Alexa Fluor® Reactive Dye Decapacks for Microarray Applications

Catalog no. A32750, A32756, A32757, A32755

Table 1. Contents and storage information.

Material*	Amount	Storage*	Stability
Each decapack contains vials of dried reactive dye packaged in one resealable pouch with a desiccant (Cat. no. A32750, A32756, A32757)	10 vials	• ≤–20˚C • Desiccate • Protect from light	When stored as directed the product is stable for at least 6 months.
Cat. no. A32755 consists of the Alexa Fluor® 555 reactive dye decapack (Cat. no. A32756) and Alexa Fluor® 647 reactive dye decapack (Cat. no. A32757) bundled together	10 vials each of Alexa Fluor® 555 and Alexa Fluor® 647 reactive dyes		
Number of assays: Each individual vial contains sufficient dye to optimally label cDNA produced from reverse transcription of 10–70 µg of total RNA or 1–5 µg of poly(A)$^+$ RNA in the presence of aminoallyl dUTP.			
*To minimize moisture contamination, keep pouches sealed and warm vials to room temperature before use.			
Approximate fluorescence excitation/emission maxima: See Table 2.			

Introduction

The Alexa Fluor® reactive dye decapacks are specially packaged succinimidyl ester dyes for labeling of DNA or RNA samples that have been modified by incorporation of aminoallyl-dUTP (Cat. no. A21664) or aminohexylacrylamido-dUTP (Cat. no. A32761). Each single-use vial contains sufficient dye to optimally label cDNA produced from reverse transcription of 10–70 µg of total RNA or 1–5 µg of poly(A)$^+$ RNA in the presence of aminoallyl dUTP. This two-step labeling method achieves extremely high and consistent labeling efficiencies, regardless of the dye chosen. It is especially useful for labeling cDNA for microarray applications, where the consistency of labeling between samples is critical for accurate interpretation of results.[1] In the first step, the amine-modified dUTP or UTP is easily and efficiently incorporated by the enzyme, without interference from bulky dyes. In the second step, the Alexa Fluor® succinimidyl ester dyes react to form a covalent bond with the introduced amine groups on the DNA or RNA. This method is highly efficient, resulting in fluorescent labeling of up to one dye every 12 bases.

Every packaging lot is rigorously tested for the ability to label aminoallyl-modified DNA to high and consistent levels. The Alexa Fluor® 488, Alexa Fluor® 555, and Alexa Fluor® 647 reactive dyes match the imaging channels of most popular microarray scanners. This manual includes an optimized protocol for incorporating aminoallyl dUTP or aminohexylacrylamido-dUTP into cDNA using a total RNA or poly(A)$^+$ RNA sample and reverse transcriptase, and then for labeling the amine-modified cDNA with the reactive dye in the decapack.

These specially packaged dyes can also be used to label amine-modified DNA or RNA created by incorporation of amine-modified dUTP or UTP using other types of enzymatic labeling reactions, although specific protocols are not included here.

Spectral Characteristics

For the best results in experiments, match the light source, excitation filters, and emission filters to the spectral characteristics of the dye. Refer to Table 2 for this information.

Before Starting

Materials Required but Not Provided

- SuperScript® III Reverse Transcriptase (Cat. no. 18080-093), or Superscript® II Reverse Transcriptase (Cat. no. 18064022), or SuperScript® Kits (Cat. no. L101402, L101602)
- 1 M NaOH
- 1 M HCl
- 3 M sodium acetate, pH 5.2
- 100% and 70% ethanol
- Nuclease-free water
- High-quality dimethylsulfoxide (DMSO)
- PureLink™ PCR Purification Kit (Cat. no. K3100-01 or K3100-02)

Experimental Protocols

Reverse Transcription

We have optimized a reverse transcription labeling protocol using 10–70 µg of total human RNA, 5 µg of anchored oligo(dT) primer ((dT) 20 VN), and SuperScript® II or SuperScript® III reverse transcriptase (Invitrogen). We have empirically determined that the ratio of dTTP to amine-modified dUTP used in this protocol, with subsequent labeling by an amine-reactive dye, results in optimally labeled samples for hybridization to microarrays.

1.1 Perform cDNA synthesis according to the reverse transcriptase manufacturer's protocol but use the following final concentrations of nucleotides: 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.15 mM dTTP, and 0.30 mM aminoallyl-dUTP. For aminohexylacrylamido-dUTP we recommend: 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.075 mM dTTP, and 0.225 mM aminohexylacrylamido-dUTP. A total reaction volume of 20–30 µL is usually sufficient.

Table 2. Spectral characteristics of the fluorescent dyes in the decapacks.

