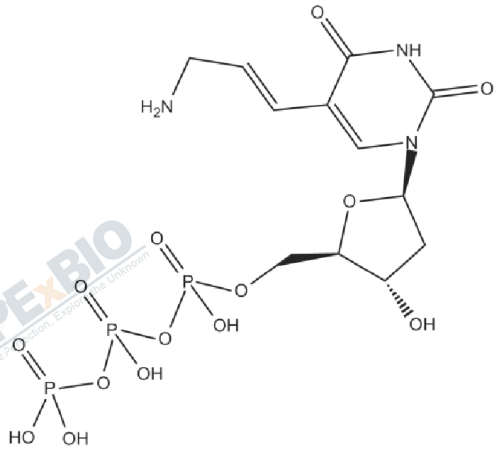
Aminoallyl-dUTP B7950

Introduction

Aminoallyl dUTP (5-(3-aminoallyl)-2′-deoxyuridine 5′- triphosphate) can be used to produce amine-modified DNA by conventional enzymatic incorporation methods such as reverse transcription, nick translation, random primed labeling, or PCR; the amine-modified DNA can then be labeled with fluorescent dye, biotin or or hapten.



Contents

1 mg or 5 mg Aminoallyl-dUTP

Important Product Information

•Store at -20°C or below. Avoid multiple freeze-thaw cycles. Aliquots of the product may be stored short-term at -20°C. Do not store in a frost-free freezer. When stored properly, the reagent should be stable for at least six months.

•Aminoallyl dUTP can be dissolved in 10 mM Tris, 1 mM EDTA, pH 8.0 or ddH2O titrated to pH 7.0-7.5 with NaOH to prepare 50 mM solution.

•Aminoallyl-dUTP can be enzymatically incorporated into DNA with Reverse Transcriptases, Taq DNA Polymerase, phi29 DNA Polymerase, Klenow Fragment, Klenow Fragment, exo- and DNA Polymerase I.

•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.

Enzymatic Incorporation Protocols

A. Calculations

1. Calculate Microliters to make a solution of a 50 mM concentration:

5 mg÷1000 mg/g÷523.22 g/M×1000 mM/M÷50 mM/L×1000000 μL/L=191.124 μL

• 523.22 = Molecular weight of Aminoallyl-dUTP

B. Reverse Transcription

We have optimized a reverse transcription labeling protocol using 5 μg of a 300-base, *in vitro*–transcribed RNA template and SuperScript™ II reverse transcriptase (Life Technologies, Inc.). We have empirically determined that the ratio of dTTP to aminoallyl dUTP used in this protocol with subsequent labeling by an amine-reactive dye, results in optimal probes for hybridization to dot blots.

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. A total reaction volume of 20 μL is usually sufficient.

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 immediately placing the reaction vial into an ice bath.

• Add 0.43 volumes of 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 a volume of 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 3M 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.

C. Nick Translation

We have optimized a nick translation labeling protocol using

1 μg of an 8 kb DNA template and the enzymes listed below. We have empirically determined that the ratio of dTTP to aminoallyl dUTP used in this protocol, with subsequent labeling by an amine-reactive dye, results in optimal probes for FISH with metaphase chromosome spreads.

2.1 Prepare 10× nick-translation buffer (0.5 M Tris-HCl, 50 mM MgCl2, 0.5 mg/mL nuclease-free BSA, pH 7.8).

2.2 Dilute a portion of the aminoallyl dUTP reagent in nuclease-free H2O to a final concentration of 0.5 mM.

2.3 Prepare on ice a DNase I stock solution by dissolving 1 mg of DNase I in 1 mL of cold 20 mM Tris-HCl,

50 mM NaCl, 1 mM dithiothreitol (DTT), 100 µg/mL nuclease-free BSA, 50% glycerol, pH 7.6. This stock solution should have an activity of approximately 2000 Kunitz units/mg. Mix gently. Do not vortex. Store aliquots at ≤–20°C.

2.4 Make a fresh 1 µg/mL working dilution of DNase I by diluting 1 µL of the 1 mg/mL DNase I stock solution (from step 2.3) into 1 mL of cold 1× nick-translation buffer. Leave on ice.

