

## Product Information

### ImmunoProbe™ Biotinylation Kit

Catalog Number **BK101**  
Storage Temperature 2–8 °C

## TECHNICAL BULLETIN

### Product Description

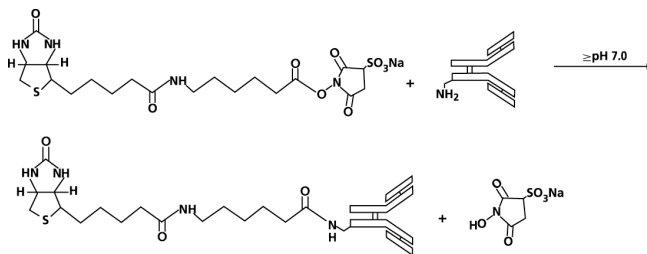
The primary application of the ImmunoProbe™ Biotinylation Kit is the preparation of biotin labeled polyclonal and monoclonal antibodies, which may be used in ELISA, immunohistochemistry, and immunoblotting. The kit may also be used for conjugation of biotin to other proteins, peptide hormones, or cytokines.

The ImmunoProbe Biotinylation Kit provides all necessary reagents for the biotinylation of proteins, a gel filtration column to isolate the biotinylated proteins, and reagents to determine the biotin:protein ratio. The kit contains sufficient reagents for at least 5 conjugations with biotinamido hexanoic acid 3-sulfo-N-hydroxysuccinimide ester (BAC-SulfoNHS) and a protein.

The avidin-biotin system provides a powerful tool for research and analysis due to the specific and high affinity ( $K_a = \sim 10^{15} M^{-1}$ ) interaction between avidin and biotin. Biotinylated antibodies, enzymes, and other protein molecules are increasingly used as specific probes in standard immunological and immunocytochemical techniques,<sup>1-5</sup> and in medical applications.<sup>3</sup> Biotinylated derivatives of peptide hormones, growth factors, and cytokines, are also useful in various research applications involving affinity purification of receptors in combination with avidin affinity chromatography.<sup>6,7</sup>

Biotinylation is often performed with *N*-hydroxysuccinimide (NHS) esters of biotin or biotinamido hexanoic acid (biotinAC, BAC).<sup>8</sup> The extended spacer arm provided by the hexanoic acid greatly improves the interaction between avidin and biotinylated macromolecules by overcoming steric hindrance at the biotin binding sites of avidin.<sup>9</sup> With the ImmunoProbe Kit, biotinylation is performed with BAC-SulfoNHS. This derivative is soluble in water and biotinylation proceeds at near neutral pH values. The reagent is particularly useful when mild reaction conditions are required for the biotinylation of sensitive biomolecules such as antibodies, enzymes, and cell surface proteins.<sup>4,5</sup>

**Figure 1.**  
Conjugation of BAC-SulfoNHS to the Amino Group of IgG



Following the labeling reaction, the biotinylated protein is separated from unreacted or hydrolyzed reagent by a fast gel filtration step. The extent of biotinylation and ratio of biotin to protein are determined by the avidin-HABA assay.<sup>10</sup> The absorption of the avidin-HABA complex at 500 nm ( $A_{500}$ ) decreases proportionally with increased concentration of biotin as the HABA dye is displaced from avidin due to the higher affinity of avidin for biotin. Using a ratio of BAC-Sulfo-NHS:IgG of 5:1 or 10:1, the level of biotin incorporation is 2–3 and 4–6 moles of biotin per mole of protein, respectively.

### Components

Sodium phosphate buffer solution 1 vial  
(Catalog Number P9693, Vial 1)  
The buffer is used for dissolving and diluting the BAC-SulfoNHS and as a suitable buffer for the biotinylation reaction.

Biotinylation Reagent 5 vials  
(Catalog Number B4430, Vial 2)  
Five vials containing 5 mg each of biotinamido hexanoic acid 3-sulfo-N-hydroxysuccinimide ester (BAC-SulfoNHS), MW = 556.8. BAC-SulfoNHS reacts with free amino groups of proteins forming stable amide bonds. For prolonged storage, keep desiccated at –20 °C.

Pronase from <i>Streptomyces griseus</i> (Catalog Number P9818, Vial 3) 1 mg/vial Used for proteolysis of the biotinylated protein, prior to determination of the level of biotinylation using the avidin-HABA assay.	1 vial
Avidin from egg white, affinity purified (Catalog Number A7066, Vial 4) Used for the determination of biotin incorporation (biotin:protein ratio) using the avidin-HABA assay.	1 vial
4-Hydroxyazobenzene-2-carboxylic acid (HABA) sodium salt, 10 mM solution (Catalog Number H6637, Vial 5) Contains 1 ml of 10 mM HABA [2-(4-Hydroxy-phenylazo) benzoic acid] in 10 mM NaOH. Serves as a dye for the determination of biotin incorporation (biotin:protein ratio) using the avidin-HABA assay. HABA binds to avidin resulting in increased absorption of the dye at a wavelength of 500 nm.	1 vial
Phosphate Buffered Saline, pH 7.4 (Catalog Number P3813) Serves as equilibration and elution buffer for the Sephadex® G-25M column and final dilution buffer of the labeled protein.	1 pkg
Gel Filtration Column - Sephadex G-25M (Catalog Number B4783) This is used for the separation of unreacted BAC-SulfoNHS and buffer exchange. The column is preswollen in water containing 0.1% Kathon® CG/ICP as preservative. The bed volume of the column is 9.1 ml and bed height is 5 cm. The maximal sample volume is 2.5 ml.	1 ea

#### Equipment and Reagents Required but Not Provided

- d-Biotin (Catalog Number B4501)
- DMSO (Catalog Number D5879)
- UV/Visible spectrophotometer
- Protein for biotinylation

#### Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

#### Preparation Instructions

0.1 M Sodium Phosphate Buffer, pH 7.2 - Reconstitute vial (Catalog Number P9693, Vial 1) with 5 ml of deionized water.

0.01 M PBS - Add contents of Phosphate Buffered Saline, pH 7.4, pouch (Catalog Number P3813) to a suitable container. Add 800 ml of distilled or deionized water and mix. Bring the final volume to 1 liter with water.

