Antibody Conjugates for Cell Biology

Antibody conjugates are extremely useful reagents for probing many biologically and chemically important molecules in vitro or in vivo. This unit presents some basic protocols for conjugating antibodies with fluorescent dyes, with biotin (see Basic Protocol 1), and with enzymes (see Basic Protocol 2). These protocols have been selected because they are relatively easy to perform and have high rates of success. The basic approach for conjugating antibodies is to derivatize amino groups on the antibody with the dye or biotin derivative of choice. Conjugation at amine sites is simple to perform, and, with a fluorescent dye, it generally yields brightly fluorescent conjugates. The conjugates retain high levels of biological activity, notwithstanding the fact that the labels react with random amines on the antibody molecule, and they may even react near or at the antigen-binding site. Labeling of the amines is the most widely used commercial method to tag antibodies. In this unit, suggestions are presented for variations on these basic protocols that are useful when quantities of available antibody are limited. Methods for estimating antibody concentration are also included (see Support Protocol).

Alternate protocols for attaching label at the carbohydrate site of the Fc portion or at the sulfhydryl groups of the hinge region, which join the two heavy chains of the antibody molecule (Fig. 16.5.1), are also possible (see Alternate Protocol 1). These alternate protocols, which entail more steps, are more complex and require more meticulous optimization. Furthermore, they are difficult to perform when <1 mg of antibody is available. Among these alternate protocols, a general procedure is described for conjugating antibodies (see Alternate Protocol 2) or enzyme (see Alternate Protocol 3) at the carbohydrate site. Although this alternate protocol is presented for antibody-enzyme conjugates only, the basic principles can easily be adapted for conjugation of antibodies with fluorophores or biotin, as detailed in the Background section. Background Information also includes references for labeling antibodies at the hinge region.

**CONJUGATING ANTIBODIES TO FLUOROPHORES OR BIOTIN**

The procedural steps for antibody-fluorophore conjugation and for antibody-biotin conjugation are quite similar. These steps consist of mixing the dye or the biotin-derived reagent at an appropriate molar ratio and pH with the antibody solution and, after incubation, purifying the conjugate from the reaction mixture by size-exclusion chromatography (UNIT 5.5) or dialysis (APPENDIX 3C). The antibody-dye or antibody-biotin conjugate can then be analyzed to determine the degree of labeling, i.e., the number of moles of dye or biotin per mole of antibody. See Critical Parameters for helpful hints.

**Materials**

- Antibody to label
- 1.0 M sodium bicarbonate (see recipe)
- Phosphate-buffered saline (PBS; see recipe)
- Probe (fluorophore or biotin)
- Anhydrous dimethylformamide (DMF) or dimethylsulfoxide (DMSO; see recipe)
- 1.5 M hydroxylamine, pH 8.0 (see recipe)
- Size-exclusion chromatography matrix: MATREX Cellufine GH25 (Amicon/Millipore), BioGel P30 (BioRad Laboratories) or equivalent matrix
- ToyoPearl HW-40F (TosoHaas)
- Reaction buffer, pH 7.5 (see recipe)
- Appropriate TLC solvent (see Table 16.5.1, optional)
Figure 16.5.1  Schematic representation of possible modes of conjugation of antibodies or their fragments to probes. An antibody or Fab fragment is shown conjugated to one or more probe molecules. The probe symbol indicates a fluorophore, biotin, or an enzyme. (A) An antibody conjugated to a probe at several amine groups; (B) antibody conjugated at carbohydrate groups in the Fc regions; (C) antibody conjugated at thiol group in hinge region; (D) Fab fragment conjugated at amine groups; (E) F(ab')_2 fragment conjugated at amine groups. The molecular weight of intact IgG molecules (with all four subunits) is 140 to 160 kDa; where heavy chains are 50 to 75 kDa, and light chains are each ~25 kDa (A-C). Papain digestion of intact IgG yields Fab fragments of ~50 kDa, consisting of one light chain and a slightly larger fragment of the heavy chain, still attached by a disulfide bond (E). Cleavage of intact IgG antibodies with pepsin yields bivalent F(ab')_2 fragments of ~105 kDa (D); and subsequent reduction of the disulfides in the hinge region of F(ab')_2 yields two Fab' fragments (not shown), each ~53 kDa.
Prepare the antibody for conjugation

1. Dissolve the antibody, if lyophilized, at 5 to 10 mg/ml or as concentrated as possible, in 0.1 M sodium bicarbonate (obtained by 10-fold dilution of a 1 M stock). If conjugating with isothiocyanates or sulfonyl chlorides of fluorophores, adjust the pH.
of the bicarbonate solution to 9.0. If the antibody to be conjugated is already in
solution in 10 to 20 mM phosphate, 0.15 M NaCl (PBS) to obtain the appropriate pH
by simply adding 1/10 of the volume of 1 M bicarbonate, with pH unmodified, or pH
9.0 as needed. If the antibody is in a buffer containing Tris, glycine or other amines,
dialyze the antibody against PBS or 0.1 M bicarbonate to “exhaustion,” because the
probes will react with any available free amine.

There is no need to modify the pH when reacting with succinimidyl esters.

**Prepare probe solution**

2. Calculate the volume of 10 mg/ml solution of probe (fluorophore or biotin) required
to react with the antibody to be labeled, as follows:

\[
\text{ml of 10 mg/ml probe} = \left(\frac{(\text{mg of Ab})}{(10 \text{ mg/ml of probe})}\right) \times (\text{MR} \times \text{MW of probe})
\]

where Ab = antibody; MW = molecular weight; and MR = incubation molar ratio of
probe to antibody.

*For example, for labeling 5 mg of antibody with a 1:10 incubation molar ratio of
carboxytetramethylrhodamine succinimidyl ester (TAMRA, SE; MW 430.5):*

\[
\text{volume of TAMRA, SE at 10 mg/ml} = (\frac{5}{10}/145,000) \times (10 \times 430.5) = 0.0148 \text{ ml.}
\]

3. Immediately prior to starting the reaction, prepare a 10 mg/ml solution of the probe
by weighing an amount of probe appropriate for the precision of the balance, for
example, 3 mg, and dissolve it in 0.3 ml of DMF or DMSO.

Sulfonyl chlorides, such as Texas Red sulfonyl chloride and Lissamine rhodamine B
sulfonyl chloride, should be dissolved in DMF, because they react with DMSO.

4. Briefly, vortex or sonicate the reactive probe until it dissolves completely.

*In general, reactive probes are very sensitive to hydrolysis; thus, solutions of any reactive
probe should not be stored. However, in some cases, the loss of reactivity during storage
at ~20°C may be within an acceptable range.*

**Label antibody**

5. While stirring, slowly add the probe solution to the antibody solution, in the amounts
determined in step 2. Mix thoroughly.

6. Incubate the reaction mixture 60 to 90 min at room temperature with gentle stirring.

7. *(Optional)* Stop the reaction by adding 1/10 vol of 1.5 M hydroxylamine hydrochlo-
ride that has been adjusted to pH 8.0 with NaOH and incubating an additional 20 to
30 min.

*Treatment with hydroxylamine is potentially useful for removing the dye or biotin from
unstable conjugates with hydroxyl-containing amino acids (Wong, 1991).*

**Purify conjugated antibody**

8. Purify the antibody from unreacted dye or biotin by size-exclusion chromatography
(*UNIT 5.5*) using PBS or reaction buffer.

