cytiva

Amersham
CyDye (Cy3/Cy5)

Product Booklet

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1 Introduction

Product codes

RPN5661

25801079

25801080

About

Reactive Dye Protocols for Post Labeling Aminoallyl-cDNA and Aminoallyl-aRNA for Microarray Applications

Important

Read these instructions carefully before using the products.

Intended use

The products are intended for research use only, and shall not be used in any clinical or *in vitro* procedures for diagnostic purposes.

Safety

All chemicals should be considered as potentially hazardous. For use and handling of the products in a safe way, refer to the Safety Data Sheets.

CAUTION
These dyes are intensely colored and very reactive. Care should be exercised when handling the dye vial to avoid staining clothing, skin, and other items.

Storage

Store at -15°C to -30°C. Do not use if desiccant capsule in foil bag is either pink or green.

Expiry

The expiry date is stated on the product label.

2 Components

12 each of the following supplied as a foil pack dispensed with 40 000 pmol dye:

- Cy™3 mono NHS ester

  40 000 pmol dye
- Cy5 mono NHS ester 40 000 pmol dye

3 Description

CyDye™ Post-Labeling Reactive Dye Pack for the generation of highly fluorescent Cy3 and Cy5 labeled probes via post labeling (amino allyl) method. The only reactive CyDye optimized for microarray probe labeling.

CyDye Post-Labeling Reactive Dye Pack provides conveniently packaged, highly reactive Cy3 and Cy5 mono functional NHS ester to label probes via the post-labeling (amino allyl) route for use in microarray applications.

The CyScribe Post-Labeling Kit RPN5660 provides a fully optimized kit containing all of the reagents for successful microarray probe labeling via the post-labeling method (see related products below).

CyDye post-labeling reactive dye pack:

- Reactive CyDye specifically optimized for microarray probe labeling.
- Higher reactivity Cy3 and Cy5 dyes - guaranteed 40 000 pmol dye per vial and >75% reactive dye content.
- 12 ready-to-use aliquots of Cy3-NHS ester and 12 ready-to-use aliquots of Cy5-NHS ester, individually dispensed and packed in foil packs for protective storage (see *Fig. 1, on page 6* and *Fig. 2, on page 6*)

The CyDye post-labeling reactive dyes are supplied in individually dispensed aliquots, containing a guaranteed 40 000 pmol of dye and >75% reactive dye. Each vial is sufficient for one labeling reaction. Wastage of excess dye and the need for storage in aliquots is thus eliminated.

(Reactive dyes are highly susceptible to degradation by air humidity once opened even if stored in closely capped tubes. Tests have shown that the content of reactive groups decreases by about 1% per day when they are dispensed into aliquots and stored. Thus stored aliquots have a very limited shelf life).

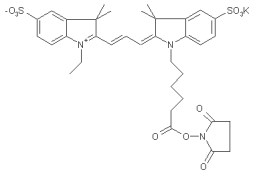

Fig 1. Cy3 mono NHS ester

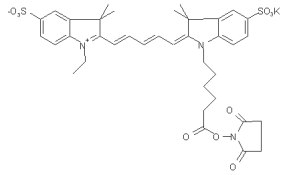

Fig 2. Cy5 mono NHS ester

The individually dispensed Cy3 and Cy5 reactive dye packs have been optimized for microarray labeling using post-labeling method. This method utilizes a chemical reaction to couple Cy3 and Cy5 reactive dyes on to synthesized cDNA containing aminoallyl groups (1). The post-labeling protocol consists of two steps. In the first step, mRNA is converted in to cDNA in the presence of aminoallyldUTP. After purification of synthesized cDNA from unincorporated nucleotides (and degraded RNA template), Cy3 and Cy5 reactive dyes are coupled to aminoallyl-cDNA (aa-cDNA) in the second step to generate probes for microarray hybridization.

The CyDye post-labeling reactive dye packs contain 40 nmoles of highly reactive Cy3 and Cy5 dyes that are individually dispensed and packed in foil packs ready to be used for aa-cDNA coupling and synthesis of microarray probes. Hybridization of Cy3 and Cy5 probes prepared using post-labeling method yields bright signals on arrays and generates highly accurate data for gene expression profiling (2). The individually packed Cy3 and Cy5 reactive dyes are also suitable for making labeled probes from invitro transcription products generated from Eberwine type (3) protocols utilizing aminoallylcontaining amplified RNA (aa-aRNA).

