

Amino Allyl cDNA Labeling Kit

cDNA Synthesis for Secondary Fluorescent Labeling

Catalog #1705

Protocol



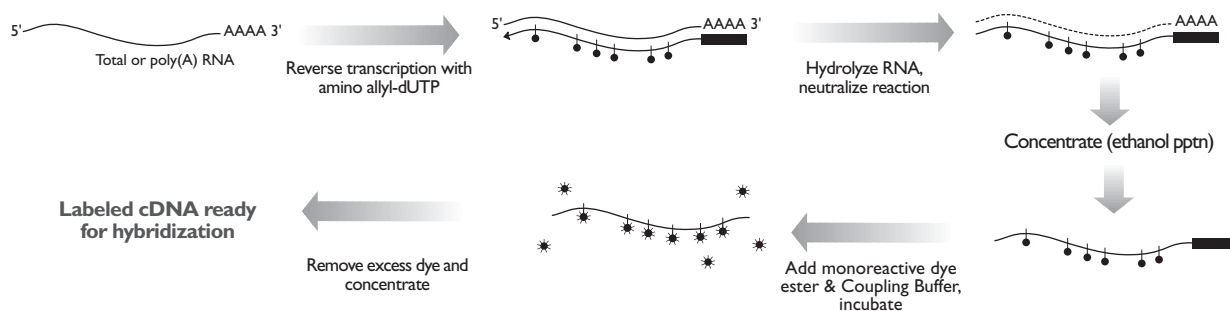
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A. Background

The Amino Allyl cDNA Labeling Kit uses a two step process to make fluorescent dye labeled cDNA for use in microarray hybridization experiments. In the first step, cDNA is made from sample mRNA by reverse transcription; one of the nucleotides (dTTP) is partially substituted with an analog containing a reactive primary amino group (i.e. amino allyl dUTP). After the reverse transcription reaction, the sample RNA is removed from the cDNA by alkaline hydrolysis, then the reaction is neutralized and the amino-modified cDNA is recovered by ethanol precipitation. In the second step, the amino modified cDNA is coupled to the fluorescent dye by incubation of the cDNA with the succinimidyl ester-derivitized reactive free dye (e.g. Cy3 or Cy5 mono-reactive NHS-ester). The coupling reaction is terminated by addition of hydroxylamine, and the reaction is passed through a NucAway Spin Column (included in the kit) to remove the free dye. If desired, paired Cy3/Cy5 labeled samples can be mixed before the spin column purification step. The labeled cDNA is then concentrated by ethanol precipitation. The kit is complete with all reagents except the ethanol and mono-reactive NHS-esters of fluorescent dyes.

Figure 1. Amino Allyl cDNA Labeling Process



B. Materials Provided with the Kit and Storage

Reagents for 15 cDNA labeling reactions.

Properly stored, the kit is guaranteed for 6 months from the date received.

The components to be stored at -20°C should be kept in a non-frost-free freezer.

Amount	Component	Storage
10 μl	RNA Control	-20°C
	Long term storage should be at -70°C	
30 μl	10X RT Buffer	-20°C
30 μl	Oligo(dT) Primers [Oligo(dT) ₁₈ , 50 μM]	-20°C
30 μl	Random Decamers (50 μM)	-20°C
15 μl	RNase Inhibitor (10 units/ μl)	-20°C
15 μl	dNTP Mix (no dTTP)	-20°C
	10 mM each: dATP, dCTP, dGTP	
15 μl	dTTP + AA dUTP Mix	-20°C
	3 mM dTTP, 3 mM Amino Allyl dUTP:	
	Amino Allyl dUTP is 5-(3-aminoallyl)-2'-dUTP	

Amount	Component	Storage
30 µl	M-MLV Reverse Transcriptase (200 units/µl)	-20°C
250 µl	3 M Sodium Acetate (pH 5.5)	-20°C
100 µl	Glycogen (5 mg/ml)	-20°C
1.75 ml	Nuclease-free Water	-20°C
60 µl	1 M Sodium Hydroxide	-20°C
500 µl	DMSO (100% dimethyl sulfoxide)	-20°C
68 µl	Coupling Buffer	-20°C
150 µl	1 M HEPES (pH 7)	-20°C
90 µl	4 M Hydroxylamine	-20°C
3.75 ml	Water for 75% ethanol Add 11.25 ml 100% ethanol before use	4°C
20 ml	Nuclease-free Water (for hydrating NucAway columns)	4°C
15	NucAway™ Spin Columns	room temp
15	2 ml wash tubes	room temp
15	1.5 ml collection tubes	room temp