*The size of the column should be selected based on the amount and concentration of the
antibody. A 10 × 400–mm column is adequate for 5 to 10 mg of antibody at 5 to 10 mg/ml,
while a 5 × 200– to 250–mm column can be used for 1 to 5 mg of antibody at ≥2 or more
mg/ml. For dyes that are relatively hydrophobic, such as rhodamines or Texas Red, it is
very useful to layer a small amount of ToyoPearl (a 2- to 3-cm layer) over the matrix used
in the column, to help retain the unreacted dye.*
If the antibody concentration is <2 mg/ml, purify the conjugate from excess probe by dialysis (APPENDIX 3C) to "exhaustion," i.e., until no free probe is detectable by TLC analysis (see step 9). Although it is more time consuming, dialysis is a very easy method to purify small, diluted amounts of antibody conjugates, avoiding the further dilution inevitable with size-exclusion chromatography performed with free-standing columns.

Alternatively, the solution of the antibody conjugate can be concentrated using a centrifugal spin filter device, with a mwco of 30 to 50 kDa. For amounts of antibody <1 mg, at the concentration of ≥1 mg/ml, separation from unreacted probe can be achieved by centrifuging the antibody, in volumes of ≤0.2 ml, on disposable spin columns, following the manufacturer’s directions. Spin columns do not cause appreciable dilution of the sample, and they offer a rapid method to obtain small amounts of purified conjugates. To avoid denaturation, dilute solutions of antibody conjugates (<1 mg/ml) should be stabilized by adding bovine serum albumin (BSA) or gelatin to a final concentration of 1 mg/ml.

9. Identify the fractions from the fluorophore-antibody column chromatography.

For dye conjugates, the antibody conjugate is the first colored fraction to elute from the column, while the unreacted fluorophore is retarded. A clear band should be visible between the conjugate and the unreacted probe. Depending on the type of fluorophore and the type of size-exclusion matrix used, the unreacted dye could appear as a single band or multiple bands, because the succinimidyl ester might be only partially hydrolyzed and/or the dye might consist of isomers. Neither the band of unreacted biotin on the top of the column nor the biotin-antibody conjugate in the eluate from a spin column can be seen directly. Consequently, the biotin conjugate eluting from the column must be identified by measuring the A_{280}.

Analyze conjugates for purity

10. For fluorescent antibodies only: Analyze the purity of the eluted antibody conjugate by TLC. Spot 1 to 2 µl of sample on a small strip (~1.5 × ~10 cm) of a silica gel thin layer chromatography sheet and run it in a small, saturated chamber in the appropriate solvent (see Table 16.5.1), in parallel with the dye as a control. Remove the strip when the solvent front reaches ~3/4 of the strip height, and let it dry.

The antibody-conjugate will remain at the origin, while the contaminating unreacted dye will migrate towards the solvent front. The spots can be seen when excited by the long wavelength light of a hand-held UV lamp. If unreacted dye is present, the conjugate can be further purified by dialysis or by spinning it on a new spin column, according to the conjugate volume. This method is not applicable to antibody-biotin conjugates.

Determine the degree of antibody labeling by the probe

For antibody-fluorophore conjugates

11a. Measure the absorbance of the conjugate at the wavelength at which the dye absorbance is maximal (A_{Dye}) and at 280 nm (A_{280}). Then calculate the dye-to-antibody ratio using the following equation:

\[
(A_{Dye}/\varepsilon_{M_{Dye}}) + [(A_{280} - A_{Dye} \times CF)/\varepsilon_{M_{A280}}] = (\text{mol of dye/mol of antibody})
\]

where \(A_{Dye} = \text{dye absorbance at peak wavelength; \(\varepsilon_{M_{A280}} = 203,000 \text{ cm}^{-1}\text{M}^{-1}\), the approximate molar extinction coefficient of the antibody (IgG) at 280 nm; \(\varepsilon_{M_{Dye}} = \text{molar extinction coefficient of the dye at the same wavelength as } A_{Dye}. CF is a correction factor equal to A_{280}(\text{for dye})/A_{Dye}.

Note that, while this formulation applies to fluorophore conjugates with intact IgG molecules, it can approximately apply as well to conjugates with antibody fragments, Fab, Fab', and F(ab')_{2} (see Fig. 16.5.1), when used with the \(\varepsilon_{M_{A280}}\) appropriate for the antibody fragment. In this case, the \(\varepsilon_{M_{A280}}\) of the antibody fragment can be calculated from the measured absorbance of antibody solution at 1.0 mg/ml.
For antibody-biotin conjugates

The normal degree of biotinylation of antibodies can be determined by taking advantage of the high affinity of biotin for avidin. The dye, HABA, interacts with avidin, yielding a complex with an absorption maximum at 500 nm. Because of its higher affinity, biotin will displace HABA, decreasing the absorbance at 500 nm ($A_{500}$) by an amount proportional to the amount of biotin present in the assay. This relation can be quantified in a standard curve.

11b. Prepare the standard curve by adding 0.25 ml of HABA to 10 ml of the 0.5 mg/ml avidin solution. Incubate 10 min at room temperature.

12b. Distribute 0.9 ml of the avidin/HABA solution into each of seven tubes. Add 0.1 ml buffer, pH 6.0, to one tube (reference) and 0.005 to 0.1 ml biotin solution to the other six tubes. Bring the final volume to 1.0 ml (when necessary) with assay buffer, mix well and incubate 10 min at room temperature.

13b. Measure the $A_{500}$ for each concentration point, using assay buffer as a blank, and subtract it from the value obtained from the reference absorption.

14b. Plot a standard curve of the nanomoles of biotin versus the decrease in $A_{500}$.

An example of such a standard curve is shown in Figure 16.5.2.

15b. To measure the degree of biotinylation of the sample, add an aliquot of biotinylated antibody of known concentration to 0.9 ml avidin-HABA complex. For example, add 0.05 to 0.1 ml of biotinylated antibody at 1 mg/ml to 0.9 ml of avidin-HABA mixture. Bring the volume to 1.0 ml with assay buffer (when necessary), incubate for 10 min, and measure the decrease in $A_{500}$. 

Figure 16.5.2 Standard curve of biotin concentration versus $\Delta A_{500}$. The standard curve for biotin assay with avidin-HABA reagent relates measured difference in absorption at 500 nm to biotin concentration.
16b. Using the standard curve, determine the nanomoles of biotin corresponding to the observed change in $A_{500}$.

The ratio between nanomoles of biotin used to displace HABA and nanomoles of antibody represents the degree of biotinylation. For example, the calculation for an IgG conjugate is:

\[
(15 \text{ nmol biotin} \times 145,000 \text{ g/mol}) \div (4.35 \text{ mg/ml antibody} \times 0.1 \text{ ml}) = 5 \text{ mol of biotin per mol of antibody}
\]

where 145,000 is the MW of the IgG antibody, and 0.1 ml is the volume of 1 mg/ml of biotinylated antibody sample.

CONJUGATION OF FLUOROPHORES OR BIOTIN WITH F(ab')$_2$ OR Fab ANTIBODY FRAGMENTS

As described in UNIT 16.4 of this series, antibody fragments that retain antibody binding activity can be generated by selective enzymatic digestion of intact antibodies. Conjugates made with antibody fragments may offer enhanced signals because their smaller size can permit penetration and reaction with the more recessed antigen sites or more extensive binding to closely located binding sites (see Critical Parameters for helpful notes).

Alternate conjugation procedure for fluorophores or biotin. In cases where conjugation with fluorophores or biotin at lysines appears to interfere with antibody binding, labeling can be performed with biotin hydrazides or fluorophore hydrazides at the carbohydrate prosthetic group, located in the Fc portion of the molecule (see Fig. 16.5.1), as described in the protocol for conjugation of antibodies with enzymes (see Basic Protocol 2). The hydrazide of the chosen fluorophore or biotin should be used in place of the enzyme, and purification of the conjugate performed as described (see Basic Protocol 2). As for the succinimidyl ester forms, biotin hydrazides are available with one (X) or two (XX) aminohexanoic acid spacers.

