

aha-dUTP and aha-dCTP

Quick Facts

Storage upon receipt:

- $\leq -20^{\circ}\text{C}$

Molecular Weight: 702.33 g/mol**Concentration:** 2 mM in TE buffer (A32760, A32768), 50 mM in TE buffer (A32761, A32769)

Introduction

Aha-dUTP and aha-dCTP (5-aminoethylacrylamido-dUTP and -dCTP) can be used to produce amine-modified DNA by conventional enzymatic incorporation methods such as reverse transcription. The amine-modified DNA can then be labeled with any amine-reactive dye. This two-step technique consistently results in a uniform and high degree of DNA labeling that is difficult to obtain by other methods. Molecular Probes also provides a wide variety of amine-reactive reagents for labeling amine-modified DNA, including succinimidyl esters of our Alexa Fluor® dyes, conventional fluorescent dyes.

Protocols are provided for producing amine-modified DNA via reverse transcription, for purifying the modified DNA, and for labeling the purified sample using succinidyl ester-based labeling reagents. These protocols yield a labeling efficiency of about ~5–8 dyes per 100 bases, which we have found to be optimal for fluorescence *in situ* hybridization (FISH) and dot blot hybridization, and especially for microarray applications, where the consistency of labeling between samples is critical for accurate interpretation of results. The wide selection of labels that can be used and the consistent labeling results also make this technique ideal for other multicolor applications, such as multicolor FISH and comparative genome hybridization (CGH).

Materials

Storage and Handling

The aha-dUTP and aha-dCTP are supplied as 500 μL of a 2 mM solution or as 50 μL of a 50 mM solution in 10 mM Tris, 1 mM EDTA (pH 8.0). Upon receipt, store the aha-dNTP at $\leq -20^{\circ}\text{C}$. When stored properly, the reagent should be stable for at least six months.

Enzymatic Incorporation Protocols

Reverse Transcription

We have optimized a reverse transcription labeling protocol using 5 μg of a 1000-base, *in vitro*-transcribed RNA template and SuperScript™ II and SuperScript™ III reverse transcriptase (Invitrogen Corp.). We have empirically determined that the ratio of dTTP to aha-dUTP and dCTP to aha-dCTP used in this protocol, with subsequent labeling by an amine-reactive dye, results in optimal probes 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: **for reactions incorporating aha-dUTP**, 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.1 mM dTTP, and 0.2 mM aha-dUTP; **for reactions incorporating aha-dCTP**, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP 0.1 mM dCTP, and 0.2 mM aha-dCTP. In both cases, a total reaction volume of 20–30 μL is usually sufficient.

1.2 Hydrolyze the RNA as follows:

- 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 immediately placing the reaction vial into an ice bath.
- Add 1 M NaOH for a final concentration of 0.30 M, mix and incubate at 65°C for 15 minutes.
- Neutralize the solution by adding 1 M HCl equal to the volume of 1 M NaOH added in the previous step.
- Add 0.1 volumes (relative to the neutralized solution) of 3 M sodium acetate (pH 5.2).

1.3 Bring the mixture to a final volume of 100 μL with nuclease-free H_2O and proceed to *Purification of Amine-Modified DNA*, below.

Purification of Amine-Modified DNA

2.1 Purify the amine-modified DNA using a QIAquick PCR Purification Kit (QIAGEN), with the following modifications:

- Substitute 75% ethanol for the wash buffer provided with the purification kit.
- Substitute 50 μL of nuclease-free H_2O for the elution buffer provided with the purification kit and perform the elution twice for 5 minutes each.

Note: These purification steps are necessary to separate the amine-modified DNA from the enzyme and from amine-containing compounds such as Tris, which would react with the amine-reactive reagent in the labeling reaction.

2.2 Precipitate the DNA by adding 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol. Freeze at -70°C for 30 minutes and then centrifuge for 15 minutes at 12K 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 will interfere with labeling.

Labeling with an Amine-Reactive Reagent

Amine-modified DNA can be easily labeled with an amine-reactive dye. We recommend the use of succinimidyl ester (also called NHS ester) reagents for this reaction. Succinimidyl ester reagents react with primary amine groups at room temperature to form an amide bond, which is stable through even the most stringent hybridization procedures. A complete description of succinimidyl ester reagents available from Molecular Probes can be found in Chapter 1 of the *Handbook of Fluorescent Dyes and Research Products*, available online at www.probes.com/handbook.

The following protocol was optimized for use with amine-modified DNA made as in *Enzymatic Incorporation Protocols*, above, and succinimidyl esters of our Alexa Fluor[®] dyes. This protocol also works well for Pacific Blue[™] and Oregon Green[®] succinimidyl esters, and should work well for succinimidyl esters of many other fluorescent dyes. However, Alexa Fluor[®] 633 succinimidyl ester should not be used, because this dye does not form bright conjugates with nucleic acids using this protocol.

3.1 Prepare Labeling Buffer by adding 1 mL of nuclease-free H₂O to 85 mg of sodium bicarbonate and vortexing the solution until the solid is completely dissolved. Labeling Buffer should be stored at $\leq -20^{\circ}\text{C}$ in small aliquots. Before use, Labeling Buffer should be thawed completely and vortexed to ensure that the bicarbonate is completely in solution. When stored properly, Labeling Buffer should be stable for at least 6 months.

3.2 Thoroughly resuspend 1–5 μg of amine-modified DNA (made in the previous section or by another technique) in 5 μL of nuclease-free H₂O, warming in a 42°C water bath for 5 minutes if necessary.