At Cytiva laboratories, we have developed two separate protocols to couple Cy3 and Cy5 reactive dyes to both aminoallyl containing cDNA (aa-cDNA) and aminoallyl amplified RNA (aa-aRNA). In protocol #1, Cy3 and Cy5 reactive dyes and aa-cDNA are mixed in 0.1 M sodium bicarbonate buffer (pH8.7) and is recommended only for cDNA coupling reactions. Protocol #2 utilizes addition of DSMO solvent to resuspend Cy3/5 reactive dyes and is optimized for aa-aRNA labeling. The labeling of CyDye reactive dyes to aa-cDNA/ aa-aRNA occurs efficiently under alkali range (pH 8.5-9.5) which is set using 0.1 M sodium bicarbonate buffer. It is important to make this buffer fresh prior to setting up labeling reactions. The recipe for making 0.1 M sodium bicarbonate buffer is found in appendix 1.

To observe bright signals on microarrays, labeling density (dye ratio) in the range of 30–60 nucleotides (one dye per every 30 to 60 unlabeled nucleotides) must be targeted for probes made from post labeling reactions. The labeling density can be controlled by titrating the amount of aa-cDNA amount (or aa-aRNA) used in the coupling step. CyDye are bright flours with high extinction coefficients and as a consequence do not

require very high density cDNA labeling for generating bright arrays. In fact, high density labeled cDNA/aRNA probes will eventually begin to reduce array signal from dye quenching effects. It is recommended not to achieve labeling density greater than 1 dye per very 30 nucleotides (dye ratios of less than 30) on microarray probes. The dye quenching effects for Cy5 are typically greater than Cy3 dye; hence more care must be taken to use Cy5 probes at optimal dye ratios.

It is recommended to always use protocol #2 for aa-aRNA labeling as Cy3/Cy5 reactive dye coupling to aRNA is usually 2-fold higher in the presence of DMSO. The coupling to aa-cDNA is not affected by DMSO and typically higher labeling yield is observed using protocol #1 with aa-cDNA.

General precautions on using Cy3 and Cy5 dyes for microarray applications

- Cy3 and Cy5 dyes are sensitive to light and must be stored and used in the dark as much as possible.
- It is highly recommended to perform all Cy3/Cy5 microarray hybridization and washing steps in the dark to prevent signal loss from dye photo-bleaching. Automated hybridization stations which perform microarray hybridization and washing steps in the dark (e.g. Cytiva, Lucidea SlidePro, 18116201) are recommended to obtain maximal signal from Cy3 and Cy5 dyes on arrays.
- The microarray slides must be completely dried immediately after the final washing step of a hybridization protocol prior to laser scanning for image acquisition. Wet arrays hybridized with Cy3/5 probes must have as little exposure to light as possible. Light exposure to arrays containing moisture or aqueous droplets will reduce signal, particularly from Cy5 channel, during scanning.
- Environmental conditions consisting of high humidity and/or high ozone levels have been shown to impact Cy5 signal and must be minimized when performing microarray hybridization and washing steps and during image acquisition.
- CyDye must be treated as toxic. Gloves must be worn at all times during handling of dyes.

4 Protocols for Post Labeling with Cy3 and Cy5 Reactive Dye Packs

Preparation of aa-cDNA or aa-aRNA

Prior to setting up the Cy3 and Cy5 reactive dye coupling reactions for microarray probe synthesis, prepare aa-cDNA or aa-aRNA according to standard methods. Purify the aminoallyl-cDNA or aa-aRNA away from unincorporated nucleotides, enzyme and buffer. Elute the aa-cDNA or aa-aRNA in water. Quantify the cDNA or aRNA yield using spectrophotometer by measuring absorbance at 260 nm. Proceed with recommended amount of aminoallyl-containing cDNA or aRNA template for Cy3 and Cy5 reactive dye coupling reactions. The CyScribe Post-Labeling Kit (RPN5660) is recommended for preparation of aa-cDNA for Cy3 and Cy5 reactive dye coupling reactions. For aa-aRNA preparation, Amino Allyl MessageAmp II aRNA amplification Kit (Ambion) is recommended and was used for Cy3 and Cy5 reactive dye protocol optimization.