METHODS TO ESTIMATE ANTIBODY CONCENTRATION

The antibody concentration can be estimated by three different methods:

Correct the $A_{280}$ of the conjugate for the $A_{280}$ due to the dye, using the correction factor of the specific dye given in Table 16.5.2 and the value, $e^M_{A_{280}} = 203,000 \text{ cm}^{-1} \text{ M}^{-1}$, for the IgG antibody. For example, conjugation of a goat anti-guinea pig IgG antibody with Alexa Fluor 488 succinimidyl ester yielded the following data, after 1:10 dilution: $A_{488} = 0.533; A_{280} = 0.314; e^M_{\text{Dye}} = 71,000 \text{ cm}^{-1} \text{ M}^{-1}$ (Table 16.5.2). In this case, the degree of labeling is

\[
0.533/71,000 \text{ cm}^{-1} \text{ M}^{-1} + [(0.314 - 0.533 \times 0.11)/203,000 \text{ cm}^{-1} \text{ M}^{-1}] = 5.96 \text{ moles of dye per mole of antibody}
\]

where 0.11 is the CF for the Alexa Fluor 488 dye (from Table 16.5.2).

Estimate the protein concentration from the initial amount, assuming a loss of 15% to 20%, or more if some precipitation occurs during purification.

Use a protein assay method (APPENDIX 3B) to measure the concentration of the conjugate. This can be problematic, however, because most protein assays suffer from interference by fluorophores.
**BASIC PROTOCOL 2**

**CONJUGATION OF ANTIBODIES WITH ENZYMES**

Conjugation of antibodies with enzymes involves crosslinking of the two proteins in successive procedural steps. The method involves (1) derivatization of the antibody with a bifunctional reagent that introduces reactive maleimide groups into the molecule and (2) derivatization of the enzyme with a thiol group that can form a stable thioether bond with the maleimide group on the antibody. This approach can be tailored to obtain either heteroconjugates consisting of one molecule of antibody and one molecule of enzyme or conjugates consisting of one antibody labeled with multiple enzyme molecules. The most commonly used enzymes are horseradish peroxidase (HRPO), alkaline phosphatase (APase), \( \beta \)-galactosidase (\( \beta \)Gase), and glucose oxidase (GO).

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**Table 16.5.2 Optical Properties of Commonly Used Fluorophores for Antibody Labeling**

<table>
<thead>
<tr>
<th>Dye</th>
<th>Abs/Em</th>
<th>( \varepsilon_{M b} )</th>
<th>CF</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor 350</td>
<td>347 nm/442 nm</td>
<td>19,000</td>
<td>0.19</td>
<td>Blue-fluorescent labeling dye with higher fluorescence per conjugated dye than AMCA.</td>
</tr>
<tr>
<td>AMCA-X</td>
<td>350 nm/448 nm</td>
<td>18,000</td>
<td>0.15</td>
<td>Widely used blue-fluorescent labeling dye. Compact structure</td>
</tr>
<tr>
<td>Marina Blue</td>
<td>365 nm/460 nm</td>
<td>20,000</td>
<td>0.20</td>
<td>Blue-fluorescent dye. Compact structure. pH-insensitive at pH &gt;6.</td>
</tr>
<tr>
<td>Cascade Blue</td>
<td>400 nm/420 nm</td>
<td>28,000</td>
<td>0.65</td>
<td>Resistant to quenching upon protein conjugation. Exhibits better spectral separation from FITC than does AMCA. Water soluble.</td>
</tr>
<tr>
<td>Cascade Yellow</td>
<td>402 nm/545 nm</td>
<td>20,000</td>
<td>0.61</td>
<td>Large Stokes shift. Useful for multicolor analysis in combination with blue-fluorescent dyes.</td>
</tr>
<tr>
<td>Pacific Blue</td>
<td>405 nm/455 nm</td>
<td>30,000</td>
<td>0.20</td>
<td>Bright blue fluorescence emission. Longer-wavelength alternative to AMCA and Marina Blue dye.</td>
</tr>
<tr>
<td>Lucifer yellow</td>
<td>428 nm/536 nm</td>
<td>11,900</td>
<td>0.30</td>
<td>Water soluble. Available as Lucifer yellow iodoacetamide.</td>
</tr>
<tr>
<td>Alexa Fluor 430</td>
<td>431 nm/541 nm</td>
<td>16,000</td>
<td>0.28</td>
<td>Large Stokes shift. One of relatively few dyes that absorb between 400 and 450 nm and have appreciable fluorescence beyond 500 nm.</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>494 nm/518 nm</td>
<td>68,000</td>
<td>0.30</td>
<td>Widely used green-fluorescent labeling dye (FITC). Absorption overlaps the 488 nm spectral line of the argon-ion laser. Prone to photobleaching. pH-sensitive from pH 5 to 8.</td>
</tr>
<tr>
<td>Alexa Fluor 488</td>
<td>495 nm/519 nm</td>
<td>71,000</td>
<td>0.11</td>
<td>Best fluorescein substitute for immunofluorescence and other labeling applications. Exceptionally photostable and pH-insensitive from pH 4 to 10. Produces brighter conjugates than FITC. Ideal for 488-nm excitation.</td>
</tr>
<tr>
<td>Oregon Green 488</td>
<td>496 nm/524 nm</td>
<td>70,000</td>
<td>0.12</td>
<td>Fluorescein substitute. pH-insensitive at pH &gt;6.</td>
</tr>
<tr>
<td>BODIPY FL</td>
<td>505 nm/513 nm</td>
<td>68,000</td>
<td>0.04</td>
<td>Insensitive to solvent polarity and pH from pH 4 to 8. Narrow spectral bandwidth. Neutral dye. Succinimidyl ester with a cysteic acid spacer is the preferred reactive form for protein conjugation.</td>
</tr>
<tr>
<td>Oregon Green 514</td>
<td>511 nm/530 nm</td>
<td>70,000</td>
<td>0.19</td>
<td>Very photostable. pH-insensitive at pH &gt;6. Absorption matched for 514-nm excitation.</td>
</tr>
</tbody>
</table>

*continued*
It should be emphasized that β-Gase contains a large number of free thiols (8 to 12 or more, according to the different preparations of the enzyme) and need not be modified; rather, it can be reacted directly in its native form with the maleimide derivatives of antibodies.

Some protein-to-protein crosslinking kits, adaptable to the conjugation of antibodies with enzymes, are commercially available (Molecular Probes, Pierce, Prozyme).
**Materials**

- HRPO, APase, GO, βGase (Boehringer Mannheim, Sigma)
- Reaction buffer, pH 7.5 (see recipe)
- TEA buffer (see recipe; for APase only)
- Succinimidyl 3-(2-pyridyldithio)propionate (SPDP, MW 312)
- Anhydrous dimethylsulfoxide (DMSO) or dimethylformamide (DMF)
- BIO-GEL P-30 (Bio-Rad) or Cellufine GH-25 (Amicon/Millipore)
- Dithiothreitol (DTT)
- Antibody to label
- Succinimidyl trans-4-(N-maleimidylmethyl)cyclohexane-1-carboxylate (SMCC, MW 334)
- Tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP, MW 287; Molecular Probes, Pierce Chemical)
- 50 mM N-ethylmaleimide (MW 125.13)
- Sephacryl S-200 for HRPO conjugates (Amersham Pharmacia Biotech or Sigma Chemical)
- BIO-GEL A-0.5m for APase conjugates (Bio-Rad Laboratories)
- BIO-GEL A-1.5m for BGase conjugates (Bio-Rad Laboratories)
- Chromatography column of appropriate size (see step 4)
- Ultrafrel centrifugal filter devices, Biomax-50K or-100K (Amicon/Millipore)
- Additional reagents and equipment for dialysis (**APPENDIX 3C**) and size exclusion chromatography (**UNIT 5.5**)

**Prepare the thiolated enzyme**

1. Dissolve or dialyze HRPO (or GO) in reaction buffer at a concentration of 10 mg/ml. Dialyze APase in TEA buffer.

2. Weigh 3 mg of SPDP, and dissolve in 0.3 ml DMF to obtain a 10 mg/ml solution. Prepare this solution fresh immediately before using.

