General Protocol of Labeling Alkyne-Modified Biomolecules with Fluorescent Dye Azides

Labeling Oligonucleotides with Dye Azides

1. Prepare the following stock solutions:
   - 200 mM THPTA [tris(3-hydroxypropyltriazolylmethyl)amine)] in water
   - 100 mM CuSO4 in water
   - Alkyne-modified oligo in water (as concentrated as possible, e.g., >10 mg/mL)
   - 100 mM sodium ascorbate in water
   - 10 mM dye azide in DMSO or water (see our website for recommended solvent)
2. Mix and vortex well CuSO4 with THPTA in a 1:2 ratio for several minutes before the reaction. This working solution is stable for several weeks when frozen.
3. To the alkyne-modified oligo solution, add an excess of dye azide (2-5 equivalents by molar ratio).
4. Add 5 equivalents of THPTA/CuSO4 working solution (from Step 1)
5. Add 10-30 equivalents of sodium ascorbate.
6. Stir, vortex or shake the reaction mixture at room temperature for 30-60 minutes.
7. Ethanol-precipitate or purify the oligo by your desired method (e.g., HPLC).

Labeling Biopolymers with Dye Azides

1. Prepare the following stock solutions:
   - 200 mM THPTA ligand in water
   - 100 mM CuSO4 in water
   - Alkyne-modified biopolymer in water (as concentrated as possible, e.g., >5 mg/mL)
   - 100 mM sodium ascorbate in water
   - 10 mM dye azide in DMSO or water (see our website for recommended solvent).
2. Incubate CuSO4 with THPTA ligand in a 1:2 ratio several minutes before the reaction. This solution is stable for several weeks when frozen.
3. To the alkyne-modified biopolymer solution, add an excess of dye azide (Loading ratio: 5-20 dye azide/alkyne).
4. Add 5 molar equivalents (referenced to dye azide) of THPTA/CuSO4.
5. Add 10 equivalents of sodium ascorbate (referenced to dye azide).
6. Stir, vortex or shake the reaction mixture at room temperature for 30-60 minutes.
7. Purify your desired molecule by gel filtration or dialysis.

Labeling Cells, Cell Lysates or Biological Samples with Dye Azides or Dye Alkynes

1. Prepare the following click solutions:
   - 100 mM THPTA ligand in aqueous buffer or water
   - 20 mM CuSO4 in water
   - 300 mM sodium ascorbate in water
   - 2.5 mM alkyne or azide labeling reagent in water or DMSO
2. For each azide- or alkyne-modified cell or cell lysate sample, add the following reagents to a 1.5 mL

microfuge tube, then vortex briefly to mix.

- 50 µL cell or cell lysate sample
- 50 µL PBS buffer
- 50 µL of 5 mM corresponding dye azide (or dye alkyne) detection reagent in DMSO or water

3. Add 10 µL of 100 mM THPTA solution, vortex briefly to mix.
4. Add 10 µL of 20 mM CuSO4 solution, vortex briefly to mix.
5. Add 10 µL of 300 mM sodium ascorbate solution to initiate the click reaction, vortex briefly to mix.
6. Protect the click reaction from light and allow it to incubate for 30 minutes at room temperature.
7. Cells or cell lysates are now click labeled and ready for downstream processing and/or analysis.

Labeling Peptides with Dye Azides

1. Prepare the following stock solutions:
   - 200 mM THPTA ligand in water
   - 100 mM CuSO4 in water
   - Alkyne-modified peptide in water or DMF (depending on your peptide solubility, >10 mg/mL if possible)
   - 100 mM sodium ascorbate in water
   - 10 mM dye azide in DMSO or water (see our website for recommended solvent)
2. Incubate CuSO4 with THPTA ligand in a 1:2 ratio several minutes before the reaction. This solution is stable for several weeks when frozen.
3. To the alkyne-modified peptide solution, add an excess of dye azide (5-10 equivalents by molar ratio).
4. Add 5-10 equivalents of THPTA/CuSO4.
5. Add 10-20 equivalents of sodium ascorbate.
6. Stir, vortex or shake the reaction mixture at room temperature for 30-60 minutes.
7. Purify your desired peptide by HPLC.

