Amine-Reactive Probes

Introduction

Molecular Probes manufactures a wide variety of amine-reactive fluorescent dyes, biotins and other haptens for conjugation to proteins, amine-modified oligonucleotides or other amine-containing compounds. This information sheet describes protocols for conjugating amine-reactive compounds to 10 mg of an IgG antibody or to 100 µg of an amine-modified oligonucleotide.

There are three major classes of commonly used reagents to label amines: succinimidyl esters (SE), including sulfosuccinimidyl esters, isothiocyanates (ITC) and sulfonyl chlorides (SC). Other amine-reactive groups include dichlorotriazines, aryl halides and acyl azides. In addition, we have developed water-soluble amine-reactive forms of some of our popular hydrophobic dyes. These dyes have an amine-reactive 4-sulfo-2,3,5,6-tetrafluorophenyl (STP) ester group and may be a good alternative for labeling biomolecules in the absence of organic solvents. For more details and a complete list of our amine-reactive compounds, please refer to our Handbook of Fluorescent Probes and Research Products, available upon request or at our Web site at www.probes.com. Further information on making bioconjugates can be found in Hermanson, Bioconjugate Techniques, Academic Press, 1996 (B-7884).

Storage and Handling

Upon receipt, reactive dyes should be stored at -20°C in a desiccator and protected from light. As lyophilized solids, reactive dyes are stable for at least six months to a year when stored properly. Reactive dyes hydrolyze readily in the presence of water and are not stable in aqueous solution. If only a portion of the dye is to be used, we recommend dissolving the dye in a volatile organic solvent, such as methanol or acetonitrile, aliquoting it into smaller portions and drying the dye for long-term storage at -20°C.

Labeling Proteins

Materials Required

Protein

Amine-reactive reagents may be conjugated with virtually any protein or peptide. The following protocol is optimized for IgG antibodies. The reaction may be scaled for any amount of protein, but the concentration of the protein should be at least 2 mg/mL for optimal results.

Reactive Dye

Succinimidyl esters are preferred for the conjugation to proteins because they form a very stable amide bond between the dye or hapten and the protein. Isothiocyanates are also commonly used, although the resulting thiourea product has been reported to deteriorate over time. Sulfonyl chlorides and acid halides are more reactive and may allow conjugation to aromatic amines. Sulfonyl chlorides form very stable sulfonamides that can survive complete protein hydrolysis, but since they are more difficult to work with, we do not recommend these for most routine conjugations with proteins.

Solvent

For the most part, reactive dyes and haptens are hydrophobic molecules and should be dissolved in high-quality, anhydrous dimethylformamide (DMF) or dimethylsulfoxide (DMSO). Caution: DMSO should not be used with sulfonyl chlorides, as it reacts with them.

Reaction Buffer

Amine-reactive reagents react with non-protonated aliphatic amine groups, including the amine terminus of proteins and the ε-amino group of lysines. The ε-amino group has a pKa of around 10.5; in order to maintain this amine group in the non-protonated form, the conjugation must take place in a buffer with slightly basic pH. It is important to avoid buffers that contain primary amines, such as Tris, as these will compete for conjugation with the amine-reactive compound. We recommend the following buffers for conjugation with amine-reactive compounds with proteins:

- 0.1–0.2 M sodium bicarbonate buffer, pH 9.0 for isothiocyanates, sulfonyl chlorides and dichlorotriazines
- 0.1–0.2 M sodium bicarbonate buffer, pH 8.3 for succinimidyl and STP esters

More specific labeling of the amine terminus may be achieved using a buffer closer to neutral pH, as the pKa of the terminal amine is lower than that of the lysine ε-amino group.

Stop Reagent (Optional)

1.5 M hydroxylamine, pH 8.5, may be used to terminate the reaction and to remove weakly bound probes. To prepare this reagent, dissolve hydroxylamine hydrochloride at 210 mg/mL in distilled water and adjust the pH to 8.5 with 5 M NaOH. Dilute the resulting solution with an equal volume of distilled water. This reagent should be freshly prepared before use (see step 1.5 below).