C. Materials Not Provided with the Kit

- Ethanol: 100%, ACS grade or better
 - Fluorescent dye mono-reactive NHS-esters
 - Cy™Dye Post-Labeling Reactive Dye Pack, Amersham Biosciences Cat #RPN 5661
 - Cy™3 mono-Reactive Dye Pack, Amersham Biosciences Cat #PA23001
 - Cy5 mono-Reactive Dye Pack, Amersham Biosciences Cat #PA 25001
- This kit was developed with CyDye™ fluorescent dyes from Amersham Biosciences, but mono-reactive NHS-esters of any label moiety should be capable of coupling to the amino modified cDNA generated with this kit.
- Optional for experimental samples, but required for the positive control reaction:
 - [α -³²P]dATP, 800 Ci/mmol in aqueous buffer
 - 10% trichloroacetic acid (TCA), glass fiber filters, and other materials for TCA precipitation and/or materials and equipment for denaturing polyacrylamide gel electrophoresis (PAGE).
 - (optional) Fluorometer to measure the fluorescent intensity of the dye labeled cDNA

D. Related Products Available from Ambion

Poly(A)Purist™ Kits Cat #1916, 1919, 1922

The Poly(A)Purist Kits (patent pending) for mRNA purification from total RNA offer a level of mRNA unmatched by other protocols. These kits use a novel methodology to not only maximize binding of poly(A) RNA to the oligo(dT) matrix, but also to minimize non-specific interactions of ribosomal RNA. The kit is available with either oligo(dT) on magnetic beads: Poly(A)Purist MAG, or with oligo(dT) cellulose: Poly(A)Purist, and MicroPoly(A)Purist.

RNA Isolation Kits see Catalog

Family of kits for isolation of total or poly(A) RNA. Included in the product line are kits using classical GITC and acidic phenol, one-step disruption/denaturation, and phenol-free glass fiber filter binding.

DNA-free™ Cat #1906

DNase treatment and removal reagents for removing contaminating DNA from RNA preparations.

SlideHyb™ Glass Array Hybridization Buffers and Glass Array Hybridization Cassette
see Catalog

There are 4 unique SlideHyb Glass Array Hybridization Buffers; they have identical salt and formamide compositions, but differ in hybridization kinetics and blocking reagents. The SlideHyb Survey Kit, for protocol optimization, contains all 4 SlideHyb Buffers plus 10% SDS and 20X SSC to make wash buffers. The SlideHyb Glass Array Hybridization Buffers are available separately in larger volumes for routine use. Ambion also offers a hybridization cassette in which glass array hybridizations can be conveniently performed.

RNase-free Tubes & Tips
see Catalog

Ambion's RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free. See our latest catalog or our web site (www.ambion.com) for specific information.

Electrophoresis Reagents
see Catalog

Ambion offers gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis. Please see our catalog or our web site (www.ambion.com) for a complete listing as this product line is always growing.

RNA Century™ Marker and RNA Century-Plus Marker Templates
Cat #7780 & 7782

Templates for the transcription of 100–500 nt and 100–1000 nt RNA molecular weight markers.

E. Amino Allyl cDNA Labeling Protocol

Make 75% ethanol solution

Add 11.25 ml of ACS grade 100% ethanol to the bottle labeled *Water for 75% Ethanol*, and check the box on the label to indicate that the ethanol has been added.

F. Experimental Design

Total RNA or poly(A) selected RNA

Either total or poly(A) selected RNA may be used as template. In general cDNA yields and size distribution are somewhat higher when poly(A) RNA rather than total RNA is used as template, although the difference may not be dramatic. *Using total RNA instead of poly(A) RNA may especially compromise the detection of rare mRNA targets.* The decision on whether to use poly(A) selected RNA or total RNA as template should be based on the availability of sample RNA, the level of sensitivity required, and empirical results obtained with your microarrays.

RNA quantity

Generally, using more template results in synthesis of more cDNA, but the increase in cDNA yield may not be proportional to the increase in input RNA.

Poly(A) selected RNA

Between 0.5 µg and 5 µg of poly(A) RNA has been used with this kit.

