Amino Allyl cDNA Labeling Kit

(Part Number AM1705)

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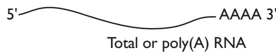
I. Introduction

A. Product Description and Background

The Amino Allyl cDNA Labeling 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 allyl-modified cDNA is recovered by ethanol precipitation. In the second step, the amino allyl-modified cDNA is coupled to the fluorescent dye by incubation of the cDNA with the succinimidyl ester-derivitized reactive free dye (e.g. Cy™3 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.

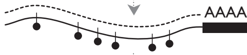
Figure 1. Amino Allyl cDNA Labeling Kit Procedure Overview

Reverse Transcribe the RNA Sample

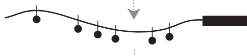


1. Mix RNA and RT primer, and denature at 75°C for ~7 min
2. Add the remaining reaction components and mix well
3. Incubate at 42°C for 1.5 hr

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

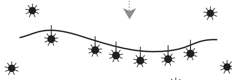


5. Add 10 μL 1 M HEPES and mix thoroughly
6. Recover the cDNA by ethanol precipitation



Couple Dye to the Amino Modified cDNA

1. Dissolve fluorescent dye in DMSO
2. Rehydrate NucAway Spin Column
3. Resuspend precipitated cDNA in 4.5 μL Coupling Buffer
4. Add 2.5 μL Nuclease-free Water
5. Add 3 μL prepared dye and incubate in the dark for 1 hr at room temp
6. Add 6 μL 4 M Hydroxylamine, mix thoroughly, and incubate 15 min in the dark at room temp



Purify and Concentrate the Dye-Labeled cDNA

1. Centrifuge rehydrated NucAway Spin Column at 750 x g for 2 min
2. Bring the labeled cDNA volume to 85 μL and pass it through the rehydrated spin column
3. Concentrate the labeled cDNA by ethanol precipitation
4. Resuspend labeled cDNA as required for your protocol

B. Materials Provided with the Kit and Storage

Reagents for 15 cDNA labeling reactions.

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

Amount	Component	Storage
10 µL	RNA Control Long-term storage should be below –70°C	–20°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) 10 mM each: dATP, dCTP, dGTP	–20°C
15 µL	dTTP + AA dUTP Mix 3 mM dTTP, 3 mM 5-(3 aminoallyl)-2'-dUTP	–20°C
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
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
15 mL	Water for 75% ethanol Add 11.25 mL 100% ethanol before use	4°C
1.75 mL	Nuclease-free Water	any temp*
20 mL	Nuclease-free Water (for hydrating NucAway columns)	any temp*
15	NucAway™ Spin Columns	room temp
15	2 mL wash tubes	room temp
15	1.5 mL collection tubes	room temp

* Store Nuclease-free Water at –20°C, 4°C or room temp.

C. Materials Not Provided with the Kit

- Ethanol: 100%, ACS grade or better
- Fluorescent dye mono-reactive NHS-esters
  – Amersham Cy™Dye Post-Labelling Reactive Dye Pack, GE Healthcare, Product Code: RPN5661
  – Amersham Cy™3 mono-Reactive Dye Pack, GE Healthcare, Product Code: PA23001
  – Amersham Cy5 mono-Reactive Dye Pack, GE Healthcare, Product Code: PA25001

  This kit was developed with Amersham Cy™Dye fluorescent dyes from GE Healthcare, 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-^{32}P]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 Applied Biosystems

Product	Description
Poly(A)Purist™ Kits P/N AM1916, AM1919, AM1922	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 web or print catalog for P/Ns	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, phenol-free glass fiber filter binding, and combination organic extraction/glass fiber filter binding kits.
DNA-*free*™ Reagents P/N AM1906	DNase treatment and removal reagents. This product contains Ambion® ultra-high quality RNase-free DNase I and reaction buffer for degrading DNA. It is ideal for removing contaminating DNA from RNA preparations. A novel reagent for removing the DNase without the hassles or hazards of phenol extraction or alcohol precipitation is also included.
SlideHyb™ Glass Array Hybridization Buffers and Glass Array Hybridization Cassette See web or print catalog for P/Ns	There are 3 unique SlideHyb Glass Array Hybridization Buffers; they have identical salt and formamide compositions, but differ in hybridization kinetics and blocking reagents. Ambion® also offers the Glass Array Hybridization Cassette for incubation of glass microarray hybridization reactions.

