continued.*	**2. Possible cause:** CyDye aliquot has been exposed to light. **Remedy:** Repeat labeling reactions with fresh CyDye aliquot. **3. Possible cause:** Samples have been exposed to light during analysis of CyDye incorporation. **Remedy:** Repeat analysis and minimize exposure of samples to light at all times. **4. Possible cause:** cDNA has been lost in purification steps. **Remedy:** Monitor the amount of cDNA before and after purification. **5. Possible cause:** Inefficient cDNA labeling due to presence of unincorporated AA-dUTP and reaction buffer after purification of amino allyl modified cDNA. **Remedy:** Repeat cDNA synthesis reactions including [**α**-^{33}P]dATP, in order to monitor purification.

Problem	Possible cause and remedy
cDNA is not fluorescent *continued.*	**6. Possible cause:** $NaHCO_3$ (used to resuspend CyDye aliquots for labeling reaction) was not stored appropriately. **Remedy:** Use freshly made $NaHCO_3$ or aliquots stored at -15°C to -30°C.
	7. Possible cause: CyDye had not been dissolved immediately before use. **Remedy:** Always prepare this solution when ready to use it.
Low recovery of labeled cDNA	**1. Possible cause:** The Ethanol concentration in the wash buffer was less than 76%. **Remedy:** Add absolute Ethanol to wash buffer prior to use and cap bottle tightly for storage.
	2. Possible cause: Poor elution of fragments from CyScribe GFX column **Remedy:** Elution buffer must cover all of the capture membrane.

8. Appendix 1

Determination of accurate A_{260} values for Cy dye-labeled nucleic acids

1. *Determine the number of Cy dye molecules incorporated per molecule of nucleic acid*

- Molar concentration of dye (moles/liter) = (absorbance of sample at λ_{max} of Cy dye) × (dilution factor) ÷ (molar extinction factor)
- Calculate moles of incorporated Cy dye based upon the molarity calculated immediately above the volume of the original, UNDILUTED nucleic acid sample:
- FOI = Frequency of Incorporation = picomoles of dye incorporated × (324.5/nanograms of cDNA probe). Probes with FOI values in the range of 20–50 generate strong signals upon hybridization. FOI value <10 will generate weak signals from poor incorporation, whereas, at FOI >70, probes may also produce reduced hybridization signals from possible quenching between CyDyes fluors.

Note: Liters are indicated to reflect the units of the molar extinction coefficient. Volumes can be transformed mathematically into ml, µl or other units.

Constants

Extinction coefficients ($M^{-1}cm^{-1}$):

Cy2, Cy3, Cy3.5	150 000
Cy5, Cy5.5, Cy7	250 000

Absorption maxima (nm):

Cy2	489
Cy3	550
Cy3.5	581
Cy5	649
Cy5.5	675
Cy7	743

9. References

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

10. Related products

Product Name	Code
CyScribe Direct™ mRNA Labeling Kit	RPN5665
illustra GFX PCR DNA and Gel Band Purification Kit	28-9034-70
illustra AutoSeq G-50 purification columns	27-5340-01
ALFexpress sizer 50–500 50 loadings	27-4539-01
Microarray hybridization buffer	RPK0325
CyScribe First-Strand cDNA Labeling Kit	RPN6200/6200X/6201/6201X/6202/6202X
Extraction of RNA and mRNA See the GE Healthcare catalogue, section 5: DNA/RNA Synthesis and Purification	

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RPN5660PL Rev G 07-2008

Amersham
CyScribe Post-Labeling Kit
Product protocol card

RPN5660/5660X

Denaturing and Annealing	Amino allyl labeling	Degradation of mRNA	GFX purification of aminoallyl-labeled cDNA
• Mix the following in a 1.5 ml eppendorf tube: – mRNA, 500 ng – Oligo(dT) primer, 1 µl – Random nonamers, 1 µl – Water to 11 µl, y µl • Heat the mix at 70 °C for 5 minutes. • Let the mix cool at room temperature for 10 minutes.	• Add the following: – 5 x CyScribe™ buffer 4 µl – 0.1 M DTT, 2 µl – 20 x nucleotide mix, 1 µl – AAdUTP, 1 µl – CyScribe reverse transcriptase, 1 µl • Mix well and incubate at 42 °C for 1.5 hours	• 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	• Add 500 µl capture buffer to CyScribe GFX™ column. • Mix labeling reaction with capture buffer in column. • Spin at full speed for 30 seconds. • Add 600 µl of 80% ethanol to CyScribe GFX column. • Spin at full speed for 30 seconds. • Repeat wash twice. • Spin at full speed for 10 seconds to remove all traces of ethanol. • Place CyScribe GFX column into amber 1.5 ml tube. • Apply 60 µl 0.1M Sodium Bicarbonate, pH 9.0. • Incubate at ambient for 1–5 minutes. • Spin at full speed for 1 minute to elute.

CyDye labeling	Quenching	GFX purification of CyDye labeled cDNA
• Resuspend one aliquot of CyDye™ NHS ester in the aminoallyl cDNA solution (60 µl in 0.1 M $NaHCO_3$, pH 9.0) • Mix well • Incubate in dark at ambient for 60–90 minutes.	• Add 15 µl 4 M Hydroxylamine to each reaction • Mix well and incubate in the dark at ambient for 15 minutes	• Add 500 µl capture buffer to CyScribe GFX column. • Mix labeling reaction with capture buffer in column. • Spin at full speed for 30 seconds. • Add 600 µl of wash buffer to CyScribe GFX column. • Spin at full speed for 30 seconds. • Repeat wash twice. • Spin at full speed for 10 seconds to remove all traces of wash buffer. • Place CyScribe GFX column into amber 1.5 ml tube. • Apply 60 µl of elution buffer. • Incubate at ambient for 1–5 minutes. • Spin at full speed for 1 minute to elute.

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