Labeling Small Organic Alkyne Molecules with Dye Azides

1. Prepare the following stock solutions:
   - 200 mM THPTA ligand in water
   - 100 mM CuSO4 in water
   - Alkyne compound in water or DMF (depending on your compound solubility, >10 mg/mL if possible,)
   - 100 mM sodium ascorbate in water
   - 10 mM dye azide in DMSO or water (see our website for recommended solvent).
2. Incubate CuSO4 with THPTA ligand in a 1:2 ratio several minutes before the reaction. This solution is stable for several weeks when frozen.
3. To the alkyne solution, add an excess of dye azide (5-10 equivalents by molar ratio).
4. Add 25 equivalents of THPTA/CuSO4.
5. Add 50 equivalents of sodium ascorbate.
6. Stir the reaction mixture at room temperature for 30-60 minutes.
7. Purify your desired molecule by chromatography or other methods.

Appendix I. HPLC Purification of Dye Oligonucleotide Conjugates

Ethanol Precipitation

Some commercial oligonucleotides often contain some interfering compounds, especially amines, such as triethylamine or Tris, and ammonium salts, we strongly recommend you to extract and precipitate the commercial oligo samples prior to initiating yourlabeling reaction. On the other hand, the labeling mixture contains labeled oligonucleotide, unlabeled oligonucleotide, hydrolyzed dyeacid and unincorporated dye SE. The impurities of hydrolyzed dye acid and unincorporated dye SE resulted from the labeling reactioncan be effectively removed by ethanol precipitation. The following protocol was optimized for the further purification of 0.1–1 mg commercial oligonucleotide sample that was purified by HPLC (3–30 A260 units).

1) Dissolve your target oligonucleotide in 100 µL of deionized water and extract three times with an equal volume ofchloroform.
2) Precipitate the oligonucleotide by adding one-tenth volume (10 µL) of 3 M NaCl and two and a half volumes (250 µL) ofcold absolute ethanol. Mix well and place at –20°C for 30 minutes.
3) Centrifuge the solution in a microcentrifuge at 10,000 to 15,000 g for 30 minutes.
4) Carefully remove the supernatant, rinse the pellet 1-3 times with cold 70% ethanol, and dry under a vacuum.
5) Dissolve the dry pellet in deionized water to achieve a final concentration of >50 µg/µL. This amine-modifiedoligonucleotide stock solution may be immediately used or stored frozen at ≤–15°C.

Purification by HPLC

Labeled oligonucleotides can be purified by reverse-phase HPLC using a standard analytical C8 or C18 column using an analytical or semi-preparative HPLC instrument. The following protocol was optimized for the further purification of 0.1–1 mglabeled oligonucleotide (3–30 A260 units).

1) Dissolve the pellet from the ethanol precipitation in 0.1 M triethylammonium acetate (TEAA).
2) Load the dissolved pellet onto the column in 0.1 M TEAA and run a linear 5–95% acetonitrile gradient over 30 minutes. *Note 1: There will be peaks that correspond to the unlabeled oligonucleotide, the labeled oligonucleotide, and the free dye.The actual order and number of these peaks depends on the length of the oligonucleotide and the purity of the sample.*

Note 2: To determine the identity of the peaks, monitor the absorbance at both 260 nm and at the absorbance maxima (λmax)for the dye. For instruments with only one detector, two small samples should be run, each monitored at a different wavelength. Unlabeled oligonucleotide will show an absorbance at 260 nm only. Both the free dye and the labeled oligonucleotide will have absorbance at both 260 nm (A260 for oligo) and at the absorbance maximum of the dye (Amax for dye); The dye-labeled oligonucleotide will have a higher A260:Amax ratio than the dye or hydrolyzed dye.

Purification by Gel Electrophoresis

1) Pour a 0.5 mm–thick polyacrylamide slab gel.
   Note: For oligonucleotides less than 25 bases in length, use 19% acrylamide, for oligonucleotides 25–40 bases, 15%acrylamide, and for oligonucleotides 40–100 bases, 12% acrylamide.
2) Resuspend the pellet from ethanol precipitation in 200 µL of 50% formamide, and incubate at 55°C for 5 minutes to disruptany secondary structure.
3) Load the warmed oligonucleotide onto the gel and load an adjacent well with 50% formamide plus 0.05% bromophenol blue.The bromophenol blue will migrate at approximately the same rate as the oligonucleotide.
   Note: You may need to use several wells.
4) Run the gel until the bromophenol blue indicator dye is two-thirds of the way down the gel.
5) Remove the gel from the glass plates and place on Saran Wrap.
6) Lay the gel on a fluorescent TLC plate.
7) Locate the labeled and unlabeled oligonucleotides by illumination with a handheld UV source.