RNase-free Tubes & Tips See web or print catalog for P/Ns	Ambion® 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 website (www.ambion.com) for specific information.
Electrophoresis Reagents See web or print catalog for P/Ns	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 P/N AM7780 & AM7782	Templates for the transcription of 100–500 and 100–1000 nt RNA molecular weight markers. Also available as pre-transcribed biotinylated RNAs (P/N AM7175, AM7180)

II. Amino Allyl cDNA Labeling Procedure

A. Before You Start

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.

B. 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.

C. Reverse Transcribe the RNA Sample

In this part of the procedure, sample RNA is reverse transcribed using amino allyl-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, *including* 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) [a-^{32}P]dATP (800 Ci/mmol, 10–20 mCi/mL)
2 µL	M-MLV Reverse Transcriptase

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

Mix thoroughly by flicking the tube 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 µL. 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 at 42°C for 1.5 hr

Incubate the reverse transcription reaction at 42°C for 1.5 hr. Note that the reaction may be extended to 2 hr if desired.

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

a. Add 4 µL 1M NaOH and mix thoroughly.

b. 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 µL 1 M HEPES and mix thoroughly

Add 10 µL 1M 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 µL aliquot of the reaction at this point and follow the instructions for TCA precipitation in section III.A on page 13.

6. Recover the cDNA by ethanol precipitation

a. Add the following to the cDNA:

Amount	Component
3.4 μL	3M Sodium Acetate
0.5–1 μL	(optional) Glycogen*
100 μL	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 μg 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) at 4°C, then carefully aspirate and discard the supernatant.

d. Wash the cDNA pellet by adding ~0.5 mL of 75% ethanol and vortexing briefly. Microcentrifuge the tube for ~5 min at room temp 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.

D. Couple Dye to the Amino Modified cDNA

Fluorescent dye

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

1. 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. Cy™ Dye Preparation Instructions

	Preparation Instructions
Amersham CyDye Post-Labeling Reactive Dye Packs (Product Code: 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.
Amersham Cy5 and Cy3 mono-Reactive Dye Packs (Product Code: 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 II.E starting on page 11) should be hydrated for 1–2 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 temp 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.e on page 9), and mix thoroughly by gentle vortexing. Centrifuge the tube briefly.

4. Add 2.5 μL Nuclease-free Water

Add 2.5 μL Nuclease-free Water to each sample and mix gently but thoroughly, then 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).

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.

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.

E. Purify and Concentrate the Dye-Labeled cDNA

NOTE

If two 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 10), 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. Bring the labeled cDNA volume to 85 µL and pass it through the rehydrated spin column

a. Bring the volume of the cDNA preparation(s) to 85 µL by adding Nuclease-free Water.

b. 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.

c. 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 sample mixture.

IMPORTANT

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

d. Place the tube with spin column in the rotor, maintaining the orientation used in the first centrifugation. Centrifuge at 750 x g for 2 min.

e. 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 ~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

a. Add the following to the labeled cDNA, and mix thoroughly:

Amount	Component
0.1 volume	3 M Sodium Acetate (~9 µL)
(0.5–1 µL)	(optional) Glycogen*
2.5 volumes	100% ethanol (~250 µL)

* If Glycogen ***was*** used in the first precipitation (step 6 on page 9), 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 9) then adding glycogen may be beneficial if <2 µg 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.

b. 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.

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 ~0.5 mL 75% EtOH and vortexing briefly. Microcentrifuge the tube for ~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.

f. The cDNA should form a small pellet, ~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 procedure for microarray hybridization.