3a. **HRPO**: To 10 mg of HRPO (0.25 µmol) in 1 ml reaction buffer, add, while stirring, 40 µl of SPDP solution, to obtain a molar ratio of reagent to HRPO of 5. Incubate 1 hr at room temperature.

3b. **APase**: To 10 mg of APase (0.07 µmol) in 1 ml of pH 7.6 TEA buffer, add 10 µl of the SPDP solution and incubate 1 hr at room temperature.

3c. **GO**: To 10 mg of GO (0.063 µmol) in 1 ml reaction buffer, add 10 µl of the SPDP solution and incubate 1 hr at room temperature.

   *Both APase and GO have many more amines available for thiolation compared to HRPO, which has only 4 to 6 amines (Welinder, 1979). Thus the molar ratio of reagent to enzyme is ~5, notwithstanding the disparity in molecular weight between the two proteins.*

4. Purify the thiolated enzyme by molecular exclusion column chromatography, using BIO-GEL P-30 or Cellufine GH-25, in reaction buffer for HRPO or TEA buffer, pH 7.6, for APase.

   *A column bed size of ~10 × 400–mm is required for 10 to 20 mg enzyme.*

**Determine the degree of thiolation**

5. Prepare a 100 mM solution of DTT by dissolving 7.7 mg of DTT in 0.5 ml of distilled water.

6. Transfer 1 ml of 0.5 to 1 mg/ml enzyme solution to a spectrophotometer cuvet. Record the $A_{280}$ and $A_{343}$ of the solution.
7. Add 50 µl of DTT solution. Mix well, incubate 3 to 5 min, and record the $A_{280}$ and $A_{343}$ again.

8. Using an extinction coefficient of 8080 cm$^{-1}$M$^{-1}$ at 343 nm (Carlsson et al., 1978), calculate the amount of pyridine-2-thione liberated during the reduction.

   *The amount of this product is equivalent to the number of thiols introduced in the protein.*

9. To determine the enzyme concentration, correct the $A_{280}$ of the enzyme solution for the absorbance contributed by 2-pyridyldithio propionate at this wavelength, according to the following equation:

   $$A_{280} \text{ of enzyme} = A_{280} \text{ of conjugate} - (\Delta A_{343} \times 5100 \text{ cm}^{-1} \text{M}^{-1}/8080 \text{ cm}^{-1} \text{M}^{-1})$$

   where $\Delta A_{343} \times 5100 \text{ cm}^{-1} \text{M}^{-1}/8080 \text{ cm}^{-1} \text{M}^{-1}$ reflects the amount of pyridine-2-thione generated during the reaction.

   For HRPO, the enzyme concentration can be determined at 403 nm using the extinction coefficient shown in Table 16.5.2, with no correction. The number of thiols per mole of enzyme can be calculated using the following equation:

   $$\text{moles of } -\text{SH groups per mole of enzyme} = (\Delta A_{343}/8080 \text{ cm}^{-1} \text{M}^{-1}) + \left\{\frac{[A_{280} - (\Delta A_{343} \times 5100 \text{ cm}^{-1} \text{M}^{-1}/8080 \text{ cm}^{-1} \text{M}^{-1})]}{\epsilon_{\text{M enzyme}}}\right\}$$

   or, for HRPO:

   $$(\Delta A_{343}/8080 \text{ cm}^{-1} \text{M}^{-1}) + \left\{\frac{A_{403}}{\epsilon_{\text{M enzyme}}}\right\}$$

10. From the preceding equations, determine the average number of moles of antibody that can be reacted with each mole of enzyme.

   *To obtain a 1:1 enzyme-antibody conjugate, the enzyme should be derivatized with 1.2 to 1.5 pyridyldithiol residues per mole. Thiolated enzymes remain stable for a few weeks when stored refrigerated in presence of 2 mM azide or, for HRPO, 0.01% (w/v) thimerosal, because azide inhibits the activity of this enzyme.*

*Prepare the antibody-maleimide derivative*

11. Dissolve or dialyze the antibody in reaction buffer.

   *The antibody should be as concentrated as possible to increase both the yield and the efficiency of the conjugation reaction. Ideally, the concentration should be 5 to 10 mg/ml; however, modifications of the labeling procedures can accommodate lower concentrations, especially when labeling small amounts of antibody (0.1 to 1.0 mg). Thus, the antibody concentration may be 2 to 10 mg/ml.*

12. Weigh 3 to 5 mg of SMCC and immediately before use dissolve it in DMSO or DMF to obtain a 10 mg/ml solution. Vortex or sonicate to assure complete dissolution.

13. While stirring, add 15 µl of SMCC solution to each 5 mg of antibody to obtain a molar ratio of reagent to antibody of $\sim$15.

   *The amount of SMCC should be tailored to the concentration of the antibody. For example: 5 mg of antibody at the concentration of 2 mg/ml would require 15 to 17 µl of the above SMCC solution, while antibody at 10 mg/ml would yield a better conjugate when using a molar ratio of SMCC of 10.*

14. Incubate 1 hr at room temperature with gentle stirring.

15. Dialyze 24 hr in the cold with four changes of 1 liter or more reaction buffer.
**Deprotect the enzyme thiol groups**

*NOTE:* It is essential that this procedure be carried out simultaneously to the labeling of the antibody with maleimide groups, because both the deprotected thiols of the enzyme and the maleimide groups of the antibody are unstable in water and are best used within a day from their preparation.

**Deprotect with DTT**

16a. Add 8.0 mg of DTT to each milliliter of the enzyme solution. Incubate 15 to 20 min at room temperature.

17a. Dialyze the thiolated enzyme 24 hr in the cold against 2 liters reaction buffer, with at least four changes of buffer to eliminate as much of the DTT as possible.

**Deprotect with TCEP**

16b. Calculate the volume of 1 mg/ml TCEP stock solution needed to obtain an incubation molar ratio of 5 moles of TCEP per mole of enzyme

\[
\mu l \text{ of TCEP} = \frac{(\text{mg of enzyme})}{(1 \text{ mg/ml TCEP})} \times \frac{286.7}{(\text{MW of enzyme})} \times 5 \times 1000
\]

where 286.7 is the MW of TCEP, 5 is the incubation molar ratio, and 1000 is a unit conversion factor. Weigh out 3 to 5 mg of TCEP powder, and dissolve it in 3 to 5 ml reaction buffer to make a 1 mg/ml solution of TCEP.

Alternatively, TCEP can be used in place of DTT to deprotect sulfhydryl groups (Getz et al., 1999). TCEP does not react with maleimides. Thus, one need not eliminate excess TCEP from the reaction mixture, avoiding the need to dialyze to exhaustion. TCEP is very reactive and should be used at an incubation molar ratio of 5 TCEP per mole of enzyme.

17b. Add the appropriate amount of TCEP stock solution to the thiolated enzyme solution, and mix well. Incubate the mixture 10 to 15 min at room temperature.

**Synthesize the antibody-enzyme conjugate**

18a. For HRPO, APase, and GO:

Transfer the antibody and the HRPO or APase from the dialysis tubes into two separate test tubes. Slowly drip the antibody solution into the enzyme solution with stirring. Incubate 1 hr at room temperature and overnight at 4°C.

18b. For βGase:

For each 5 mg of antibody dissolve 15 mg of enzyme in 1.5 ml reaction buffer and proceed as in step 18a.