Total RNA

Between 5 and 50 µg of total RNA has been used successfully in this procedure. Use 20 µg total RNA initially, then use more RNA in subsequent experiments if needed to increase hybridization signal from your microarrays.

RNA quality

Template RNA should be intact, essentially free of DNA contamination, and free of RT inhibitors such as heme, salts, phenol, proteins, etc.

Oligo(dT) or random primers

When total RNA is used as template, use only the Oligo(dT) Primers, not the Random Decamers. Random Decamers can prime cDNA synthesis from ribosomal RNA, which would compromise the production of cDNA from the intended mRNA templates. This could potentially decrease sensitivity and contribute to higher background signal on the array.

When poly(A) RNA (i.e. mRNA) is used as template, either or both types of primers may be used. cDNA yields are usually slightly higher when Random Decamers or a combination of Random Decamers and Oligo(dT) Primers are used, compared to using Oligo(dT) Primers alone.

The decision as to which type(s) of RT primer to use may also be influenced by the type of immobilized nucleic acids on the microarray. For microarrays that contain sequences from the 3' untranslated regions of mRNAs, it may be more appropriate to use only Oligo(dT) Primers, so that the cDNA synthesized will be targeted to those regions.

Another consideration is the extent to which the poly(A) RNA is contaminated with ribosomal RNA (rRNA); if the RNA preparation contains significant amounts of rRNA, it may be better to use only Oligo(dT) Primers.

G. Reverse Transcribe the RNA Sample

In this part of the procedure, sample RNA is reverse transcribed using amino alkyl-modified dUTP to produce cDNA modified with reactive primary amines.

Negative control

If you intend to assess the fluorescent intensity of the labeled cDNA from this procedure, prepare a negative control reaction that lacks either the template RNA, the reverse transcriptase, or both. Carry this minus-RNA/minus-RT control reaction through the dye coupling and clean-up steps. The negative control will be used to establish a baseline (background) fluorescent intensity value.

1. Mix RNA and RT primer, and denature at 75°C for ~7 min

a. In an RNase-free microfuge tube at room temp, mix the following:

Amount	Component
0.5–5 µg	poly(A) selected RNA (or 5–50 µg total RNA)
1 µl	Oligo(dT) Primers and/or Random Decamers*
-- µl	Nuclease-free Water: Bring the volume to 20 µl final volume, <i>including</i> the reaction components to be added in the next step.

* To use both Oligo(dT) Primers and Random Decamers, add 1 µl of each primer to the mixture.

b. Denature the template RNA

Heat the RNA, RT primer(s), and water to 75°C (±5°C) for 7 min; the exact time of heat denaturation is not critical and may be adjusted (5–10 min) depending on the amount of template used. This heat denaturation step should not be done in the presence of the 10X RT Buffer or the nucleotides.

After heating, briefly centrifuge the tube to collect the contents at the bottom, and keep the tube(s) at room temperature.

2. Add the remaining reaction components and mix well

Amount	Component
2 µl	10X RT Buffer
1 µl	RNase Inhibitor
1 µl	dNTP Mix (no dTTP)
1 µl	dTTP + AA dUTP Mix
(1 µl)	*(optional) [α - ³² P]dATP (800 Ci/mmol, 10–20 mCi/ml)
2 µl	M-MLV Reverse Transcriptase

* Add [α -³²P]dATP to the reaction if you intend to assess the yield and size of cDNA synthesized.

Mix thoroughly by flicking or gentle vortexing, then centrifuge briefly to collect the reaction at the bottom of the tube.

**NOTE:**

Keep the M-MLV Reverse Transcriptase and the RNase Inhibitor on ice while setting up the reaction.

**NOTE:**

The reaction can be scaled up to 50 μ l. When scaling up the RT reaction, be sure to also scale up the volumes of reagents used in steps 4, 5, and 6 below.

3. Incubate reaction at 42°C for 1.5 hr

The reaction may be extended to 2 hr if desired.

4. Add 4 μ l 1 M NaOH, mix thoroughly, and incubate at 65°C for 15 min

This treatment removes the template RNA by alkaline hydrolysis. It is important to remove the RNA template for efficient dye coupling in the next part of the procedure.

5. Add 10 μ l 1 M HEPES and mix thoroughly

The HEPES neutralizes the reaction.