The labeled cDNA may alternatively be resuspended in ~10 µL 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.

III. Assessing Reaction Products

A. TCA Precipitation

If [α-^{32}P]dATP was added to the reverse transcription reaction, the reaction efficiency can be determined by trichloroacetic acid (TCA) precipitation. TCA is used to separate unincorporated nucleotides from nucleic acids. Following is an example of how to assess the reaction by TCA precipitation. The specific details (e.g. size of tubes, amounts of carrier and sample) are arbitrary and can be varied according to user preference.

1. TCA precipitate and count incorporated radiolabel

a. After adding HEPES to neutralize the cDNA in step 5 on page 8 (but before the ethanol precipitation in the next step), remove 1 µL of the reaction and mix thoroughly with ~100 µL of 1 mg/mL carrier DNA (for example, sheared salmon sperm DNA).

b. Add this mixture to ~2 mL of 10% TCA in a borosilicate tube (13 x 100 mm or 12 x 75 mm), vortex briefly to mix, and place on ice for ~5 min.

c. Assemble a glass fiber filter (e.g. Whatman GF/C filter circle) in a vacuum manifold, and wet it with 10% TCA solution

d. Collect the precipitated cDNA by vacuum filtration. Wash the filter twice with ~2 mL each time of 10% TCA. Use one of the TCA washes to rinse residual sample from the tube and apply this to the filter. Follow the TCA washes with a 95% ethanol wash (~3 mL).

e. Remove the filter from the vacuum manifold and immerse it in an aqueous scintillation cocktail (if using a toluene-based scintillation cocktail, the filter must be dried before counting). Count the TCA-precipitated material in a scintillation counter.

2. Count the total radiolabel in the reaction

a. Make a 1:100 dilution of 1 µL of the [α-^{32}P]dATP used in the reaction, and count 1 µL of the dilution in scintillation cocktail.

b. Multiply this number by 100 to determine the total cpm of [α-^{32}P]dATP present in the reaction. (Counting 1 µL of undiluted label will exceed the reliable range of the scintillation counter.)

3. Calculate the percent radiolabel incorporation

a. Multiply the cpm obtained from the 1 µL of TCA-precipitated cDNA (step 1.e on page 13) by 34 (the final reaction volume after base hydrolysis and neutralization) to determine the total number of cpm of cDNA produced.

b. Divide this number by the total cpm of [α-^{32}P]dATP used in the reaction (step 2.b on page 13). This will give the % incorporation of ^{32}P into cDNA, which is assumed to reflect the % incorporation of the total dNTP pool into cDNA.

Example

1 µL of the 34 µL reaction was TCA precipitated and found to have 20034 cpm.
The total cpm incorporated into cDNA was 34 x 20034 = 681156.
1 µL of a 1:100 dilution of [α-^{32}P]dATP was found to have 76406 cpm.
The total cpm in the reaction was 100 x 76406 = 7640600.
The % incorp'n of [α-^{32}P]dATP into cDNA is 681156 ÷ 7640600 = 8.91%.

Expected radiolabel incorporation

The % incorporation of ^{32}P dATP is related to cDNA yield: the higher the percent incorporation, the higher the yield of cDNA. The percent ^{32}P incorporation using this kit, however, is expected to be relatively low (usually <10%) because the concentration of unlabeled dATP in the reverse transcription reaction is high relative to the concentration of radiolabeled dATP. (This is in contrast to reaction conditions used to make small mass amounts of high specific activity radiolabeled probes, in which the concentration of the corresponding unlabeled nucleotide is very low).