Cat. no.	Fluorescent dye	λ_{max} (nm)*	Em (nm)†	ε_{dye} ($cm^{-1}M^{-1}$)‡	CF_{260}§	Spectrally Similar Dyes
A32750	Alexa Fluor® 488	495	519	71,000	0.3	Cy™2, fluorescein (FAM), fluorescein isothiocyanate (FITC)
A32756, A32755	Alexa Fluor® 555	555	565	150,000	0.04	Cy™3, tetramethylrhodamine (TRITC, TAMRA)
A32757, A32755	Alexa Fluor® 647	650	670	239,000	0.00	Cy™5

*Absorbance maximum for the dye. †Emission maximum for the dye. ‡Extinction coefficient for the dye. §Correction factor = A_{260} for the free dye / A_{max} for the free dye.

1.2 Hydrolyze the RNA using the following protocol:

- Place the reverse transcription reaction at 95°C for 5 minutes to inactivate the reverse transcriptase and denature the RNA:cDNA hybrids. Snap cool by placing the reaction immediately into an ice bath.
- Add 0.43 volumes of 1 M NaOH for a concentration of 0.30 M, mix and incubate at 65°C for 15 minutes.
- Neutralize the solution by adding a volume of 1 M HCl equal to the volume of 1 M NaOH added in the previous step.
- Add 0.11 volumes (relative to the neutralized solution) of 3 M sodium acetate (pH 5.2).

Purifying Amine-Modified DNA

2.1 Bring the reaction mixture (from step 1.2) to a final volume of 100 µL with nuclease-free water. Purify the amine-modified DNA using a PureLink™ PCR Purification Kit (Cat. no. K3100-01 or K3100-02), following the instructions in the kit.

2.2 Perform an ethanol precipitation. This step is very important because it removes trace amines from the reaction mixture, which will interfere with the labeling reaction. Precipitate the amine-modified DNA by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol. Freeze at –80°C for 30 minutes and then centrifuge for 15 minutes at 12,000 rpm. Wash the pellet with 70% ethanol and allow it to air-dry.

Do not use ammonium acetate for precipitation as the residual ammonium ions interfere with the reactive dye labeling step. The addition of ~20 µg of glycogen helps to precipitate the DNA and generally results in greater DNA recovery.

Labeling with a Reactive Fluorescent Dye

3.1 Prepare the amine-modified DNA by thoroughly dissolving the amine-modified DNA (made in the previous section or by another labeling technique) in 5 µL of nuclease-free water, warming in a 42°C water bath for 5 minutes, if necessary.

3.2 Prepare labeling buffer by adding 25 mg of sodium bicarbonate to 1 mL of nuclease-free water and vortex the solution until the solid is completely dissolved. Store the labeling buffer at ≤–20°C in single-use aliquots. When properly stored, labeling buffer is stable for at least 6 months.

3.3 Add 3 µL of labeling buffer (prepared in step 3.2) to the amine-modified DNA (prepared in step 3.1).

3.4 Prepare fresh reactive dye by dissolving one vial of the reactive dye in 2 µL of high-quality DMSO. Vortex for about 10 seconds to ensure that the dye is completely dissolved. It may be useful to remove the label of the vial to see the dye more clearly. Once the reactive dye is dissolved, perform the reaction immediately. Dissolved dye cannot be saved for another day.

3.5 Add 8 µL of the amine-modified DNA (from step 3.3) to the dissolved reactive dye (from step 3.4). Vortex briefly to ensure that the reaction is well mixed.

3.6 Incubate the reaction in the **dark** at room temperature for 1 hour.

Purifying Dye-Labeled DNA

4.1 Add 80 µL of nuclease-free water and 10 µL of 3 M sodium acetate (pH 5.2) to the reaction

mixture and purify the labeled DNA using a PureLink™ PCR Purification Kit (Cat. no. K3100-01 or K3100-02), following the instructions in the kit.

4.2 Perform an ethanol precipitation of the labeled DNA as before (step 2.2). Resuspend the DNA pellet directly in deionized water.

Calculating the Labeling Efficiency and Concentration of Nucleic Acid

The relative efficiency of a labeling reaction can be evaluated by calculating the approximate ratio of bases to dye molecules.

This ratio can be determined, as described below, by measuring the absorbance of the nucleic acid at 260 nm and the absorbance of the dye at its absorbance maximum (λ_{max}). The calculations are based on the Beer-Lambert law:

$A = \varepsilon \times \text{path length (cm)} \times \text{concentration (M)}$

where ε is the extinction coefficient in $cm^{-1}M^{-1}$. The absorbance measurements can also be used to determine the concentration of nucleic acid in the sample. Values needed for these calculations are found in Tables 2 and 3. Alternatively, the ratio can be determined by using our Base:Dye Ratio Calculator on probes.invitrogen.com. Optimal labeling for hybridization to microarrays is 1 dye for every ~12–20 bases. Higher levels of labeling interfere with hybridization.