19. Stop the reaction for HRPO, GO, and APase conjugates with the addition of N-ethylmaleimide (NEM) at a final concentration of 50 µM, by diluting the NEM solution 1:1000 in the conjugate reaction mixture. Incubate 30 min at room temperature.

*The conjugate is now ready to be purified by column chromatography, immediately or after overnight storage at 4°C.*

*The reaction of βGase cannot be stopped because of the large number of –SH groups of this enzyme.*

**Purify the antibody-enzyme conjugate**

20. Concentrate the antibody-enzyme conjugate in a centrifugal filter device at the speed recommended by the manufacturer.
21. Pack appropriate size columns (e.g., 1 x 60-cm for 10 mg of protein in the conjugate reaction mixture) with the appropriate matrix.

For purification of HRPO-antibody conjugate use Sephacryl S-200 in reaction buffer. For GO and APase-antibody conjugates use Bio-Gel A-0.5m in 3 M NaCl/30 mM TEA/1 mM MgCl₂, pH 7.6; for BGase Bio-Gel A-1.5m in reaction buffer, with the addition of 3 mM MgCl₂ and 2 mM 2-mercaptoethanol.

22. Load the conjugate onto the column, elute with the buffer used to pack the column, collecting 1-ml fractions.

The first peak to elute, measurable by absorbance at 280 nm, or 403 nm for HRPO conjugates, corresponds to the conjugate. However, each fraction should be checked for antibody binding and enzymatic activity in an ELISA-type (APPENDIX 3) or other appropriate assay.

An alternative method to purify antibody-enzyme conjugates from the unreacted enzyme relies on the affinity of the Fc portion of the antibody for nickel chelates. An affinity column based on this principle is available from Pierce Chemical. The Fc region complexes with nickel on the column while the unconjugated enzyme passes through. The bound antibody-enzyme conjugate can be eluted by lowering the pH, by increasing the salt concentration, or by simply adding EDTA to the elution buffer. A detailed procedure for using this affinity column accompanies the product. This column cannot be used for F(ab')₂ or Fab fragments, as they are devoid of the Fc region (Hermanson, 1996a).

CONJUGATION AT THE CARBOHYDRATE SITE OF THE ANTIBODY

The sugar constituents of glycoproteins contain vicinal hydroxyl groups that can be oxidized to dialdehydes by treatment with periodic acid or its salts. The dialdehydes formed easily react with amines, including the hydrazide form of fluorophores, the ε-amino group of lysines, and the amino terminus of proteins. The carbohydrate-containing portion of an antibody molecule is located in the Fc region; thus, the periodate oxidation method of conjugating antibodies allows labeling at a specific location, remote from the antigen binding site.

CAUTION: Some monoclonal antibodies are not glycosylated and cannot, therefore, be conjugated by this procedure.

Materials

- Antibody to label in solution (see Basic Protocol 1, step 1)
- 0.1 M acetate buffer, pH 6.0
- 20 mM sodium metaperiodate in acetate buffer, pH 6.0, ice cold
- Reaction buffer, pH 7.5 (see recipe)
- 100 mM aqueous sodium cyanoborohydride, freshly prepared
- HRPO, APase, BGase, GO
- Column chromatography matrices (see Basic Protocol 2)

NOTE: Protect the sample from light during the entire procedure.

1. Dialyze the antibody at 2 to 10 mg/ml in 0.1 M acetate buffer, pH 6.0, at 4°C.

2. Keeping the dialyzed antibody on ice, add an equal volume of ice-cold metaperiodate solution. Incubate 2 hr in the dark at 4°C.

3. Purify the oxidized antibody: If the antibody is dilute (<1 mg/ml), dialyze it against the same buffer, protecting it from light. If the antibody is concentrated, purify it on a desalting column (Bio-Gel P-30 or equivalent) in free-standing or spin-column form (Basic Protocol 1, step 8) to remove the iodate that is produced during the oxidation.
4. Prepare a solution of the enzyme of choice in reaction buffer in the amount needed to obtain an incubation molar ratio of enzyme to antibody of 2 to 10, according to the type of conjugate desired.

   For instance, if a 1:1 ratio of probe to antibody is desired, use an incubation molar ratio of 2; otherwise, the higher the desired enzyme-antibody ratio, the higher will be the necessary incubation molar ratio.

5. Slowly add the antibody to the enzyme solution with stirring and incubate the mixture for 2 hr at room temperature with gentle stirring.

6. (Optional) To stabilize the imine bond into a substituted hydrazide, add sodium cyanoborohydride to a final concentration of 10 mM, adding 1/10 vol of a 100 mM stock solution of sodium cyanoborohydride in distilled water. Incubate for 2 hr at 4°C.

   Some researchers consider the imine bond between the sugars on the antibody and the amines on the enzymes to be relatively unstable.

7. Purify the conjugate by any of the chromatography methods (see Basic Protocol 2, step 21).

**CONJUGATION AT THE CARBOHYDRATE SITE OF THE ENZYME**

Since HRPO and GO are glycoproteins, Alternate Protocol 2 can be modified by oxidizing the sugar on the enzyme and, following removal of excess reagent, adding it to the native antibody solution. The steps, molar ratios, and times suggested in Alternate Protocol 2 can be applied to this “reversed” version of the procedure. GO is a flavoenzyme consisting of two 80-kDa subunits that are rich in carbohydrates and joined by a disulphide bond (O’Malley and Weaver, 1972). The conjugation method of choice for GO involves oxidizing the enzyme with periodate at its carbohydrate site. The antibody can then be conjugated to GO through one or more of its amino groups (Rodwell et al., 1986; Husain and Bieniarz, 1994; Hage et al., 1997).

**REAGENTS AND SOLUTIONS**

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

**Hydroxylamine, 1.5 M, pH 8.0**

   4.2 g hydroxylamine, hydrochloride
   25 ml H₂O
   Adjust the pH to 8.0 with 5 M NaOH
   Add H₂O to 40 ml
   Prepare fresh

**Phosphate-buffered saline (PBS)**

   1.1 g K₂HPO₄
   0.43 g KH₂PO₄
   9 g NaCl
   900 ml H₂O
   Adjust the pH to 7.2 or 7.4, if necessary, with 1 M sodium bicarbonate (see recipe)
   Add H₂O to 1 liter
   Store 1 week at 4°C

   The final concentrations are 0.01 M potassium phosphate, 0.15 M NaCl, pH 7.2.
**Reaction buffer**

- 11.5 g Na$_2$HPO$_4$
- 2.6 g NaH$_2$PO$_4$
- 5.8 g NaCl
- 900 ml H$_2$O

Adjust the pH to 7.5 with 5 M NaOH
Add H$_2$O to 1 liter
Store 2 weeks at 4°C

*For labeling IgM, adjust the pH to 7.2.*

**Sodium bicarbonate, 1.0 M**

- 8.3 g NaHCO$_3$
- 100 ml H$_2$O

Store 2 weeks at 4°C

*The resulting pH is generally 8.3 to 8.5
This bicarbonate solution is used to raise the pH of PBS.*

**TEA buffer**

For each liter of buffer, weigh 175 g of NaCl and dissolve it in 750 ml H$_2$O. Add 4.08 ml of TEA (98%, $\rho = 1.12$ g/ml). Prepare a stock solution of 0.5 M MgCl$_2$ and 0.05 M ZnCl$_2$ by weighing 10.16 g of MgCl$_2$ and 0.68 g of ZnCl$_2$ and dissolving them in distilled water to obtain a final volume of 100 ml. Add 2.0 ml of this stock to the NaCl-TEA solution. Adjust the pH to 7.6 with 3 M HCl, and bring the volume to 1.0 liter with H$_2$O. Store at 4°C. For prolonged storage (>1 week), add azide to 2 mM final concentration.