B. Denaturing Polyacrylamide Gel Electrophoresis

1. Prepare a denaturing %5 acrylamide gel

Gel matrix is 5% acrylamide/8M urea gel in 1X TBE (Tris Borate EDTA)

15 mL is gel solution for one 13 cm x 15 cm x 0.75 mm gel

- Mix the following:

for 15 mL	Component
7.2 g	Urea (high quality) (Ambion® P/N AM9902)
1.5 mL	10X TBE
1.9 mL	40% Acrylamide (acryl: bis-acryl = 19:1) (Ambion® P/N AM9022, AM9024)
to 15 mL	water

- Stir at room temp until the urea is completely dissolved, then add:

120 µL	10% ammonium persulfate
16 µL	TEMED

- Mix briefly after adding the last 2 ingredients, which will catalyze polymerization, then pour the gel immediately.
- Use 1X TBE as the gel running buffer.

2. Prepare 1–2X denaturing gel loading buffer

This gel loading buffer is also available from Ambion®: Gel Loading Buffer II, P/N AM8546G.

Concentration	Component
95 %	formamide
0.025 %	xylene cyanol
0.025 %	bromophenol blue
18 mM	EDTA
0.025 %	SDS

3. Choose molecular weight markers for the gel

Suitable markers are in vitro transcripts of single-stranded RNA, for example those made with Ambion Century™-Plus Marker Templates, P/N AM7782. The mobility of single-stranded RNA and single-stranded cDNA of several hundred bases in length is approximately equal on denaturing polyacrylamide gels.

4. Load samples and run the gel

a. **Heat denature samples in loading buffer**
Mix 1 µL of sample with ~8 µL 1–2X denaturing gel loading buffer, and heat for 5 min at ~90°C.

b. **Load samples on the gel**
Flush the wells of the gel with 1X TBE to remove urea, and load the samples.

c. **Electrophoresis conditions**
Run the gel at about 20 V/cm gel length; for a 13 cm long gel this will be about 250 V. Alternatively, denaturing acrylamide gels of this size can be run at ~25 mAmp, constant current.

Stop the electrophoresis when the leading dye band (bromophenol blue) is near the bottom of the gel; this is usually about 1 hour.

5. Autoradiography

Transfer the gel to filter paper, cover it with plastic wrap and expose it to film for several hours to overnight with an intensifying screen.

6. Expected appearance of autoradiograph

- A successful Amino Allyl cDNA Labeling reaction should yield product that is mostly larger than 750 bases. The cDNA should migrate as a fairly compact band and >90% of it should migrate near the top of the gel (see Figure 2).
- The recovery of labeled cDNA by ethanol precipitation (at step 6 on page 9) can be estimated by comparing the intensity of the cDNA before and after ethanol precipitation.
- The extent to which unincorporated dNTPs were removed can be determined by comparing the intensity of the unincorporated [α-^{32}P]dATP (which runs near the bottom of the gel) between samples taken before and after ethanol precipitation. Typically, there will be very little or no unincorporated [α-^{32}P]dATP in the postprecipitation sample.

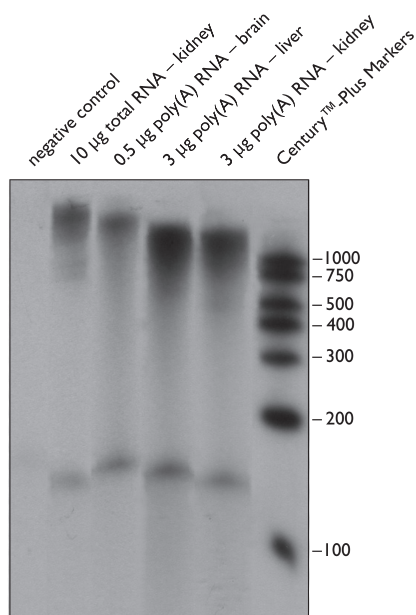


Figure 2. Amino Allyl cDNA Labeling Reactions Using Total RNA and Different Amounts of Poly(A) Selected RNA.

The indicated RNA samples were used in the Amino Allyl cDNA Labeling Kit; the negative control lacked both RNA and reverse transcriptase. A mixture of Oligo(dT) Primers and Random Decamers was used as primers, and 1 µL of [a-32P]dATP was added to trace label the reactions. After alkaline hydrolysis and neutralization, 1 µL aliquots of the reactions were removed and analyzed on a denaturing polyacrylamide gel as described in section III.B. Radiolabeled molecular size markers were generated from the Ambion® Century™-Plus Marker Templates (P/N AM7782). The unincorporated label that was present at the bottom of the autoradiograph is not shown.

