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Methods for Enzymatic Incorporation of ChromaTide™ UTPs

Introduction

Our fluorophore-labeled ChromaTide™ UTPs can be enzymatically incorporated into probes for use in many molecular biology applications. The use of ChromaTide nucleotides with different fluorescence emission profiles allows for multicolor applications. We have developed the following protocol for incorporating these fluorescent nucleotides into hybridization probes. Depending on your application, some further optimization of the reaction conditions may be necessary.

Labeling with T3, T7 or SP6 RNA Polymerase

Introduction

To make labeled RNA probes, the sequence of interest is cloned into a plasmid vector downstream of the transcriptional promoter recognized by the T3, T7 or SP6 bacteriophage RNA polymerase. Upon addition of the appropriate polymerase, in the presence of ChromaTide UTP and unlabeled nucleotides, RNA is synthesized using the sequence of interest as a template. As the RNA is synthesized, the ChromaTide UTP is incorporated into the growing strand to make a fluorophore-labeled sense or antisense probe.

Materials Required

- ChromaTide UTP, 1 mM solution
- Linearized template DNA, we typically use 500 ng
- T3, T7 or SP6 enzyme
- ATP, CTP, GTP, UTP, (10 mM solutions)
- 10X Transcription buffer (T3 and T7 polymerase buffer: 200 mM Tris-HCl, pH 8.0, 40 mM $MgCl_2$, 10 mM spermidine, 250 mM NaCl; SP6 polymerase buffer: 200 mM Tris-Cl, pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine)
- RNase inhibitor, such as RNasin®
- EDTA, 0.2 M, pH 8.0
- DNase I

Preparation of the Template

1.1 For optimal results, use DNA purified by phenol/chloroform extraction and ethanol precipitation.

1.2 Linearize the template using a restriction enzyme. The restriction enzyme chosen should cut immediately downstream of the probe sequence so that runoff transcripts of a unique size will be produced. Restriction enzymes that generate 5′ overhangs are best. Enzymes that leave 3′ overhangs should be avoided because they may serve as initiation sites for the RNA polymerase, leading to synthesis of RNA complementary to the desired probe.

1.3 Remove the restriction enzyme from the linearized template with two rounds of phenol/chloroform extraction followed by ethanol precipitation.

Transcription Reaction

2.1 The following ingredients should be added together before addition of the template DNA to avoid precipitation of the DNA by the concentrated spermidine present in the 10X transcription buffer.

- 1.0 µL 10 mM ATP
- 1.0 µL 10 mM CTP
- 1.0 µL 10 mM GTP
- 0.75 µL 10 mM UTP
- 2.5 µL 1 mM ChromaTide UTP
- 2.0 µL 10X Transcription buffer
- 1.0 units RNase inhibitor
- RNase-free dH_2O to bring the final volume (including template and enzyme) to 20 µL

Mix well, and then add:

- ~500 ng linearized DNA template
- 2–4 units T3, T7 or SP6 RNA polymerase

Mix gently by tapping the tube, centrifuging in a microcentrifuge for a couple of seconds if necessary to make sure all of the reaction mixture remains at the bottom of the tube.

2.2 Incubate the reaction for 2 hours at 37°C for T3 or T7, or at 40°C for SP6.

2.3 Add 10 units of RNase-free DNase I and incubate for 15 minutes at 37°C.

2.4 Stop the reaction by adding 2 µL of 0.2 M EDTA, pH 8.0 and briefly vortexing. Store at -20°C until needed.

Purification (optional)

Overview

It may by necessary to remove unincorporated nucleotides from the reaction mixture prior to use of the labeled products in subsequent experiments. The procedure described below has been used successfully with all of the labeled nucleotides. Ethanol precipitation is not recommended as an alternative step, because with some conjugates it may not efficiently remove the nucleotides from the reaction mixtures.