Important considerations for setting up Cy3 and Cy5 reactive dye coupling reactions

- Tris-based buffers commonly used for eluting cDNA or aa-aRNA at the final purification steps must be avoided as amines on Tris-base compete with aa-cDNA/aa-aRNA for dye coupling.
- 0.5–2 µg of aa-cDNA or 3–10 µg of aa-aRNA can be used to label from a single vial of Cy3 and Cy5 reactive dyes (RPN5661).

- To observe bright signals from Cy3 and Cy5 dyes on microarrays, labeling density of 30–60 (one dye per every 30 to 60 unlabeled nucleotides) must be targeted for probe synthesis. Initially, titration of template amount may be required to hit this range depending on the method used to generate aa-cDNA or aa-aRNA.
- Make sure the reactive dye reaches ambient temperature before opening the vial (~20 minutes). After opening the vial, spin at maximum speed to collect all dye molecules at bottom of the tube.
- The final reaction volume for coupling reactions is indicated as 40 µL. The volumes may be reduced down to 20 µL by reducing the appropriate components, if desired.
- Use only RNase free reagents when working with RNA and when setting up aa-aRNA coupling reactions. Wear gloves at all times to prevent degradation of RNA.
- Quantify aa-cDNA yield using a spectrophotometer at an absorbance wavelength of 260 nm and use known quantity of template for labeling.
- The CyDye reactive dye coupling to aminoallyl-containing cDNA/aRNA template only occurs under alkali conditions between pH 8.5–9.5 which is set using 0.1 M sodium bicarbonate buffers. Make this buffer fresh prior to setting up labeling reactions (see Chapter Appendix).

Protocol #1 - For Cy3 and Cy5 Reactive Dye Coupling to aa-cDNA

Cy3 and Cy5 reactive dyes have high solubility in aqueous buffers and can be readily dissolved by adding 0.1 M sodium bicarbonate buffer (pH 8.7) directly to each vial. For coupling to aa-cDNA, dissolving Cy3 and Cy5 reactive dye with DMSO is not required. Maximal coupling of Cy3/5 reactive dyes to aa-cDNA template has been observed when both reactive dye and aa-cDNA are mixed together only in 0.1 M sodium bicarbonate buffer (pH 8.6–9.0). The CyScribe Post-Labeling Kit (RPN5660) is recommended for preparation of aa-cDNA for Cy3 and Cy5 reactive dye coupling reactions.

Step	Action
1	Spin dry the prepared aminoallyl-cDNA (aa-cDNA) to ~5 µL. Do not over-dry to a pellet as cDNA may become difficult to resuspend. It is important to guard the aa-cDNA from over-drying to completion to preserve the entire template for coupling.
2	Bring up the volume of aa-cDNA to 40 µL by adding appropriate volume of 0.1 M sodium bicarbonate buffer (pH 8.7). Pipette several times to completely mix/dissolve the aa-cDNA.
3	Open a vial of Cy3 and/or Cy5 reactive dye pouch after it has reached room temperature. Spin at maximum speed to bring contents to bottom of the tube.

Step	Action
4	Add the entire 40 µL of aa-cDNA (in 0.1 M sodium bicarbonate buffer) directly to a single tube of Cy3 and/or Cy5 reactive dye. Pipette up-and-down several time to resuspend the reactive dye/aa-cDNA mix.
5	Briefly spin at maximum speed using microcentrifuge to bring all contents to bottom of the tube.
6	Incubate at room temperature for 90 minutes in the dark to allow Cy3 and/or Cy5 reactive dye to couple to aa-cDNA.
7	Add 15 µL of 4 M Hydroxylamine to the reaction.
8	Incubate for 15 minutes at room temperature in the dark.
9	Proceed to purification of Cy3- and Cy5-labeled cDNA probes. Use of CyScribe GFX™ Purification Kit (27960601) is recommended for Cy3- and Cy5-probe purification. Quantify probe yield using a spectrophotometer at absorbance wavelength of 260 nm to measure cDNA yield and at absorbance wavelengths of 550 nm and 650 nm to quantify Cy3 and Cy5 dye incorporation, respectively.
10	Store labeled probes in the dark at -20ºC until needed for hybridization.

