

## Methods for Enzymatic Incorporation of ChromaTide® OBEA-dCTPs

C21555 ChromaTide® Alexa Fluor® 488-7-OBEA-dCTP  
C21556 ChromaTide® Alexa Fluor® 546-16-OBEA-dCTP  
C21558 ChromaTide® Alexa Fluor® 594-7-OBEA-dCTP  
C21559 ChromaTide® Alexa Fluor® 647-12-OBEA-dCTP

### Quick Facts

#### Storage upon receipt:

- $\leq -20^{\circ}\text{C}$
- Protect from light
- Avoid freeze-thaw cycles

#### Concentration:

- 1 mM in TE buffer

### Introduction

ChromaTide® OBEA-dCTPs can be enzymatically incorporated into probes for many molecular biology applications. The variety of fluorescence emission profiles enables ChromaTide nucleotides to be used for multicolor applications. Protocols are provided for the incorporation of ChromaTide OBEA-dCTPs into hybridization probes by using nick translation or reverse transcription; the reaction conditions may need to be optimized for your application.

### Materials

#### Contents

- ChromaTide OBEA-dCTP, 1 mM solution

Each ChromaTide OBEA-dCTP nucleotide is supplied in a 50  $\mu\text{L}$  unit size as 1 mM solution in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). ChromaTide OBEA-dCTP nucleotides are >95% pure as determined by HPLC spectrophotometric analysis.

#### Storage Conditions

Upon receipt, store the ChromaTide OBEA-dCTP at  $\leq -20^{\circ}\text{C}$  and protected from light. When stored properly, these reagents are stable for up to 2 years. AVOID REPEATED FREEZING AND THAWING.

#### Materials Required but Not Provided

- Reagents required for the enzymatic incorporation reaction, including unlabeled nucleotides (see protocols below).
- QIAquick® PCR Purification Kit (Qiagen).

### Enzymatic Labeling Protocols

#### Labeling by Reverse Transcription

We have optimized a reverse transcription labeling protocol using 20–30  $\mu\text{g}$  of total human RNA, 5  $\mu\text{g}$  of anchored oligo (dT) primer ((dT)<sub>20</sub> VN) and SuperScript™ II reverse transcriptase (Invitrogen). We have empirically determined that the ratio of dCTP to ChromaTide OBEA-dCTP used in this protocol results in optimally-labeled samples for hybridization to microarrays.

#### 1.1 Synthesize ChromaTide OBEA-dCTP-labeled cDNA.

Perform reverse transcription according to the manufacturer's protocol, using the following **final** concentrations of nucleotides: 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dTTP, 0.025 mM dCTP and 0.175 mM ChromaTide OBEA-dCTP.

**1.2 Digest the RNA template.** Add 1  $\mu\text{L}$  (2 U) of RNase H to the reaction, and incubate at 37°C for 30 minutes.

Note: The RNA does not need to be digested when using the reaction mixture for a hybridization experiment. However, the RNA needs to be digested in order to obtain an accurate measurement of the base:dye ratio (see **Product Notes**). DO NOT digest the RNA by base hydrolysis because this will destroy the fluorophore.

**1.3 Purify the labeled DNA.** Add the appropriate amount of nuclease-free deionized water (dH<sub>2</sub>O) to bring the reaction mixture to a final volume of 100  $\mu\text{L}$ . Then, following the manufacturer's instructions, use a QIAGEN QIAquick PCR Purification Kit to purify the labeled DNA. Performing the wash step of the QIAquick protocol 2–3 times helps to reduce background. Other spin-column methods may also work well, but ethanol precipitation is not recommended, because free nucleotides may not be effectively removed from the reaction mixtures.

## Labeling by Nick Translation

We have optimized a nick-translation labeling protocol using 1  $\mu\text{g}$  of an 8 kb DNA template and the enzymes listed below. We have empirically determined that the ratio of dCTP to ChromaTide OB EA-dCTP used in this protocol results in optimal probes for fluorescence *in situ* hybridization (FISH) to metaphase chromosome spreads.

**2.1 Dilute the ChromaTide OB EA-dCTP.** Dilute a portion of the ChromaTide OB EA-dCTP in nuclease-free  $\text{dH}_2\text{O}$  to a final concentration of 0.2 mM.

**2.2 Prepare 10X nick-translation buffer.** This buffer is 0.5 M Tris-HCl, 50 mM  $\text{MgCl}_2$ , 0.5 mg/mL nuclease-free bovine serum albumin (BSA), pH 7.8.

**2.3 Prepare a DNase I stock solution.** On ice, dissolve 1 mg of DNase I in 1 mL of cold 20 mM Tris-HCl, 50 mM NaCl, 1 mM dithiothreitol (DTT), 100  $\mu\text{g}/\text{mL}$  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  $\leq -20^\circ\text{C}$ .

**2.4 Prepare a fresh DNase I working solution.** Dilute 1  $\mu\text{L}$  of the 1 mg/mL DNase I stock solution (from step 1.1) into 1 mL of cold 1X nick-translation buffer. Leave on ice.