C. Assessing the Dye Coupling Reaction in a Fluorometer

The dye coupling step can be assessed by reading the fluorescent intensity of the labeled cDNA in a fluorometer.

a. Take 10–20% of each labeling reaction (including the minus-RNA/minus-RT negative control reaction) and dilute to 50 µL (or to the volume required by the fluorometer) with water.

b. Set the fluorometer for the dye used in the labeling reaction.
   - For Cy5 labeled samples, use an excitation wavelength of 648 nm and an emission wavelength of 670 nm.
   - For Cy3 labeled samples, use an excitation wavelength of 550 nm and an emission wavelength of 592 nm.

Expected result

The fluorescent intensity of the labeled cDNA produced from 5 µg of poly(A) RNA template should be at least 10-fold greater than that of the minus-RNA/minus-RT negative control reaction. It is important that the sample be applied carefully to the NucAway column (see step 2 on page 11) to properly remove the free dye. If a significant amount of free dye is recovered, the fluorescent intensity of the labeled cDNA may not be 10-fold higher than the negative control.

The absolute fluorescent intensity values for Cy3 labeled samples (both experimental and negative control samples) are typically significantly lower than for Cy5 labeled samples.

D. Spectrophotometric Analysis of Dye Incorporation

1. Determine the appropriate dilution for spectrophotometer readings

Dilute 5 µL labeled cDNA in 95 µL 10 mM Tris-HCl, 1 mM EDTA (TE) (a 1:20 dilution). Mix well, place in a quartz cuvette and measure the A_{260} in a UV-Vis spectrophotometer.

If the A_{260} is below 0.1 reduce the dilution factor and check the A_{260} again. To obtain an accurate reading at both 260 nm and at the maximum absorbance wavelength for the dye, the cDNA dilution should result in an A_{260} between 0.1 and 1.0.

2. Measure the cDNA absorbance at 260 nm and at the absorbance max for the dye

Blank the instrument with the TE used for making dilutions. Measure the absorbance of each sample at 260 nm (A_{260}) and also at the maximum absorbance wavelength for the dye used in the coupling reaction (A_{dye}).

Absorbance maximum	Dye type	Extinction coefficient*
550 nm	Cy3	150,000
650 nm	Cy5	250,000

* Extinction coefficient (ε) at λ_{max} in $cm^{-1}M^{-1}$

3. Calculate the number of dye molecules incorporated per 1000 nt

- With these absorbance values (A_{dye} and A_{260}) in hand, use the convenient dye incorporation calculator on our web site. Find it at: www.ambion.com/techlib/misc/aama_dye_calc.html
- Alternatively use this formula to estimate the number of dye molecules incorporated per 1000 nt of labeled aRNA.

$$\frac{\text{\# dye molecules}}{\text{1000 nt}} = \frac{A_{dye}}{A_{260}} \times \frac{9010\ M^{-1}\ cm^{-1}}{\text{dye extinction coefficient}} \times 1000$$

IV. Troubleshooting

A. Positive Control Reaction

An RNA Control is provided with the Amino Allyl cDNA Labeling so that users can test whether the kit is working properly and as a troubleshooting tool; it is a mammalian poly(A) RNA sample. In the positive control reaction, the RNA Control is reverse transcribed with a trace ^{32}P label so that the reaction products can be easily quantitated and characterized. The yield of cDNA can be determined by TCA precipitation, and cDNA size can be assessed by polyacrylamide gel electrophoresis. The cDNA produced may also be used to assess the efficiency of the dye coupling step.