Purification

A typical labeled antibody can be easily separated from free dye using a gel filtration column, such as Sephadex® G-25 or equivalent, equilibrated with the buffer of your choice. For much smaller or larger proteins, other gel filtration columns may be more appropriate.
**Labeling Procedure**

This procedure may be scaled up or down, maintaining the same molar ratios of reagents. It is important to consider that the number and surface position of the amines will vary greatly among proteins and even among different IgGs, as will the reactivity of the dyes. We therefore recommend that whenever possible three different degrees of labeling be tried, using three different molar ratios of the reactive reagent to protein, and that future protocols be based on the amount of reagent that gives the most satisfactory results for your specific protein. Reviews by Brinkley and by Haugland provide comprehensive survey of procedures and reagents for protein conjugate preparation.1,2

1.1 Dissolve ~10 mg of the protein in 1 mL of 0.1 M sodium bicarbonate buffer. The protein concentration in the reaction should usually be 5–20 mg/mL. The kinetics and success of the reaction are highly concentration dependent. Concentrations lower than 2 mg/mL will greatly decrease the efficiency of the reaction.

Protein solutions must be free of any amine-containing substances such as Tris, glycine or ammonium ions. Antibodies that have been previously dissolved in buffers containing amines can be dialyzed against 10–20 mM phosphate-buffered saline (PBS), and the desired pH for the reaction can be obtained by adding 0.1 mL of 1 M sodium bicarbonate buffer (pH 8.3–9.0) for each mL of antibody solution. It is not necessary to remove azide from previously prepared protein solutions prior to conjugation.

1.2 Dissolve the amine-reactive compound in DMF or (except for sulfonyl chlorides) DMSO at 10 mg/mL. For a typical reaction, 5 mg of dye can be dissolved in 0.5 mL of DMF or DMSO. It is important that the dye be dissolved immediately before starting the reaction as reactive compounds are not very stable in solution. Stock solutions of dyes with an STP ester can be prepared in deionized water. Briefly sonicate or vortex.

1.3 While stirring or vortexing the protein solution (step 1.1), slowly add 50–100 µL of the reactive dye solution (step 1.2). This volume corresponds to 0.5–1 mg of amine-reactive dye. In general, about one-fourth to one-third of the reactive dye will conjugate to the protein. This percentage may be higher with isothiocyanates. Variations due to the different reactivities of both the protein and the labeling reagent may occur, which may necessitate optimization of the dye-to-protein ratio used in the reaction.

1.4 Incubate the reaction for 1 hour at room temperature with continuous stirring. For sulfonyl chlorides, incubate at 4°C, with continuous stirring.

1.5 Optional: Stop the reaction by adding 0.1 mL of freshly prepared 1.5 M hydroxylamine, pH 8.5. For Rhodol Green and Rhodamine Green conjugates, the hydroxylamine-containing reaction should be incubated overnight at 4°C. Otherwise, the hydroxylamine-containing reaction should be incubated for one hour at room temperature.

Treatment with hydroxylamine at this stage is required to remove the trifluoroacetyl protecting groups of Rhodamine Green™ (R-6112) succinimidyl ester. Hydroxylamine may also remove dye from unstable conjugates with tyrosine, serine, threonine and histidine.

1.6 Separate the conjugate from unreacted labeling reagent. We recommend using a Sephadex G-25 or equivalent gel filtration column (10 × 300 mm) equilibrated with PBS or buffer of choice. The excluded fraction, corresponding to the first fluorescent band to elute, will be the conjugate.

If you are conjugating a dilute antibody, to avoid further dilution you may want to purify the conjugate by extensive dialysis. If you prefer to purify your conjugate by column chromatography, after elution, add bovine serum albumin (BSA) or any other stabilizer of choice to a final concentration of 1–10 mg/mL to prevent denaturation.

1.7 Storage of protein conjugate. In general, conjugates should be stored under the same conditions used for the parent protein. For storage in solution at 4°C, sodium azide (2 mM final concentration) should be added as a preservative. Since azide is an inhibitor of horseradish peroxidase (HRP), thimerosal should be substituted as a preservative for conjugates that are derived from HRP or that will be used for experiments in which HRP is present. Removal of preservatives prior to use may be necessary to avoid inhibitory effects in applications in which conjugates are added to live cell specimens.