Separation of Labeled Nucleic Acids from Unincorporated Nucleotides

3.1 Add 2 µL of 10 mg/mL tRNA as carrier and water or TE (RNase-free) to make a final volume of 50 µL. Purify the labeled RNA probe using a gel filtration column, such as a Sephadex® G50, a BioRad Bio-Gel® P-30 or a Princeton Separations Centri-Sep™ column. These columns efficiently remove the unincorporated ChromaTide UTPs without degrading the RNA probes. Other spin column methods may also work well. Autoclave the columns first to render them RNAse free as suggested in their literature.

3.2 Follow the manufacturer's instructions in centrifuging the column. After spinning the column, most of the color will remain on the column. The eluate containing the labeled probe will have very little color because most of the ChromaTide UTP will not have been incorporated into the probe. Recoveries are typically ~80% or better, based on visual estimates from agarose gel analysis, before and after purification.

3.3 The reaction product can be concentrated using a Microcon™ Microconcentrator (Amicon, Inc., Beverly, MA) following the manufacturer's instructions. Some of the reaction product will likely be lost in this step (~20%).

3.4 The results of the reaction may be analyzed by running ~10% of the reaction on a 1% agarose gel. The fluorescent product can be viewed on a typical UV transilluminator and photographed using Polaroid® 667 black-and-white print film and an appropriate filter, such as the SYBR® Green/Gold photographic filter (S-7569). To assess the purity of the probe, first view the unstained gel, then stain the gel with a sensitive gel stain like SYBR Green II or SYBR Gold stain to detect unlabeled RNA species. It is also possible to measure the base:dye ratio to assess the degree of labeling (see below).

3.5 Dilute the purified riboprobe immediately into an appropriate hybridization buffer. Alternatively, store the probe in a non–frost-free -20°C freezer until needed. Avoid repeated freeze/thaw cycles.

Measuring the Base:Dye Ratio for Fluorophore-Labeled Nucleic Acids

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:

$$A = \epsilon \times \text{path length} \times \text{concentration},$$

where ϵ is the extinction coefficient in $cm^{-1}M^{-1}$. Values needed for the calculations are found in Table 1.

Measuring the Base:Dye Ratio

4.1 Measure the absorbance of the RNA–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 are given in Table 1.

Table 1. Absorption characteristics for fluorescent dyes.

Fluorescent Dye	λ_{max} (nm) *	ϵ_{dye} ($cm^{-1}M^{-1}$) †	CF_{260} ‡
Alexa Fluor 488	492	62,000	0.30
Alexa Fluor 546	555	104,000	0.21
BODIPY FL	504	68,000	0.00
BODIPY TMR	535	57,800	0.15
BODIPY TR	588	55,000	0.11
Fluorescein	494	30,000	0.32
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.

- To perform these measurements, the RNA–dye conjugate should be at a concentration of at least 5 µg/mL. Depending on the dye you use 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 plate 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.

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 (CF_{260}). Use the CF_{260} values given in Table 1 in the following equation:

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

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

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

where ϵ_{dye} is the extinction coefficient for the fluorescent dye (found in Table 1) and ϵ_{base}, the average extinction coefficient for a base in RNA, is equal to 8250 $cm^{-1}M^{-1}$. Note that since that you are calculating 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 your 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, using 340 g/mol for the MW_{base} in RNA:

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

Troubleshooting the RNA Labeling Protocol

5.1 If you do not see a fluorophore-labeled RNA product of the appropriate length, determine first if there is any reaction product visible with post-staining with SYBR Green II or SYBR Gold stain (or ethidium bromide) when 10% of the reaction is analyzed on a gel. This will tell you whether the polymerase itself is working.

5.2 For certain templates, the ratio of labeled UTP to unlabeled UTP in the reaction may have to be altered. For our typical template, we use a 1:3 molar ratio of labeled to unlabeled UTP. If the product of the reaction is too short, the polymerase may be stalling after addition of the Chromatide UTP. In such cases, a full length probe can be obtained by using a lower ratio of labeled to unlabeled nucleotide.

5.3 Occasionally, BODIPY® TR 14-UTP forms a blue-colored insoluble precipitate when added to the reaction mixture. The precipitate can simply be centrifuged into a pellet to remove it before proceeding with the transcription reaction.

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