Measuring the Base:Dye Ratio

5.1 Measure the absorbance of the nucleic acid–dye conjugate at 260 nm (A_{260}) and at the λ_{max} for the dye (A_{dye}). Measure the background absorbance at 260 nm and λ_{max}, using buffer alone, and subtract these numbers from the raw absorbance values for the sample. The λ_{max} values for the fluorophores used in the decapacks are given in Table 2.

- To perform these measurements, the nucleic acid–dye conjugate should be at a concentration of at least 5 µg/mL. Depending on the dye used and the degree of labeling, a higher concentration may be required.
- For most applications, it will be necessary to measure the absorbance of the entire sample using a conventional spectrophotometer with a 100 µL or 200 µL cuvette or an absorbance microplate reader with a microplate, and then recover the sample after taking the measurement.
- Use a clean, nuclease-free cuvette or microplate that does not block UV light. Note that most plastic disposable cuvettes and microplates have significant absorption in the UV.

5.2 Correct for the contribution of the dye to the A_{260} reading. Most fluorescent dyes absorb light at 260 nm as well as at their λ_{max}. To obtain an accurate absorbance measurement for the nucleic acid, it is necessary to account for the dye absorbance using a correction factor (CF_{260}). Use the CF_{260} values given in Table 2 in the following equation:

$$A_{base} = A_{260} - (A_{dye} \times CF_{260})$$

5.3 Calculate the ratio of bases to dye molecules. Use the following equation:

$$\text{base:dye} = (A_{base} \times \varepsilon_{dye}) / (A_{dye} \times \varepsilon_{base})$$

where ε_{dye} is the extinction coefficient for the fluorescent dye (found in Table 2) and ε_{base} is the average extinction coefficient for a base in double stranded DNA (dsDNA) or RNA (found in Table 3). Note that since the calculation is a ratio, the path length has canceled out of the equation.

Measuring the Concentration of Nucleic Acid

The absorbance values A_{260} and A_{dye} may also be used to measure the concentration of nucleic acid in the sample ([N.A.]). To obtain an accurate measurement for a dye-labeled nucleic acid, a dye-corrected absorbance value (A_{base}) must be used, as explained in step 5.2. In addition, for concentration measurements, the path length (in cm) is required. If the path length of the cuvette or of the solution in a microplate well is unknown, consult the manufacturer. Follow steps 5.1 and 5.2 above and then use the following equation:

$$[\text{N.A.}]\ (\text{mg/mL}) = (A_{base} \times MW_{base}) / (\varepsilon_{base} \times \text{path length})$$

Table 3. Values for a base in different nucleic acids.

Nucleic Acid	ε_{base} ($cm^{-1}M^{-1}$)*	MW_{base}†
dsDNA	6600	330
RNA	8250	340

*Average extinction coefficient for a base. †Average molecular weight of a nucleotide residue (g/mol).

Reference

1. J Bacteriology 183, 7027 (2001).

Product List **Current prices may be obtained from our website or from our Customer Service Department.**

Cat. no.	Product Name	Unit Size
A32750	Alexa Fluor® 488 reactive dye decapack *for microarrays* *set of 10 vials*	1 set
A32756	Alexa Fluor® 555 reactive dye decapack *for microarrays* *set of 10 vials*	1 set
A32757	Alexa Fluor® 647 reactive dye decapack *for microarrays* *set of 10 vials*	1 set
A32755	Alexa Fluor® 555 and Alexa Fluor® 647 reactive dye decapacks *for microarrays* *set of 2 x 10 vials* *includes A32756 and A32757 decapacks*	1 set
Related Products		
A21664	aminoallyl dUTP (5-(3-aminoallyl)-2´-deoxyuridine 5´-triphosphate, trisodium salt) *2 mM in TE*	500 µL
A32761	aha-dUTP (5-aminohexylacrylamido- dUTP) *50 mM in TE buffer*	50 µL
K3100-01	PureLink™ PCR Purification Kit	50 reactions
K3100-02	PureLink™ PCR Purification Kit	250 reactions
L101402	SuperScript® Indirect cDNA labeling	30 reactions
L101602	SuperScript® Indirect RNA Amplification Kit	20 reactions
18064-022	SuperScript® II Reverse Transcriptase	2,000 units
18080-093	SuperScript® III Reverse Transcriptase	2,000 units

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