3.3 Add 3 μL of Labeling Buffer (prepared in step 3.1) to the amine-modified DNA.

3.4 Make a stock solution of the amine-reactive reagent by dissolving it in an appropriate solvent at a concentration of 30 $\mu\text{g}/\mu\text{L}$. For Alexa Fluor[®] succinimidyl esters, use the actual weight of the dye, without regard to its stated reactivity. Vortex for about 10 seconds to ensure that the dye is completely dissolved. It may be useful to remove the label of the vial in order to see the dye more clearly. Approximately 2 μL of the stock solution is sufficient to label 1–5 μg of amine-modified DNA.

- Most fluorescent dyes dissolve well in dimethylsulfoxide (DMSO). Alexa Fluor[®] 350, Alexa Fluor[®] 568, and Pacific

Blue[™] dyes should be dissolved in dimethylformamide (DMF). Use only high quality solvents with a very low water content. DMSO is especially hygroscopic and will absorb water from the air very readily.

- Once the reactive dye has been dissolved, the reaction should be performed immediately. If high quality anhydrous solvent is used, any excess reactive dye may be stored at $\leq -20^{\circ}\text{C}$, desiccated and protected from light. However, some loss of activity may occur. Succinimidyl ester reagents will hydrolyze in the presence of water or amine-containing impurities in the solvent. Great care should be exercised to prevent exposure to water during storage. The vial should be warmed to room temperature before opening to prevent condensation of water inside the vial.

3.5 Add 2 μL of the dissolved dye (made in step 3.4) to the tube containing the amine-modified DNA and Labeling Buffer. Vortex thoroughly (15 seconds) to ensure that the reaction is well-mixed.

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

3.7 Add 80 μL of nuclease-free H₂O and 10 μL of 3 M sodium acetate (pH 5.2) to the reaction mixture and purify the labeled DNA using QIAquick PCR Purification Kit (QIAGEN), following the instructions in the kit, except perform three washes instead of one.

3.8 Precipitate the labeled DNA as before (step 2.2).

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 by measuring the absorbance of the nucleic acid at 260 nm and the absorbance of the dye at its absorbance maximum (λ_{max}) and by using the Beer-Lambert law:

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

where ϵ is the extinction coefficient in $\text{cm}^{-1}\text{M}^{-1}$. 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 1 and 2.

Measuring the Base:Dye Ratio

4.1 Measure the absorbance of the DNA–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 many commonly used fluorophores are given in Table 1.

- To perform these measurements, the DNA–dye conjugate should be at a concentration of at least 5 $\mu\text{g}/\text{mL}$. 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 either a conventional spectrophotometer with a 100 or 200 μL cuvette or an absorbance microplate reader with a microplate.

Table 1. Absorption characteristics for commonly used dyes.

Fluorescent Dye	λ_{\max} (nm) *	ϵ_{dye} (cm ⁻¹ M ⁻¹) †	CF ₂₆₀ ‡
Alexa Fluor® 350	345	18,400	0.25
Alexa Fluor® 488	492	62,000	0.30
Alexa Fluor® 532	525	82,300	0.24
Alexa Fluor® 546	555	104,000	0.21
Alexa Fluor® 555	555	150,000	0.04
Alexa Fluor® 568	576	93,000	0.45
Alexa Fluor® 594	588	80,400	0.43
Alexa Fluor® 647	650	239,000	0.00
Alexa Fluor® 660	660	107,000	0.00
Alexa Fluor® 680	680	164,000	0.00
BODIPY® 630/650	632	100,900	0.09
BODIPY® 650/665	651	101,800	0.07
BODIPY® FL	504	68,000	0.00
BODIPY® TMR	535	57,800	0.15
BODIPY® TR	588	55,000	0.11
Cascade Blue®	400	27,000	0.18
Fluorescein	494	80,000	0.32
Oregon Green® 488	494	80,000	0.31
Pacific Blue™	410	36,000	0.15
Rhodamine Green™	500	78,000	0.24
Tetramethylrhodamine	550	100,000	0.27
Texas Red®	593	85,000	0.23

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

Table 2. Average values for bases in different nucleic acids.

Nucleic Acid	ϵ_{base} (cm ⁻¹ M ⁻¹) *	MW _{base} †
dsDNA	6600	330
ssDNA	8919	330

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

- Use a cuvette or microplate that does not block UV light and that is clean and nuclease-free. Note that most plastic disposable cuvettes and microplates have significant absorption in the UV.

4.2 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 therefore necessary to account for the dye absorbance using a correction factor (CF₂₆₀). Use the CF₂₆₀ values given in Table 1 in the following equation:

$$A_{\text{base}} = A_{260} - (A_{\text{dye}} \times \text{CF}_{260})$$

4.3 Calculate the ratio of bases to dye molecules using the following equation:

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

where ϵ_{dye} is the extinction coefficient for the fluorescent dye (found in Table 1) and ϵ_{base} is the average extinction coefficient for a base in double-stranded DNA (dsDNA) or single-stranded DNA (ssDNA) (found in Table 2). 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} , and the Beer-Lambert law may also be used to measure the concentration of nucleic acid in the sample ([N.A.]). In order 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 4.2. 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 4.1 and 4.2 above and then use the following equation:

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

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

Cat #	Product Name	Unit Size
A32768	aha-dCTP (5-aminohexylacrylamido-dCTP) *2 mM in TE*	500 μ L
A32760	aha-dUTP (5-aminohexylacrylamido-dUTP) *2 mM in TE*	500 μ L
A32769	aha-dCTP (5-aminohexylacrylamido-dCTP) *50 mM in TE*	50 μ L
A32761	aha-dUTP (5-aminohexylacrylamido-dUTP) *50 mM in TE*	50 μ L

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