**IMPORTANT!**

If the reaction was trace labeled with [α - 32 P]dATP, and you want to determine the percent of 32 P incorporated into cDNA, remove a 1 μ l aliquot of the reaction at this point and follow the instructions for TCA precipitation.

6. Recover the cDNA by ethanol precipitation

a. Add the following to the cDNA:

Amount	Component
3.4 μ l	3M Sodium Acetate
0.5–1 μ l	(optional) Glycogen*
100 μ l	100% ethanol

* The addition of glycogen at this step may contribute to background in some microarray hybridization protocols. Normally the labeled cDNA will precipitate efficiently without glycogen added as carrier, but adding glycogen may be beneficial if <2 μ g of template RNA was used in the reverse transcription.

b. Mix well, incubate at least 30 min at -20°C or colder.

c. Microcentrifuge for 15 min at maximum speed (12,000 x g or higher) at 4°C , then carefully aspirate and discard the supernatant.

d. Wash the cDNA pellet by adding \sim 0.5 ml of 75% ethanol and vortexing briefly. Microcentrifuge the tube for \sim 5 min at room temperature or 4°C , and carefully aspirate and discard the supernatant.

e. To remove the last traces of ethanol, re-centrifuge the tube containing the cDNA pellet briefly to collect all residual fluid at the bottom of the tube. Then use a fine-bore pipet tip, a short-bevel syringe needle, or a drawn-out Pasteur pipet and bulb to gently aspirate away the residual fluid.

H. Couple Dye to the Amino Modified cDNA**Fluorescent dye**

This kit was developed with CyDye fluorescent dyes from Amersham Pharmacia Biotech, but the coupling reaction is chemically compatible with mono-reactive NHS esters of other fluorescent dyes and labeling moieties.

I. Dissolve fluorescent dye in DMSO**CAUTION!**

DMSO is hygroscopic; to prevent it from becoming contaminated with water from the atmosphere, warm the tube to room temperature before opening, and recap it immediately after use.

Table 1.

Amersham Biosciences Cy™ Dyes	Preparation Instructions
CyDye Post Labelling Reactive Dyes (Cat #RPN5661)	These dyes are supplied ready-to-use, in single-use quantities. Resuspend one vial with 3 µl of DMSO and keep in the dark at room temp for up to 1 hr until you are ready to use it.
FluoroLink Cy5 and Cy3 monofunctional dye 5-pack (Cat #PA23001, PA25001)	These dyes are supplied in relatively large aliquots; resuspend one vial in 45 µl of DMSO. Store dissolved dye in the dark at -20°C.

- 2. Rehydrate NucAway Spin Column**
The NucAway Spin Columns that will be used in the next part of the procedure (section **I** starting on page 6) should be hydrated for at least 1 hr (up to 2 hr) just before use.
Add 650 µl of Nuclease-free Water (from the 25 ml bottle) to the NucAway Spin Column, cap the column, vortex vigorously for ~10 sec, tap out air bubbles, and store upright at room temperature for 1–2 hr.
- 3. Resuspend precipitated cDNA in 4.5 µl Coupling Buffer**
Add 4.5 µl Coupling Buffer to the precipitated cDNA (from step **6.c** on page **5**), and mix thoroughly by gentle vortexing. Centrifuge the tube briefly.
- 4. Add 2.5 µl Nuclease-free Water**
Mix gently but thoroughly and centrifuge the tube briefly.
- 5. Add 3 µl prepared dye and incubate in the dark for 1 hr at room temp**
a. Add 3 µl of the fluorescent dye from step **1** to each sample.
b. Mix thoroughly by brief gentle vortexing and centrifuge the tube briefly to collect the liquid.
c. Incubate 1 hr at room temperature in the dark (i.e. wrap the tube in foil and/or put it in a drawer or a dark room).
- 6. Add 6 µl 4 M Hydroxylamine, mix thoroughly, and incubate 15 min in the dark at room temp**
This quenching step terminates the coupling reaction. It prevents cross-coupling of free dyes between 2 reactions if they are mixed prior to spin-column purification.
a. Add 6 µl 4 M Hydroxylamine, mix thoroughly by brief gentle vortexing and centrifuge the tube briefly to collect the liquid.
b. Incubate 15 min at room temperature in the dark.