1. Positive control reverse transcription reaction set-up

If the positive control reaction will be dye labeled, include a negative control reaction that lacks the RNA Control and reverse transcriptase. This negative control will serve as a baseline for measuring the fluorescent intensity of the labeled RNA Control.

a. Mix RNA, RT primers, and water:

Amount	Component
10 µL	RNA Control
1 µL	Oligo(dT) Primers
1 µL	Random Decamers

b. Heat to 75°C for 7 min.

After the heat treatment, briefly centrifuge the reaction to collect the contents.

c. Add the remaining reaction components and mix gently but thoroughly:

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	[a-^{32}P]dATP (800 Ci/mmol, 10–20 mCi/mL)
2 µL	M-MLV Reverse Transcriptase

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

d. **Incubate at 42°C for 90 min.**

e. **Add 4 μL 1 M Sodium Hydroxide, mix thoroughly, and incubate at 65°C, 15 min.**

f. **Add 10 μL 1 M HEPES and mix thoroughly.**

g. **Remove a 1 μL sample for denaturing PAGE analysis.**
Remove 1 μL of the reaction, and save on ice or at –20°C for gel analysis (step 5 on page 20).

2. TCA precipitation of the positive control reaction

TCA precipitate and count 1 μL of the reaction. Also count 1 μL of the 1:100 dilution of [α-^{32}P]dATP, and calculate the % incorporation as described in section III.A on page 13. The positive control reaction should incorporate at least 5% of the ^{32}P.

3. Recover the cDNA by ethanol precipitation

a. **Add following to the cDNA:**

Amount	Component
3.4 μL	3M Sodium Acetate
1 μL	Glycogen
100 μL	100% EtOH

b. **Mix well, incubate for at least 30 min at –20°C, and microcentrifuge for 15 min at maximum speed at 4°C.**

c. **Carefully aspirate and discard the supernatant, and wash the cDNA pellet with ~0.5 mL 75% EtOH.**
(See section II.C step 6 on page 9 for detailed instructions).

d. **Continue with dye labeling of the cDNA (next step), or if the positive control reaction will *not* be dye labeled:**
Resuspend the pellet in 32 μL Nuclease-free Water (this will give the same nominal concentration of cDNA in the before-and-after ethanol precipitation samples, assuming that 1 μL of the initial 34 μL reaction was used for TCA precipitation and 1 μL was saved for gel analysis). Analyze 1 μL of the sample by denaturing PAGE as described in step 5 below.

4. (optional) Couple dye to the positive control cDNA

NOTE

If desired, an aliquot of the sample can be removed for gel analysis prior to the dye coupling step, but because of the small volume of the reaction at this point (step II.D.4 on page 10), even a 0.5 μL aliquot would represent a much larger proportional amount of the reaction compared to the 1 μL aliquots reserved for gel analysis at other steps.

a. Follow the procedure in section *II.D. Couple Dye to the Amino Modified cDNA* starting on page 9.

b. Continue with the column purification of the dye labeled cDNA (section II.E, steps 1 and 2 on page 11), but do not ethanol precipitate the cDNA.

c. Adjust the volume of the sample if needed, and measure its fluorescent intensity (and that of the negative control) in a fluorometer as outlined in section III.C on page 16.
   - If desired remove an aliquot for gel analysis.
   - Recovery can also be assessed by removing a second aliquot and measuring its radioactivity in a scintillation counter. (TCA precipitation is not required since unincorporated [α^{32}-P]dATP will have already been removed.)

5. Denaturing PAGE analysis of positive control reaction

Gel analysis of the positive control reaction will indicate whether the yield and size of the cDNA is as expected and will also provide information about cDNA recovery and removal of unincorporated nucleotides. Aliquots (1 μL) of the positive control reaction can be taken at any or all of 3 steps for gel analysis:

- After the neutralization at the end of the reverse transcription (step 1.g on page 19)
- After the first ethanol precipitation (step 3.d on page 19, see also note in step 4 above)
- After the dye coupling procedure

Instructions for denaturing PAGE are in section III.B starting on page 14.