**Determining the Degree of Labeling**

You may need to optimize the labeling efficiency to achieve the desired results in your application. The relative efficiency of a labeling reaction can be determined by measuring the absorbance of the protein at 280 nm and the absorbance of the dye at its absorbance maximum (λmax). Using the Beer-Lambert law: A = ε × path length × concentration, where ε is the extinction coefficient in cm⁻¹M⁻¹, one can calculate the approximate number of dye molecules per protein molecule. A correction needs to be made for the absorbance of the dye at 280 nm. In the case of a biotinylated conjugate, the degree of labeling can be measured with HABA reagent as described in the protocol of our FluoroReporter® Biotin-XX Labeling Kit (F-2610) and elsewhere.3

2.1 Measure the absorbance of the protein–dye conjugate at 280 nm (A_{280}) and at the λmax for the dye (A_{max}). Dilute the protein–dye conjugate to approximately 0.1 mg/mL. Dilute only as much as you need to make the measurement. The λmax values for commonly used fluorophores are given in Table 1. Please consult our Handbook of Fluorescent Probes and Research Products or our Web site (www.probes.com) for information about other dyes.

2.2 Determine the concentration of the protein in mg/mL.

a. Correct for the contribution of the dye to the absorbance at A_{280}:

\[
A_{protein} = A_{280} - A_{max} (CF)
\]

\[
CF = \frac{A_{280 \text{ free dye}}}{A_{max \text{ free dye}}}
\]

CF values for commonly used fluorophores are listed in Table 1.

b. Calculate the protein concentration assuming 1.4 A_{protein} units = 1 mg/mL. This value is correct for IgG antibodies, but may be different for your protein.
2.3 Calculate the degree of labeling (D.O.L.): 

\[ DOL = \frac{A_{\text{max}} \times MW}{[\text{protein}] \times \epsilon_{\text{dye}}} \]

where MW = the molecular weight of the protein, \( \epsilon_{\text{dye}} \) = the extinction coefficient of the dye at its absorbance maximum and the protein concentration is in mg/mL.

### Labeling Amine-Modified Oligonucleotides

#### Materials Required

**Oligonucleotide**

The oligonucleotide must be synthesized with an amine group on the 5’ end. The following protocol has been optimized for use with 100 µg of oligonucleotide.

**Reactive Dye**

Succinimidyl esters are preferred for the conjugation to oligonucleotides because they are easy to use and form a very stable amide bond between the dye or hapten and the amine-modified oligonucleotide. For DNA sequencing applications, the BODIPY dyes are useful because they are isomerically pure and cause little perturbation to the mobility of DNA fragments. We also offer 5-FAM, 6-TAMRA, 6-ROX, 6-HEX, 6-TET and 6-JOE, the traditional fluorophores used in oligonucleotide labeling and automated DNA sequencing applications. The Alexa Fluor series of dyes provides very bright, photostable fluorescence; however, note that the fluorescence of the Alexa Fluor 633 dye appears to be quenched by nucleic acids, so this dye should not be used to label oligonucleotides.

**Solvent**

For the most part, reactive dyes and haptens are hydrophobic molecules and should be dissolved in high-quality, dimethylsulfoxide (DMSO) before reaction with amine-modified oligonucleotides.

**Reaction Buffer**

Amine-reactive reagents will react with the non-protonated amine group on the modified oligonucleotide. In order to maintain this amine group in the non-protonated form, the conjugation must take place in a buffer with slightly basic pH. For optimal results we recommend using a tetraborate buffer at pH 8.5, rather than the bicarbonate buffers recommended for protein conjugations. It is important to avoid buffers that contain primary amines, such as Tris, as these will compete for conjugation with the amine-reactive compound.

#### Reaction Protocol

The protocol has been optimized for labeling 100 µg of an 5'-amine-modified oligonucleotide, 18 to 24 bases in length. Slightly shorter or longer oligonucleotides may be labeled by the same procedure; however, adjustments to the protocol may be necessary for greatly shorter or longer oligonucleotides. The reaction may be scaled up or down as long as the concentration of each component is not changed. The procedure has not been tested with oligonucleotides containing more than one amine. Following the labeling reaction, the conjugate may be purified from the reaction mixture by preparative gel electrophoresis or reverse-phase HPLC.