**2.5 Prepare the reaction mixture.** 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  $\mu\text{L}$ :

- 18.5  $\mu\text{L}$   $\text{dH}_2\text{O}$
- 5  $\mu\text{L}$  10X nick-translation buffer (from step 2.2)
- 5  $\mu\text{L}$  0.1 M DTT
- 4  $\mu\text{L}$  d(GAT)TP mix (0.5 mM dGTP, 0.5 mM dATP, 0.5 mM dTTP)
- 1.2  $\mu\text{L}$  0.2 mM dCTP
- 8.8  $\mu\text{L}$  0.2 mM ChromaTide OB EA-dCTP (from step 2.1)
- 1  $\mu\text{L}$  DNA template, 1  $\mu\text{g}/\mu\text{L}$
- 5  $\mu\text{L}$  DNase I, 1  $\mu\text{g}/\text{mL}$  (from step 2.4)
- 1.5  $\mu\text{L}$  DNA polymerase I, 10 U/ $\mu\text{L}$

**2.6 Incubate at  $15^\circ\text{C}$  for 2 hours.**

**2.7 Purify the labeled DNA.** Following the manufacturer's instructions, use a QIAGEN QIAquick PCR Purification Kit to purify the labeled DNA. Performing the wash step of the QIAquick protocol 2–3 times helps to reduce background. Other spin-column methods may also work well, but ethanol precipitation is not recommended, because free nucleotides may not be effectively removed from the reaction mixtures.

## Measuring the Base:Dye Ratio for Fluorophore-Labeled DNA

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

**Table 1.** Absorption characteristics of fluorescent dyes.

Fluorescent Dye	$\lambda_{\text{max}}$ (nm) *	$\epsilon_{\text{dye}}$ ( $\text{cm}^{-1}\text{M}^{-1}$ ) †	$\text{CF}_{260}$ ‡
Alexa Fluor 488	492	62,000	0.30
Alexa Fluor 546	555	104,000	0.21
Alexa Fluor 594	588	80,400	0.43
Alexa Fluor 647	650	239,000	0

\* Absorption maximum for the fluorophore. † Extinction coefficient for the dye.

‡ Correction factor =  $A_{260}$  for the free dye/ $A_{\text{max}}$  for the free dye.

nucleic acid at 260 nm and the absorbance of the dye at its absorbance maximum ( $\lambda_{\text{max}}$ ) and by using the Beer–Lambert law:

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

where  $\epsilon$  is the extinction coefficient in  $\text{cm}^{-1}\text{M}^{-1}$ . Values needed for the calculations are found in Tables 1 and 2.

## Measuring the Base:Dye Ratio

**3.1** Measure the absorbance of the DNA–dye conjugate at 260 nm ( $A_{260}$ ) and at the  $\lambda_{\text{max}}$  for the dye ( $A_{\text{dye}}$ ). Measure the background absorbance at 260 nm and at  $\lambda_{\text{max}}$  using buffer alone, and subtract these numbers from the raw absorbance values for the sample. The  $\lambda_{\text{max}}$  values for the 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  $\mu\text{L}$  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.

**3.2** 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  $\lambda_{\text{max}}$ . To obtain an accurate absorbance measurement for the nucleic acid, it is therefore necessary to account for the dye absorbance using a correction factor ( $\text{CF}_{260}$ ). Use the  $\text{CF}_{260}$  values given in Table 1 in the following equation:

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

**3.3** 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  $\epsilon_{\text{dye}}$  is the extinction coefficient for the fluorescent dye (found in Table 1) and  $\epsilon_{\text{base}}$  is the average extinction coefficient for a base in double stranded DNA (dsDNA), long single-stranded

**Table 2.** Average extinction coefficients for a nucleotide residue in different nucleic acids.

Nucleic Acid	$\epsilon_{\text{base}}$ (cm <sup>1</sup> M <sup>-1</sup> ) *	MW <sub>base</sub> †
dsDNA	6600	330
ssDNA	8919	330
oligonucleotide	10,000	330

\* Average extinction coefficient for a base. † Average molecular weight for a nucleotide residue in a nucleic acid (g/mol).

DNA (ssDNA) or oligonucleotides (found in Table 2). Note that because that you are calculating a ratio, the path length has canceled out of the equation.

### Measuring the Nucleic Acid Concentration

The absorbance values ( $A_{260}$  and  $A_{\text{dye}}$ ) and the Beer–Lambert law may also be used to measure the nucleic acid concentration in your sample. In order to obtain an accurate measurement for a dye-labeled nucleic acid, a dye-corrected absorbance value ( $A_{\text{base}}$ ) must be used, as explained in step 8.2. 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 3.1 and 3.2 above and then use the following equation:

$$\text{Concentration (in mg/mL)} = (A_{\text{base}} \times MW_{\text{nt}}) / (\epsilon_{\text{base}} \times \text{path length})$$

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