*This buffer is used for APase only.
Final concentrations are 3.0 M NaCl, 30 mM TEA, 1.0 mM MgCl$_2$, 1.0 mM ZnCl$_2$, pH 7.6.*

**COMMENTARY**

**Background Information**

**Antibodies**

The use of antibodies as probes has become widespread in the biosciences. In immunocytochemical applications, the use of labeled secondary antibodies is limited by the number of targets to be detected by different antibodies. This is due to the fact that secondary antibodies, even when purified by immunoabsorption against related species, nonetheless exhibit significant residual cross-reactivity when more than two antibodies are used to stain multiple features of the same sample. Thus, to detect multiple structures or functions simultaneously, direct labeling of primary antibodies with fluorophores, biotin, or enzymes is essential. Biotinylation of antibodies allows detection with fluorescently labeled avidin (or analogous molecules), generally without interference from the secondary antibody or antibodies required in the experimental procedure. Investigators new to the field might refer for general information on the theory of fluorescence and applications of fluorescent probes to the introduction to the topic by Johnson (1996; see also UNIT 4.2) and for avidin-biotin technology to the review by Wilchek and Bayer (1990).

For procedures employing fluorescent labeling of primary antibodies, it is important to select probes with the highest quantum yield, because signals from these antibodies lack the amplification obtained with the secondary antibodies. Signal amplification can be achieved by conjugating antibodies with enzymes that use fluorogenic or colorimetric substrates. These substrates should be of the precipitating type for histological preparations or soluble for ELISA and other microplate assays.

Very useful antibody labeling kits, containing detailed instructions, premeasured reactive fluorophores or biotin are now available from a few commercial sources (Amersham Pharmacia Biotech, Molecular Probes, Pierce). These kits generally allow conjugations of 0.2 to 10 mg of an antibody. They are ideal for researchers who need to perform labeling procedures only occasionally or do not have the necessary equipment (e.g., separation columns and solvent for the la-
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16.5.16

Antibody Lefevre et al., 1996). Antibody conjugates (Banks and Paquette, 1995; Voloshina et al., 1999), Cy dyes, prepared by sulfonylating coumarins or rhodamines (Panchuk-Voloshina et al., 1999), Cy dyes, prepared by sulfonylation of Texas Red and that of Lissamine rhodamine B. The reactive form of fluorophores is extremely labile in water. Consequently, in conjugation with proteins the reaction rate is strongly counteracted by the hydrolysis rate of the dye, making reproducibility of the reaction difficult. Also sulfonyl chlorides require relatively basic conditions, pH 9.0 to 9.5, to react with amines.

2. Sulfonyl halides. The most commonly used probes in this category are the sulfonyl chloride of Texas Red and that of Lissamine rhodamine B. This reactive form of fluorophores is extremely labile in water. Consequently, in conjugation with proteins the reaction rate is strongly counteracted by the hydrolysis rate of the dye, making reproducibility of the reaction difficult. Also sulfonyl chlorides require relatively basic conditions, pH 9.0 to 9.5, to react with amines.

3. Succinimidyl esters. Reagents of this form react more selectively with aliphatic amines than the isothiocyanates by forming stable carbamate bonds at pH 7.5 to 8.5 (Haugland, 1996a). Most fluorophores, including fluorescein, rhodamine, Cy3, Cy5, and the new Alexa Fluor dyes, are available as succinimidyl esters (Haugland, 1999). These are highly preferred because of their greater selectivity for amino groups (Wong, 1991), relatively slow hydrolysis rate (Wong, 1991), and high reactivity at lower pH (Hermanson, 1996b), closer to physiological pH. Because of their still high reactivity at pH 7.2, succinimidyl esters can be used efficiently to label IgM antibodies, which tend to denature when the pH is above 7.4.

The author strongly recommends using fluorescein, tetramethylrhodamine, and Texas Red in their succinimidyl ester forms. In the past, fluorescein and tetramethylrhodamine were available only as isothiocyanates, broadly known in their abbreviated forms as FITC and TRITC, respectively. Texas Red was available only as its sulfonyl chloride, which has an extremely rapid rate of hydrolysis in buffer. The new, succinimidyl ester forms of these dyes are preferable, because they will generally yield more reproducible and stable antibody conjugates (Banks and Paquette, 1995; Lefevre et al., 1996).

In determining the degree of antibody labeling in fluorophore conjugates, one should note that the values, both of the extinction coefficients and of the correction factors described in Table 16.5.2, are only approximate. These values are not corrected for the change in extinction coefficients that may occur when the dyes are conjugated to proteins. This change in extinction coefficient can vary from protein to protein, and it can vary with the number of dyes reacted with the protein, due to interference between dye molecules (Ravdin and Axelrod, 1977). Thus, determination of the degree of labeling is relative and approximate. Nonetheless, it is invaluable when comparing different preparations of the same antibody conjugate and for determining the relative optimal number of moles of fluorophores per mole of antibody for particular biological applications.

Phycobiliproteins

This unit does not include protocols for conjugation of antibodies with phycobiliproteins—large, highly fluorescent pigments that are isolated from the photosynthetic apparatus of algae. The conjugation procedure of phycobiliprotein to antibodies is very similar to Basic Protocol 2. Molecular Probes offers a protocol for conjugation with phycobiliproteins, and details are published (Haugland, 1996b). A kit that greatly facilitates phycobiliprotein conjugation is available from Prozyme, although it does not lend itself to optimizing the procedure. The Protein-to-Protein Crosslinking Kit from Molecular Probes could be used for this purpose. However, obtaining the phycobiliprotein of interest, R-phycoerytin (R-PE) or allophycocyanin (APC), already derivatized with 1 to 2 pyridyl disulfide groups per molecule is strongly recommend. This is more economical than preparing and optimizing the activated protein.

Other fluorophores

Fluorophores with enhanced water solubility and fluorescence output have been developed by introducing sulfonic groups in the rings of their molecule. This procedure also confers negative charges that help to decrease interaction between fluorophores and to minimize the loss of probe fluorescence that generally occurs upon conjugation to biological molecules. The excitation wavelengths for these sulfonated fluorophores span the entire spectrum from excitation in the UV range to the 633-nm excitation of the HeNe laser. They include the Alexa Fluor dyes, prepared by sulfonating coumarins or rhodamines (Panchuk-Voloshina et al., 1999), Cy dyes, prepared by sulfonating carbocyanines (Mujumdar et al.,...
1989, 1993, 1996), and Cascade Blue, which is a pyrenetrisulfonic acid derivative (Whitaker et al., 1991). Each of these dyes generally offers enhanced signal-to-noise ratios. Many of these dyes form conjugates that tolerate a higher number of moles of dyes per mole of antibody in comparison with the non-sulfonated ones, while avoiding quenching of the fluorescence or precipitation of the antibody (Panchuk-Voloshina et al., 1999). In rare cases the negative charges on these dyes may cause nonspecific interaction with positive charges of the cells under investigation. In these cases, which unfortunately can be determined only experimentally, the nonsulfonated form of the dye (e.g., TAMRA, instead of Alexa Fluor 546, or Texas Red-X, instead of Alexa Fluor 594) should be used. Fluorescein itself carries two negative charges, and in cases of background problems the neutral BODIPY FL can be tried as an alternative.

**Biotin**

Biotin is a small, ubiquitous molecule that acts as a coenzyme of carboxylases. Due to its strong affinity for avidin and avidin-like molecules ($K_d = 1.3 \times 10^{-15} \text{ M at pH 5.0}$), it is broadly used as a label that can be probed with fluorescent or enzyme-linked avidin or streptavidin. Avidin (MW = 66 kDa) and streptavidin (MW = 60 kDa), both tetrameric proteins with ~33% homology in their amino acids composition, can each bind four molecules of biotin (Green, 1975).