### Table 1. Spectral characteristics of common dyes. Values for other dyes may be found at our Web site (www.probes.com).

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* Absorbance and fluorescence emission maxima in nm. † Extinction coefficient at \( \lambda_{\text{max}} \) in cm\(^{-1}\)M\(^{-1}\). ‡ Correction factor (\( A_{280} \) free dye / \( A_{\lambda_{\text{max}}} \) free dye). § Measured at pH 8.0. NA = not applicable. ** Measured at pH 12.0.
3.1 Purify the amine-modified oligonucleotide. To ensure that
the oligonucleotide is free of interfering compounds, especially
amines, such as triethylamine or Tris, and ammonium salts, we
strongly recommend extracting and precipitating the sample
prior to initiating the labeling reaction. We suggest the follow-
ing protocol for 0.1–1 mg oligonucleotide (3–30 A260 units).

- Dissolve the oligonucleotide in 100 µL dH2O and extract
  three times with an equal volume of chloroform.
- Precipitate the oligonucleotide by adding one-tenth volume
  (10 µL) of 3 M NaCl and two and a half volumes (250 µL)
  of cold absolute ethanol. Mix well and place at -20°C for
  30 minutes.
- Centrifuge the solution in a microcentrifuge at ~12,000 g for
  30 minutes.
- Carefully remove the supernatant, rinse the pellet once or
twice with cold 70% ethanol and dry under vacuum.
- Dissolve the dry pellet in dH2O to achieve a final concentra-
tion of 25 µg/µL (4.2 mM for an 18-mer). This amine-modified
oligonucleotide stock solution may be stored frozen at -20°C.

3.2 Prepare 0.1 M sodium tetraborate, pH 8.5 labeling
buffer. Make a 0.1 M sodium tetraborate buffer by dissolving
0.036 g of sodium tetraborate decahydrate for every mL of water.
Adjust pH with HCl to 8.5. This labeling buffer should be made
as close as possible to the time of labeling. Alternatively, it may
be divided into small aliquots and frozen immediately for long-
term storage. Exposure of this solution to air for a long time will
result in carbon dioxide absorption, which will change the pH of
the buffer.

3.3 Dissolve 250 µg of the amine-reactive compound in 14 µL
DMSO. Allow amine-reactive compound to come to room tem-
perature before opening the vial. Do not heat. For dinitrophenyl
(DNP) succinimidyl ester, use 160 µg for 100 µg of oligonucleo-
tide. For fluorescein, tetramethylrhodamine, Marina Blue, FAM
or TAMRA succinimidyl ester, use 200 µg for 100 µg of oligo-
nucleotide. Dissolve the material by pipetting up and down,
washing the sides of the vial. Texas Red compounds tend to pre-
cipitate easily and may require longer times to completely dissolve.

It is important that the amine-reactive label be freshly prepared
for each labeling reaction as reactive compounds are not stable in
solution.

3.4 To the vial containing the reactive label in DMSO, add:
- 7 µL dH2O
- 75 µL labeling buffer (step 3.2)
- 4 µL of a 25 µg/µL oligonucleotide stock solution (step 3.1)

The reaction mixture may have a grainy appearance, but this
should not adversely affect the conjugation. We strongly advise
against attempting to improve the solubility of the label, because
modifying the composition of the mixture can drastically reduce
the labeling efficiency. The reaction may be scaled up or down
as long as the concentration of each component is not changed.
Do not add more dye than recommended, as excess dye will not
improve the labeling efficiency and may make the purification
more difficult.

3.5 Incubate the reaction for at least six hours (or overnight
if more convenient) at room temperature. Place the vial on a
shaker oscillating at low speed or gently vortex mix or tap the vial
every half hour for the first two hours to ensure that the reaction
remains well mixed. Do not mix violently, as material may be
left on the sides of the vial. After six hours, 50–90% of the
amine-modified oligonucleotide molecules should be labeled.
Allowing the incubation to proceed overnight does not necessarily
result in a greater labeling efficiency.