2.5 Add the following to a microfuge tube in the order indicated, adjusting the volume of water if necessary to achieve a final volume of 50 µL:

• 21.5 µL nuclease-free H2O

• 5 µL 10× nick-translation buffer

• 5 µL 0.1 M DTT

• 4 µL d(GAC)TP mixture (0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP)

• 1 µL 0.5 mM dTTP

• 6 µL 0.5 mM aminoallyl dUTP (from step 2.2)

• 1 µL DNA template, 1 µg/µL

• 5 µL DNase I, 1 µg/mL (from step 2.4)

• 1.5 µL DNA polymerase I, 10 U/µL

2.6 Incubate at 15°C for 2 hours. Bring to a final volume of 100 µL by adding 50 µL nuclease-free H2O and proceed to Purification of Amine-Modified DNA, below.

Purification of Amine-Modified DNA

3.1 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 H2O 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 amine-containing compounds, such as Tris, which would react with the amine-reactive reagent in the

labeling reaction.

3.2 Precipitate the DNA by adding 1/10 volume 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 with amine reactive dyes or haptens.

Labeling with an Amine-Reactive Reagent

The amine-modified DNA can be easily labeled with an amine-reactive dye, biotin or hapten.

The following protocol was optimized for use with aminoallyl-modified DNA made as in Enzymatic Incorporation Protocols, above. This protocol works well for Pacific Blue™ and Oregon Green® succinimidyl esters, and should work well for many other fluorescent dyes and biotin.

However, Alexa Fluor 633 succinimidyl ester should not be used, because this dye does not form bright conjugates with nucleic acids using this protocol.

4.1 Prepare Labeling Buffer by adding 1 mL of nuclease-free H2O to 25 mg of sodium bicarbonate and vortexing the solution until the solid is completely dissolved. Labeling Buffer should be stored at ≤–20°C in aliquots. Before use, Labeling Buffer should be thawed completely and vortexed to ensure that the bicarbonate is completely in solution. When properly stored, Labeling Buffer should be stable for at least 6 months.

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

Note: Although not essential, we recom mend that, prior to labeling, nick-translated amine-modified DNA be denatured for 5 minutes at 95°C and then snap cooled on ice. We have found that denaturation of nick-translated amine-modified DNA improves the subsequent labeling of the amine-modified DNA with amine-reactive reagents by 10–20%.

4.3 Add 3 µL of Labeling Buffer (made in 4.1) to the amine-modified DNA.

4.4 Make a stock solution of the amine-reactive reagent by dissolving it in an appropriate solvent at a concentration of about 30 µg/µL. Vortex for about 10 seconds to en sure 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 ≤–20°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.

4.5 Add 2 µL of the dissolved dye (made in step 4.4) to the tube containing the amine-modified DNA and Labeling Buffer. Vortex briefly to ensure that the reaction is well-mixed. DO NOT spin the tube to collect the solution in the bottom of the tube, but instead, let it settle by gravity.

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

4.7 Add 80 µL of nuclease-free H2O and 10 µL of 3M sodium acetate (pH 5.2) to the reaction mixture; purify the labeled DNA using QIAquick PCR Purification Kit (QIAGEN), performing three washes instead of one.

4.8 Precipitate the labeled DNA as before (step 3.2).

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 (λmax) and by using the Beer-Lambert law:

$A = \varepsilon \times$ path length (cm) $\times$ 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 1 and 2.

Measuring the Base:Dye Ratio

5.1 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 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 either a conventional spectrophotometer with a 100 or 200 µL cuvette or an absorbance microplate reader with a microplate.

• 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.

5.2 Correct for the contribution of the dye to the A260 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:

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

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

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

where ε_{dye} is the extinction coefficient for the fluorescent dye (found in Table 1) and ε_{base} is the average extinction co efficient 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.

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	30,000	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
Cy3	570	150,000	0.08
Cy5	670	250,000	0.05

*Absorbance maximum for the fluorophore; † Extinction coefficient for the dye;
‡ Correction factor = A260 for the free dye/Amax for the free dye.

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 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:

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

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).