Iminobiotin, a modified biotin with lower affinity for avidin, is occasionally used in place of biotin to prepare conjugates that can be separated by affinity chromatography. The succinimidyl ester of iminobiotin can be used to effect labeling of a tag only transiently because the iminobiotin-avidin complex dissociates at low pH (Hoffman et al., 1980; Orr, 1981). Affinity purification of biotinylated conjugates is also possible with monomeric avidin, which has a lower affinity for biotin than the tetrameric form and is now commercially available (Pierce Chemical). Nitroavidin, another form of monovalent avidin with a lower affinity for biotin, can be prepared by selectively nitrating three of the tyrosines, each located at the active site of avidin or streptavidin subunits, leaving just one site available for biotin binding (Morag et al., 1996). Another commercially available form of reactive biotin is the DNP biocytin succinimidyl ester, which carries the colorimetric properties of dinitrophenyl (DNP) and the avidin-binding properties of biotin (Briggs and Punfili, 1991; Haugland, 1996c). With this reagent, direct spectrophotometric measurement of the number of moles of biotin per mole of antibody can be achieved by using the molar extinction coefficient of DNP (15,000 cm$^{-1}$M$^{-1}$ at 364 nm). This reagent (MW = 862 Da) is larger than most common fluorophores and the reactive biotins described above. Thus, more reagent is necessary to obtain an efficient incubation molar ratio of reagent to antibody during the reaction, because larger molecules react more easily with surface groups and have difficulty reaching internal lysines. However, the presence of the DNP group on the conjugate offers a second tag on the target and the conjugate can be further probed with an anti-DNP antibody (Haugland, 1996c).

**Enzymes**

Antibody-enzyme conjugates are probably the most widely prepared antibody-based reagents because they are broadly used in diagnostic clinical tests. HRPO conjugates represent ~70% to 80% of the antibody-enzyme conjugates in common use, while APase makes up for most of the remainder; βGase and GO represent ~2%, and they are used primarily in research. The major applications of enzyme-conjugated antibodies lie in immunocytotoxicomical studies of cells or tissue slices and in enzyme-linked immunoabsorbent assays (ELISA) for quantifying and detecting a wide variety of analytes. The activity of the conjugated enzyme causes the accumulation of measurable enzyme product in the test system, offering amplification of the signal and high assay sensitivity.

It is important to consider the experimental application for which the antibodies are to be conjugated with the enzymes. For cytological or histological studies, conjugates comprised of one enzyme and one antibody molecule are preferable, because, in general, larger conjugates are accompanied by higher background signals. The MW of IgG is ~150 kDa, and IgM is often >850 kDa. Enzymes can also be rather large (e.g., glucose oxidase (GO, MW = 160 kDa), alkaline phosphatase (APase, MW = 140 kDa), β-galactosidase (βGase, MW = 540 kDa)). Consequently, the mass of the antibody-enzyme conjugate can be considerable, especially when multiple enzyme molecules are bound to one antibody molecule. In contrast, for ELISA-based tests, in which only the total signal is essential, larger enzyme-antibody aggregates often increase the sensitivity of the assay.

The simplest method (and one of the oldest) to prepare enzyme-antibody conjugates involves mixing the two conjugate components and adding a “condensation” reagent, generally glutaralde-
Antibody by measuring absorption (Carlsson et al., 1978), the number of free thiols obtained per molecule of enzyme permits one to promptly determine the number of free thiols generated on forming thioether bonds between a maleimide group on the antibody and a free thiol on the enzyme. These two reactive groups are obtained by derivatizing the conjugate components with the heterobifunctional crosslinking reagents, SMCC and SPDP. This approach is “controllable” and easier to optimize than other methods. Similar methods that involve reaction of the maleimide crosslinking reagent to the enzyme and conjugation of the sulfhydryl crosslinking reagent to the antibody have been also reported. This “reverse” method, however, is recommended only when S-acetyltioglycolic acid N-hydroxysuccinimide ester (SATA) is used as the thiolating reagent, because iiminothiolane yields very unstable free thiol groups, and SPDP entails reduction with DTT or TCEP, which might disrupt the disulfide groups of the antibody. Furthermore, because deprotection of the thiol in SATA requires only exposure to hydroxylamine (Duncan et al., 1983), the antibody molecule is less likely to be altered. However, one should also consider that assaying for the free thiols generated with either iiminothiolane or SATA requires a relatively large amount of antibody. In contrast, the release of pyridyl-2-thione from SPDP reduction permits one to promptly determine the number of free thiols obtained per molecule of enzyme by measuring absorption (Carlsson et al., 1978), yielding the precise number of reactive sites. This offers two advantages: (1) the enzyme can be derivatized with only 1 to 2 sulfhydryl groups in order to obtain mostly heteroconjugates of one enzyme per antibody molecule, and (2) the activity of the enzyme can be disrupted to a lesser degree when only 1 or 2 amines are derivatized.

The protocols presented in this unit for linking enzymes to antibodies can be tailored to the antibody-specific application by modulating the number of reactive crosslinking groups with which the antibody or the enzyme is modified. When free thiols are available, as with β-Gase, no chemical modification of the enzyme is necessary. Enzymes derivatized with only 1 to 1.5 thiol and antibodies carrying only a few maleimide groups generally yield heteroconjugates, most of which have one enzyme and one antibody. By introducing a larger number of thiols on the enzyme or a larger number of maleimide groups on the antibody, it is possible to obtain small aggregates consisting of one or more antibody molecule conjugated to two or more enzyme molecules.

Critical Parameters

**Preparation of the antibody for conjugation**

Before being conjugated to a probe, antibodies should be as free as possible of other proteins. Association with extraneous proteins can arise from inadequate purification of the antibody or from stabilizers added to diluted antibody preparation such as bovine serum albumin (BSA) or gelatin. The proper buffer and pH for the antibody conjugation can be obtained by dialysis or by adding 1 M bicarbonate when the antibody is dissolved in PBS. When antibodies are in PBS solution, the pH can be easily changed by adding 1/10 volume of 1 M sodium bicarbonate at pH 8.3 or 9.0, depending on which probe is used. This obviates the need for dialysis, accompanied by losses of material during manipulation of the antibody solution. One should note, however, that the buffer in which the antibody is dissolved should contain no primary amines (e.g., TRIS, glycine, or traces of ammonium sulfate), because they will react with the amine-reactive probe.

**Solubility, storage, and stability**

Most dyes, biotin, and cross-linking reagents are poorly soluble in buffers, and they require solubilization in an organic solvent such as dimethylformamide (DMF) or dimethylsulfoxide (DMSO). Some of the most recently introduced dyes that exhibit greater fluorescence output and photostability (Cy and Alexa Fluor series) have...
improved or good solubility in water, because of the sulfonic groups introduced in the ring of their molecule (Panchuk-Voloshina et al., 1999). Some labeling reagents are available as sulfosuccinimidyl esters, with a sulfonic acid as part of the reactive group, which increases water solubility but may decrease the overall conjugation efficiency. However, in the author’s experience, dissolving the labeling reagent as a concentrated solution in DMF or DMSO (10 to 20 mg/ml) and then adding it to the antibody solution in amounts such that the solvent does not exceed 10% of the reaction mixture, does not generally decrease the antibody binding activity. The presence of the solvent, in fact, helps to maintain the labeling reagent in solution and favors the efficiency of the conjugation reaction.