Purification of Labeled Oligonucleotide

Following the reaction, the labeling mixture contains labeled
oligonucleotide, unlabeled oligonucleotide and unincorporated
dye (or biotin or DNP). The labeled oligonucleotide can be puri-
fied from the reaction mixture by preparative gel electrophoresis
or reverse-phase HPLC. Regardless of the purification method
selected, ethanol precipitation is recommended as the first step.

Ethanol precipitation of labeled oligonucleotide. Precipitate
the reaction mixture with ethanol as follows: Add one-tenth vol-
ume of 3 M NaCl and two and a half volumes of cold absolute
ethanol to the reaction vial. Mix well and place at -20°C for
30 minutes. Centrifuge the solution in a microcentrifuge at
~12,000 × g for a full 30 minutes. Loss of sample may occur if
the centrifugation is not long enough. Carefully remove the
supernatant, rinse the pellet once or twice with cold 70% ethanol
and dry briefly. If the labeled oligonucleotide becomes completely
dry, it will be difficult to redissolve.

- Some unreacted labeling reagent may have precipitated over
the course of the reaction or may be stuck on the walls of the
reaction vial. This material should be completely redissolved
by extensive vortex mixing before centrifugation. Redissolving
the labeling reagent ensures that the precipitated oligonucle-
otide will be minimally contaminated with unreacted label.
- In some cases, the labeled oligonucleotide may have already
precipitated onto the walls of the reaction tube. This precipi-
tate will not dissolve with the addition of NaCl and ethanol — the
precipitated product will remain on the walls of the tube,
however the free dye will dissolve and be eliminated. After
centrifugation and rinsing, the pellet should be soluble.
- Some reactions may benefit from a second ethanol precipita-
tion in order to adequately eliminate unreacted dye. In par-
icular, when using tetramethylrhodamine (TAMRA), you
should redissolve the oligonucleotide pellet in dH2O and
repeat the ethanol precipitation. This extra ethanol precipita-
tion step is necessary because the tetramethylrhodamine labeling
reagent has a tendency to adhere nonspecifically to the
oligonucleotide. Similarly, a second ethanol precipitation is
often appropriate when using the Texas Red-X, BODIPY
564/570, BODIPY 581/591 or BODIPY 630/650-X dyes.

Purification by HPLC. Labeled oligonucleotides can be
purified by reverse-phase HPLC using a standard analytical
(4.6 × 250 mm) C8 column. Dissolve the pellet from the ethanol
precipitation in 0.1 M TEAA (triethylammonium acetate). Load
the dissolved pellet onto the column in 0.1 M TEAA and run a
linear 5–65% acetonitrile gradient over 30 minutes. This gradient
is a 2% increase in acetonitrile per minute. For oligonucleotides
labeled with very hydrophobic dyes, like Texas Red dye, you can
achieve good separation running a faster gradient with up to a 3%
increase per minute. For separation of oligonucleotides labeled
with more hydrophilic dyes, like Marina Blue dye, run a slower
gradient, about 1% increase in acetonitrile per minute. In all
cases, the unlabeled oligonucleotide will migrate fastest, followed
by the labeled oligonucleotide and finally the free dye. For more
Purification by gel electrophoresis. To purify the labeled oligonucleotide by gel electrophoresis, pour a 0.5 mm–thick polyacrylamide slab gel. 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. Resuspend the pellet from ethanol precipitation in 200 mL of 50% formamide, and incubate at 55°C for 5 minutes to disrupt any secondary structure. Load the warmed oligonucleotide onto the gel (you may need to use several wells) 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. Run the gel until the bromophenol blue indicator dye is two-thirds of the way down the gel. Remove the gel from the glass plates and place on Saran Wrap™. Lay the gel on a fluorescent TLC plate. Locate the labeled and unlabeled oligonucleotides by illumination with a handheld UV source. Fluorophore-labeled oligonucleotides will show fluorescence when illuminated with UV light. Cut out the band containing the labeled oligonucleotide and purify by the “crush-and-soak” method or other suitable method. For more details, please refer to Sambrook J., Fritsch E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory (1989).

References


Contact Information

Further information on Molecular Probes’ products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Leiden, the Netherlands. All others should contact our Technical Assistance Department in Eugene, Oregon.

Please visit our Web site — www.probes.com — for the most up-to-date information.

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