6. Expected results of gel analysis of the positive control reaction

The bulk of the cDNA made from the RNA Control will migrate as a wide band near the top of a 5–6% denaturing acrylamide gel (see Figure 2 on page 16). The majority of the reaction products should be larger than 750 nt.

B. Poor Signal from Microarray Hybridization

Poor signal from microarray hybridization can be due to a number of causes, for example insufficient or degraded template RNA, or poor cDNA labeling. Poor results could also be caused by damaged or poor-quality microarrays, or suboptimal hybridization and/or washing conditions.

A complete discussion of factors affecting microarray analysis is beyond the scope of this manual, but useful information can be found on a number of websites (for example the DeRisi lab site, at http://www.microarrays.org/) and in microarray users' groups (for example, the NIH/NCI array users group, Microarray-User-L, which can be accessed and joined at http://NCIDCS@list.nih.gov/). The following suggestions may be useful for troubleshooting problems with cDNA synthesis.

1. Problems with the input RNA

Low yields (and/or production of low molecular weight cDNA) from an experimental RNA template, in the context of a successful cDNA synthesis reaction with the RNA Control, indicates a problem with the input RNA.

RNA quality

The quality of input RNA can be assessed by denaturing agarose gel electrophoresis and ethidium staining (for total RNA), or by Northern blot analysis (for poly(A) RNA). On denaturing agarose gels intact RNA migrates as distinct, sharp 28S (~5 kb) and 18S (~2 kb) ribosomal RNA bands, and the intensity of the 28S band should be greater (by ~2-fold) than the 18S band. Even poly(A) RNA often has enough residual ribosomal RNA to determine sample integrity by denaturing agarose gel electrophoresis.

RNA purity

Another issue related to template RNA is the possible presence of inhibitors of reverse transcriptase. The template RNA should be dissolved in nuclease-free water, or in Tris-EDTA buffer (10 mM Tris pH 7–7.5, 0.1–1 mM EDTA); do not include SDS or other detergents in the RNA preparation.

UV absorbance can be used to analyze RNA purity: the ratio of A_{260}:A_{280} of pure RNA should be between 1.9 and 2.1. The absorbance ratio is not, however, an indication of whether or not the RNA is intact.

2. Insufficient RNA template

Use poly(A) RNA, or use more RNA in the reaction

In general, greater sensitivity for detecting low abundance mRNAs can be achieved by using more template RNA, and by using poly(A) rather than total RNA in the reverse transcription. Yield of cDNA is generally significantly higher when poly(A) selected RNA is used as template rather than total RNA. Use 0.5–5 µg poly(A) RNA. If you are using total RNA, up to 50 µg can be used in the Amino Allyl cDNA Labeling reaction.

V. Appendix

A. Safety Information

The MSDS for any chemical supplied by Applied Biosystems or Ambion is available to you free 24 hours a day.

IMPORTANT

For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from product-specific links on our website at the following address: www.ambion.com/techlib/msds
- Alternatively, e-mail your request to: MSDS_Inquiry_CCRM@appliedbiosystems.com. Specify the catalog or part number(s) of the product(s), and we will e-mail the associated MSDSs unless you specify a preference for fax delivery.
- For customers without access to the internet or fax, our technical service department can fulfill MSDS requests placed by telephone or postal mail. (Requests for postal delivery require 1–2 weeks for processing.)

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

B. Quality Control

Functional Testing

The Control RNA is reverse transcribed following the instructions in section *IV.A. Positive Control Reaction* starting on page 18. The resulting cDNA is then labeled with both Cy3 and Cy5 fluorescent dyes following the procedure. The cDNA is analyzed by TCA precipitation and polyacrylamide gel analysis. The fluorescence intensity of the cDNA is shown to be at least 8 fold greater that the corresponding negative control reaction.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with 25 ng labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with 300 ng supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with 40 ng labeled *Sau*3A fragments of pUC19, and analyzed by PAGE.

Protease testing

Meets or exceeds specification when a sample is incubated with 1 µg protease substrate and analyzed by fluorescence.