Typical Cy3- and Cy5 -cDNA Labeling Data

Following are examples of results obtained from two separate labeling reactions starting with 1500 ng of aa-cDNA and adding single vial of Cy3 and Cy5 reactive dyes. The probes were purified and yield quantified using a spectrophotometer. The aa-cDNA was prepared from human skeletal muscle mRNA using reverse transcription reaction as outlined in the CyScribe Post-Labeling Kit Instruction Manual (RPN5660).

Probe	O.D. 260 nm	O.D. 550 nm	O.D. 650 nm	Probe Volume, µL
Cy3-cDNA	0.41	0.173		80
Cy5-cDNA	0.32		0.260	80

Calculations from Cy3 and Cy5 labeling reaction:

Cy3 Reaction:

cDNA Yield: 0.41 * 37 µg/mL * 70 µL = 1214 ng

Cy3–cDNA Probe Yield: 0.173/0.15 M^{-1} (dye coefficient) * 80 µL = 92 pmoles of probe

Dye Ratio: [(1214 *1000)/324.5]/92 = 41 (labeling density of one Cy3 dye per 41 unlabeled nucleotides).

Cy5 Reaction:

cDNA Yield: 0.32 * 37 µg/mL * 80 µL = 947 ng

Cy5–cDNA Probe Yield: 0.26/0.25 M^{-1} (dye coefficient) * 80 µl = 83 pmoles of probe

Dye Ratio: [(947 *1000)/324.5]/83 = 35 (labeling density of one Cy5 dye per 35 unlabeled nucleotides).

Where cDNA length average is assumed to be 1000 bases and 324.5 is average molecular weight of nucleotide in cDNA.

Protocol #2 - Coupling Cy3 and Cy5 Reactive Dye to aminoallyl containing amplified RNA (aa-aRNA)

The individually dispensed CyDye packs can be used to label invitro transcription products made from Eberwine-type reactions that incorporate aminoallyl-UTP into aRNA. Cy3 and Cy5 reactive dye coupling to aa-aRNA is highly efficient when DMSO solvent (50%) is present during the labeling reaction. Typically, at least 2-fold higher yields are obtained from aa-aRNA labeling reactions when DMSO is included with 0.1 M sodium bicarbonate buffer (4). It is recommended to use entire contents Cy3 and Cy5 reactive dyes for a single labeling reaction, increasing aa-aRNA amounts to control labeling density.

As a guideline, 3–10 µg of aminoallyl-incorporated aRNA (aa-aRNA) is a good starting amount to label from a single tube of Cy3 or Cy5 reactive dye to make probes for hybridization on oligonucleotidebased arrays. For aa-aRNA preparation, Amino Allyl MessageAmp II aRNA amplification Kit (Ambion) is recommended and was used for Cy3 and Cy5 reactive dye protocol optimization. The template (aa-aRNA) amount may need to be titrated to hit desired labeling density, with optimal dye ratios being 30–60 for acquiring bright arrays.

NOTICE
RNase free reagents and consumable are absolutely required to prevent degradation of RNA.

Step	Action
1	Spin dry the aa-aRNA to ~5 µL. Do not over-dry to a pellet as aa-aRNA will become difficult to resuspend. It is important to guard the aa-aRNA from over-drying to preserve the entire template for coupling.
2	Bring up the volume of aa-aRNA to 20 µL by adding 0.1 M sodium bicarbonate buffer (pH 8.7). Pipette several times to completely dissolve/mix the aa-aRNA template. At least 25% (v/v) of reaction volume must contain 0.1 M sodium bicarbonate buffer.
3	Open Cy3 or Cy5 reactive dye pouches after it has reached room temperature. Spin at maximum speed to bring contents to bottom of the tube.
4	Add 20 µL of DMSO (Sigma D-8418) to each single tubes of Cy3 or Cy5 reactive dyes. Resuspend the dye by pipetting several times. Spin to collect all contents to bottom of the tube.
5	Add the entire 20 µL of Cy3 or Cy5 reactive dye (in DMSO) to aa-aRNA tube to bring total volume to 40 µL. If less than 20 µL of Cy3 or Cy5 reactive dye is used, make-up the remaining volume with DMSO. The final DMSO concentration must be at least 50% in the reaction. Pipette several time to mix the Cy3 or Cy5 reactive dye/aa-aRNA mix.