**IMPORTANT!**

The dye coupling reaction can be assembled in the light, but must be incubated in the dark.

- d. Store remaining dye solution at -20°C in a non frost-free freezer protected from light.

I. Purify and Concentrate the Dye Labeled cDNA

**NOTE:**

If 2 samples labeled with different fluorescent dyes will be mixed during microarray hybridization, it may be desirable to mix them before the spin column purification to ensure equal sample recovery from both samples. Mixing the samples, however, will make it impossible to separately assess the dye coupling efficiency for each label.

- 1. Centrifuge rehydrated NucAway Spin Column at 750 x g for 2 min**
Remove the bottom cap from the rehydrated NucAway Spin Column (from step **2** on page 6), place the column in a 2 ml wash tube (supplied) and centrifuge at 750 x g for 2 min to remove excess interstitial fluid. (750 x g corresponds to 3000 rpm on the Eppendorf Model 5415 microcentrifuge.) Note the orientation of the NucAway Spin Column in the rotor.

2. Pass the labeled cDNA through the rehydrated spin column

- Bring the volume of the cDNA preparation(s) to 85 μ l by adding Nuclease-free Water.
- Discard the 2 ml wash tube from the previous step, put the NucAway Spin Column into a 1.5 ml collection tube (supplied), and remove the upper cap from the column.



NOTE:

Make sure that none of the labeled cDNA runs down the side of the tube bypassing the column matrix, because this will result in failure to remove some of the free dye.

Slowly and carefully apply the labeled cDNA directly to the center of the gel bed at the top of the column without disturbing the gel surface or touching the sides of the column with the pipet or reaction mixture.

- Place the tube plus spin column in the rotor, maintaining the orientation used in the first centrifugation. Centrifuge at 750 \times g for 2 min.
- The dye labeled cDNA will run through to the 1.5 ml tube. Free dye is retained in the column matrix. The volume of flow-through should be approximately the same as the volume of sample applied to the column (plus-or-minus \sim 30%). Discard the spin column and continue with the procedure.
After spin column purification, the labeled cDNA should only be slightly colored, if at all. If it has a strong red (Cy3) or blue (Cy5) color to it, then the dye removal procedure most likely failed. Most of the color should remain in the column matrix.

3. Concentrate the labeled cDNA by ethanol precipitation

- Add the following to the labeled cDNA, and mix thoroughly:

Amount	Component
0.1 volume (0.5–1 μ l)	3 M Sodium Acetate (\sim 9 μ l) (optional) Glycogen*
2.5 volumes	100% ethanol (\sim 250 μ l)

* If Glycogen *was* used in the first precipitation (step 6 on page 5), then it will remain in the cDNA, and shouldn't be added to this precipitation.
If glycogen *was not* used in the first precipitation (step 6 on page 5) then adding glycogen may be beneficial if <2 μ g of template RNA was used in the reverse transcription, but it may contribute to background in some microarray hybridization protocols. Normally the labeled cDNA will precipitate efficiently without glycogen added as carrier.

- Incubate at least 30 min at -20°C or colder. The cDNA may be stored in ethanol for at least a week prior to recovering it for use in microarray hybridization.
- Microcentrifuge for 15 min at maximum speed (12,000 \times g or higher) at 4°C , then carefully aspirate and discard the supernatant.
- Wash the cDNA pellet by adding \sim 0.5 ml 75% EtOH and vortexing briefly. Microcentrifuge the tube for \sim 5 min at room temperature or 4°C , and carefully aspirate and discard the supernatant.
- To remove the last traces of ethanol, re-centrifuge the tube containing the cDNA pellet briefly to collect all residual fluid at the bottom of the tube. Then use a fine-bore pipet tip, a short-bevel syringe needle, or a drawn-out Pasteur pipet and bulb to gently aspirate away the residual fluid.
- The cDNA should form a small pellet, \sim 1–2 mm in diameter, which is visibly red (for Cy5) or blue (for Cy3).

4. Resuspend labeled cDNA as required for your protocol

The labeled cDNA can now be resuspended according to the protocol for microarray hybridization.

The labeled cDNA may alternatively be resuspended in \sim 10 μ l 10 mM EDTA or Nuclease-free Water and stored dark at -20°C .



IMPORTANT!

Minimize exposure of the labeled cDNA to ambient light during this and all subsequent steps to avoid photobleaching.