All labeling reagents should be stored desiccated at -20°C. Antibody conjugates of fluorophores, biotin, or enzymes can be stored frozen in small aliquots, avoiding repeated freezing and thawing. The defrosted aliquots should be stable at 4°C for ≥1 week. Labeled antibodies generally maintain the storage requirements of their native form. However, introduction of dyes or biotin to the molecule, with concomitant loss of the positive charges of the tagged lysines or addition of enzyme molecules, may favor aggregation. Therefore, it is good practice to centrifuge antibody conjugates of fluorophores substituting the positive charged amines. The Fab fragment (Fig. 16.5.1E), tolerates two or three fluorescein or Alexa Fluor 488 molecules well, but only one Texas Red or rhodamine. F(ab')2 fragments (Fig. 16.5.1D) can be labeled with three to four fluorescein molecules or one to two Texas Red molecules. Consequently, when using Fab fragments, one should use smaller molar ratios of reactive probe to antibody than those recommended in Table 16.5.1 for native antibodies. Precipitated antibodies generally cannot be recovered.

Choosing the appropriate fluorophore

The choice of fluorophore for a particular application should be based on the following considerations. When the fluorescence microscope is the detection instrument, for single-color experiments the choice should be dictated by the spectral properties of the dye relative to the excitation source, the brightness of the probe, and its photostability. Photostability is less essential in flow cytometry experiments. For multiple-color experiments, minimal spectral overlap among the chosen fluorophores is essential. For samples with pronounced autofluorescence, longer wavelength emission dyes are better than the green fluorescence–emitting ones because the autofluorescence background will be lower. Table 16.5.2 describes the optical properties of the most commonly used fluorophores for the labeling of antibodies (also see APPENDIX IE).

Choosing the appropriate incubation molar ratio of fluorophore or biotin to antibody

The factors that determine the number of moles of probe per mole of antibody (degree of labeling) that will be present in the purified conjugate are:

1. Reactivity of the dye. This varies from dye to dye; it is pH and temperature dependent, and it must be taken into consideration when planning the reaction molar ratio of reagent to protein to use.

2. Concentration of the antibody. As in any chemical reaction, the reagent concentrations determine the rate and efficiency of the reaction and will generate higher yields of conjugate at higher concentrations.

3. Structure of the antibody. Although the Fc portion of the antibody molecule is relatively preserved, the amino acid composition of the remaining regions is quite varied and contains lysine residues in different numbers and positions. Consequently, the reactivity of each antibody is different. When possible, three or more molar incubation ratios of dye, biotin, or crosslinking reagent to antibody should be tried.
to obtain a degree of labeling of the probe-antibody conjugate that will work best in the specific application. Table 16.5.1 gives guidelines of suggested incubation molar ratios of probe to antibody for different concentrations of antibody. The guidelines are very general and individual antibodies may require customized variations. As previously mentioned, it is desirable to try three different degrees of labeling or, if that is not possible, aim for a moderate degree of labeling of three to four fluorophore moles per mole of antibody. Also note that it is possible to label an antibody for the second time, but it is not possible to reduce the moles of dye per antibody following a conjugation because they are covalently attached.

### Choosing the appropriate biotin-binding protein

Avidin is a highly positively charged glycoprotein, rich in mannose and glucosamine. Thus, it may interact nonspecifically with negatively charged molecules within the cell or with sugar receptors of the cell membrane. For this reason, a chemically modified avidin, deglycosylated, and with a lower isoelectric point has been developed. It is commercially available in both unconjugated and conjugated forms under the trademark, NeutrAvidin (Haugland, 1999). Streptavidin is not glycosylated, and it generally yields less nonspecific binding than avidin. However, streptavidin contains the amino acid sequence Arg-Gly-Asp (RYD) that mimics the binding peptide of fibronectin (RGD), a universally recognized domain of the extracellular matrix. Thus, although it is rare, nonspecific interaction is possible also with streptavidin (Alon et al., 1990), in which cases, avidin or its NeutrAvidin form may be preferable.

### Choosing the appropriate biotinylation reagent

As described for conjugations with fluorophores, the most broadly used reactive biotin for conjugation with antibodies is the succinimidyl ester form. Three types of succinimidyl esters are available: either biotin derivatized directly at the carboxyl with the reactive group or with a 7- or

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<th>Dye</th>
<th>PBS</th>
<th>Slow-Fade</th>
<th>SlowFade Light</th>
<th>ProLong</th>
<th>Vecta-Shield</th>
<th>Cyto-seal</th>
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<td>nt</td>
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</table>

<sup>a</sup>Fading was measured for 90 sec.

<sup>b</sup>Abbreviations: nt: not tested; PBS: phosphate-buffered saline.

<sup>c</sup>Notes: In addition to testing anti-photobleaching reagents listed in the table, the authors tested the performance of the following anti-photobleaching reagents when used with the dyes in the Alexa Fluor series: Crystal Mount, Gel Mount, Immuno-Mount, Fluor-Save, Clarion, Aqua-Poly Mount, Mowiol. Aqua-Poly Mount and Mowiol worked well with Alexa Fluor 546.

---

**Table 16.5.3** Performance of Commercially Available Anti-Photobleaching Reagents<sup>a,b</sup>

<table>
<thead>
<tr>
<th>Reagent</th>
<th>PBS</th>
<th>Slow-Fade</th>
<th>SlowFade Light</th>
<th>ProLong</th>
<th>Vecta-Shield</th>
<th>Cyto-seal</th>
<th>Others&lt;sup&gt;b,c&lt;/sup&gt;</th>
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</tr>
</tbody>
</table>

<sup>a</sup>Fading was measured for 90 sec.

<sup>b</sup>Abbreviations: nt: not tested; PBS: phosphate-buffered saline.

<sup>c</sup>Notes: In addition to testing anti-photobleaching reagents listed in the table, the authors tested the performance of the following anti-photobleaching reagents when used with the dyes in the Alexa Fluor series: Crystal Mount, Gel Mount, Immuno-Mount, Fluor-Save, Clarion, Aqua-Poly Mount, Mowiol. Aqua-Poly Mount and Mowiol worked well with Alexa Fluor 546.
14-atom spacer between the carboxyl and the reactive group (called biotin-X and biotin-XX, respectively). The addition of a spacer favors the efficiency of formation of the complex between avidin and the biotinylated antibody, possibly by facilitating the interaction of the biotin group with the hydrophobic crevice of the avidin binding site (Gretch et al., 1987; Hnatowich et al., 1987). Biotin-X and biotin-XX can be used interchangeably, although in this laboratory the authors have found that biotin-XX gives a somewhat higher signal in ELISA (Haugland and You, 1998). Sulfo succinimidyl esters of biotin, which are highly water soluble are also commercially available. However, this form is generally less reactive and requires a higher molar incubation ratio of reagent to antibody.

**Fluorescence intensity in histological preparations**

For detection by microscopy, either conventional or confocal, the resistance of the fluorophores to photobleaching is a critical concern. It is very frustrating to see an image disappear as soon as it is in focus. There is considerable variation in the performance of anti-photobleaching products as it is in focus. There is considerable variation in the performance of anti-photobleaching products. The author has tried many of the commercially available anti-photobleaching reagents in cellular preparations stained with the most commonly used fluorophores. The results from this study are reported in Table 16.5.3.

**Anticipated Results**

The protocols in this unit are established and have a high probability of success. The exact degree of labeling depends on the antibody to be labeled and the application for which it is used. The reactions may be scaled up or down.

**Time Considerations**

Fluorophore or biotin conjugation and purification of the conjugated antibody will take ∼3 hr. Checking the degree of labeling will take 30 to 60 min. Longer times (∼2 days) are required when working with very dilute antibodies that require dialysis for purification. Conjugation with enzymes and purification of the conjugated antibody will take ∼5 days.

**Literature Cited**


Antibody

Conjugates for

Cell Biology

16.5.22

Supplement 6

Current Protocols in Cell Biology

Contributed by Rosaria P. Haugland
Molecular Probes, Inc.
Eugene, Oregon