Labeling set-up:	
aa-aRNA template	10 µL
0.1 M Sodium Bicarbonate Buffer, pH 8.7	10 µL

Step	Action

Cy3 or Cy5 reactive dye in DMSO[1]	20 µL
Total Volume	40 µL

[1] Final DMSO concentration must be at least 50% in the labeling reaction.

6 Briefly spin at maximum speed to bring all contents to bottom of the tube.

7 Incubate at room temperature for 90 minutes in the dark to allowCy3 or Cy5 to couple to aa-aRNA.

8 Add 15 µL of 4 M Hydroxylamine to the reaction.

9 Incubate for 15 minutes at room temperature in the dark.

10 Proceed to purification of Cy3- or Cy5-aRNA probes using RNase-free reagents. Quantify probe yield using a spectrophotometer at absorbance wavelength of 260 nm to measure aRNA yield and at absorbance wavelengths of 550 nm and 650 nm to quantify Cy3 and Cy5 dye incorporation, respectively.

11 Store labeled probes in the dark at -70ºC until needed for hybridization.

Typical aRNA Labeling Data

Shown below are results from labeling 5 µg of aa-aRNA with single pouch of Cy5 reactive dye. The probes were purified, diluted 11-fold and yield quantified using a spectrophotometer.

Probe	O.D. 260 nm	O.D. 650 nm	Probe Volume, µL
Cy5-aRNA	0.42	0.38	20

Calculations:

aa-RNA Yield: 0.42 * 11 * 37 mg/mL * 20 µL = 3.4 µg

Cy5-aRNA Probe Yield: 0.38/0.25 M^{-1} (dye coefficient) *11 * 20 µL = 334 pmoles of probe

Dye Ratio: [(3440 *1000)/324.5]/334 = 32 (labeling density of one Cy5 dye per 32 unlabeled nucleotides).

5 Troubleshooting guide

Problem	Possible causes/solutions
Cy3 orCy5 probe labeling yield was poor.	• 0.1 M sodium bicarbonate buffer pH incorrect. The Cy3 and Cy5 reactive dye coupling reaction occurs under alkali conditions between the pH ranges of 8.5–9.5. Make fresh 0.1 M sodium bicarbonate buffer and pH to 8.7. Repeat coupling reaction. • Labeled probe was lost during purification. Check if there is absorbance at 260 nm. If no cDNA or aRNA was recovered then the labeled probe was likely lost during purification. Switch to CyScribe GFX Purification Kit for cDNA probe purification. • Insufficient 0.1 M sodium bicarbonate buffer was present in the coupling reaction. For efficient coupling of Cy3 and Cy5 reactive dyes to aa-cDNA or aa-aRNA, at least 25% of reaction volume (v/v) must consist of buffer, otherwise, labeling yields may be inconsistent or low. • The aminoallyl cDNA synthesis reaction was poor. The Cy3 and Cy5 reactive dyes couple to amine groups on cDNA generated by incorporation of aminoallyldUTP during first strand cDNA synthesis step. Quantify the aa-cDNA after synthesis. If the cDNA synthesis results in low yield, then fewer aminoallyl groups will be present for CyDye couplings. Repeat the aa-cDNA synthesis step, quantify the yield and repeat the reactive dye coupling reactions. Same applies when working with aa-aRNA template. Very low yield from invitro transcription reaction will impact CyDye reactive dye coupling. • mRNA or aa-aRNA degradation occurred. Analyze the cDNA or aRNA probes on denaturing 10% acrylamide gel to see if the products size distribution is between 0.3–2 Kb. If there are signs of degradation of RNA, then repeat invitro transcription step. If the cDNA distribution is small, then repeat aa-cDNA preparation.

Problem	Possible causes/solutions
Template (cDNA/ aRNA) yield dropped after labeling reaction	• Coupling volume reduced to small volumes. Reducing coupling volumes to less than 40 µL will impact cDNA/aRNA recovery during the purification step. Increase during the purification step. Increase coupling volume to recommended level of 40 µL.
Low CyDye signals from array	• Low Cy3/Cy5 dye incorporation into probes. Quantify probe yield using spectrophotometer at absorbance wavelength of 260 nm to measure cDNA/aRNA yield and at absorbance wavelengths of 550 nm and 650 nm for Cy3 and Cy5 incorporation, respectively. Use recommended amounts of probes for hybridization. • Dye ratios are too high. Make sure labeling density is in the range of 30–60. High dye ratios means incorporation was too low to observe bright signals on arrays. • Over labeling of probes. Over labeling can reduce signals from dye quenching effects. High density labeled probes is also difficult to purify using standard resins leading to low yields of cDNA or aRNA for hybridization. • Hybridized slides were wet during scanning. The microarray slides must be completely dried after hybridization and washing steps prior to scanning to prevent signal loss from dye bleaching. Avoid exposure to light during hybridization and washing steps of protocol.

6 Appendix

Preparation of 0.1 M sodium bicarbonate buffer (pH 8.6–9.0)

Step	Action
1	Add 4.2 g of $NaHCO_3$ to a litre flask.
2	Add 500 mL of water, and mix with a magnetic stirrer until the $NaHCO_3$ dissolves. The pH must be near 8.5.
3	Adjust the pH to 8.7 by slowly adding 10–30 mL solution of 0.1 M Na_2CO_3 and monitoring the pH using a pH meter.
4	Filter-sterilize the buffer. Autoclave if working with aa-aRNA.

7 Related products

CyScribe Post-Labeling Kit	24 reactions	RPN5660

- includes optimized, individually dispensed reactive Cy3 and Cy5 dyes
- highly efficient and even incorporation of Cy3 and Cy5 reactive dyes
- bright and even signals with low backgrounds
- features new enzyme-CyScribe which gives excellent yields of cDNA
- prime with anchored oligo (dT) and/or random primers
- as little as 100 ng of mRNA template can be used

- optimized reagents all in one kit, including proprietary microarray hybridization buffer

CyScribe post labeling kit contents

CyScribe reverse transcriptase, nucleotide mix, aa-dUTP, anchored oligo (dT), random nonamers, 5 × CyScribe reaction buffer, 0.1 M DTT, 12 ready-to-use aliquots of Cy3-NHS ester and 12 ready-touse aliquots of Cy5-NHS ester, nuclease free water, control RNA, 0.24 to 9.5 kb RNA ladder, microarray hybridization buffer, optimized protocols.

Also available for microarray probe labeling:

CyScribe Direct mRNA Labeling Kit	4 reactions	RPN5665
CyScribe First-Strand cDNA Labeling Module	25 reactions	RPN6200
CyScribe First-Strand cDNA Labeling Kit with 25 nmol Cy3dUTP and 25 nmol Cy5dUTP	50 reactions	RPN6201
CyScribe First-Strand cDNA Labeling Kit with 25 nmol Cy3dCTP and 25 nmol Cy5dCTP	50 reactions	RPN6202

CyDye nucleotides		
Cy3 dCTP	25 nmol	PA53021
Cy5 dCTP	25 nmol	PA55021
Cy3 dUTP	25 nmol	PA53022
Cy5 dUTP	25 nmol	PA55022
Cy3.5 dCTP	25 nmol	PA53521
Cy5.5 dCTP	25 nmol	PA55521
Cy3 dCTP	250 nmol	PA53031
Cy5 dCTP	250 nmol	PA55031
Cy3 dUTP	250 nmol	PA53032
Cy5 dUTP	250 nmol	PA55032

Value packs	
Cy3 dCTP 25 nmol × 5 plus Cy5 dCTP 25 nmol × 5	PA55321
Cy3 dUTP 25 nmol × 5 plusCy5 dUTP 25 nmol × 5	PA55322

Probe quantification	
Ultrospec™ 3100 *Pro* with CyDye probe specific algorithm	80211231 / 32 / 37 / 38
Microarray Hybridization Buffer	RPK 0325

8 References

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3. Use of this material to perform services for a fee for third parties, including contract research and drug screening.

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RPN5661PL AF V:4 05/2021