Pronase Solution - Reconstitute pronase (Catalog Number P9818, Vial 3) in 1.0 ml of deionized water. Note: Store Pronase Solution in 0.1–0.2 ml aliquots at –20 °C. Long term storage of the Pronase Solution at 2–8 °C is not recommended.

Avidin Solution - Reconstitute avidin (Catalog Number A7066, Vial 4) with 19.4 ml of 0.01 M PBS.

HABA Solution – 10 mM solution is supplied ready-to-use.

#### Storage/Stability

Store the kit at 2–8 °C.

#### Procedures

##### Protein Labeling

The following procedure describes the preparation of biotin labeled IgG. The molar ratio in the reaction mixture is 5:1 or 10:1 of Biotinylation Reagent (BAC-SulfoNHS, MW = 556.8) to IgG (MW = 150,000). These ratios result, in most cases, in 2–3 moles and 4–6 moles of biotin per mole of protein, respectively. This procedure can be modified if a protein of a different molecular mass or a different amount of IgG is used in the labeling reaction

Notes: The level of biotin labeling may vary from one protein to another, in part due to the different number of lysine residues available for modification.

Protein concentration should be kept high (~10 mg/ml). At lower protein concentrations, the biotinylation reaction is less efficient.

1. Prepare 1 ml of IgG solution at 10 mg/ml in 0.1 M Sodium Phosphate Buffer, pH 7.2. The A<sub>280</sub> of an 1.0 mg/ml IgG solution is 1.4 (1.0 cm pathlength).

Notes: Protein solutions should not be prepared in buffers containing amines such as Tris and glycine, or sodium azide since they inhibit the labeling reaction. If the buffer contains amines or sodium azide, dialyze against 0.1 M sodium phosphate, pH 7.2.

When biotinylating antibodies, the starting material should be free of contaminating serum proteins. Either affinity isolated antibodies or IgG fractions  $[(\text{NH}_4)_2\text{SO}_4$  cut or Protein A purified] are generally acceptable.

2. Dissolve the contents of one vial of BAC-SulfoNHS (Vial 2) with 30  $\mu\text{l}$  of DMSO and then add 0.1 M Sodium Phosphate Buffer, pH 7.2 (Vial 1) to a final volume of 0.5 ml. Vortex well. The resulting concentration of the BAC-SulfoNHS solution is 10 mg/ml.

**Notes:** The solution of BAC-SulfoNHS should be prepared fresh before labeling as it is not stable in aqueous solutions.

Biotinylation with BAC-SulfoNHS can be carried out in 100% aqueous solutions. However, greater solubility can be achieved when the reagent is first dissolved in a small amount of DMSO.

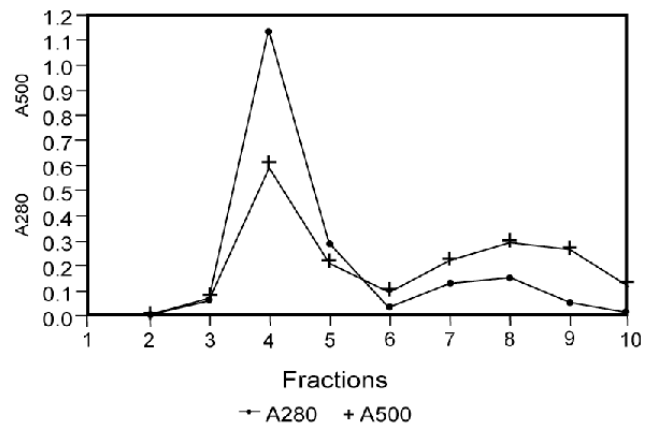
3. Immediately add 19  $\mu\text{l}$  (5:1 ratio) or 38  $\mu\text{l}$  (10:1 ratio) of the Biotinylation Reagent to 1 ml of the IgG solution with gentle stirring.
4. Incubate with gentle stirring for 30 minutes at room temperature or 2 hours at 2–8 °C.

#### Isolation of labeled protein

1. Support the gel filtration column over a suitable container (100 ml).
2. Remove the cap from the top of the column, cut open lower tip of column, and let excess liquid flow through. The column will not run dry.
3. Equilibrate the column with 30 ml of 0.01 M PBS, 6 washes of 5 ml each. If the column is not used immediately, close with top and bottom caps and store the column at 2–8 °C.
4. Apply reaction mixture to the column and collect flow-through (Fraction 1).
5. Elute column with 9 ml of 0.01 M PBS collecting 1 ml fractions (9  $\times$  1 ml).
6. Monitor protein content by measuring absorbance at 280 nm.

7. Pool fractions containing the protein. If collecting 1 ml fractions, the labeled protein should be present in fractions 3, 4, and 5. Do not pool fractions after fraction 6. A typical elution profile is shown in Figure 2. Figure 2 also shows typical  $A_{500}$  values obtained for each fraction in the avidin-HABA assay.

**Figure 2.**  
Typical Elution Profile



8. Wash the column with 30 ml of 0.01 M PBS, 6 washes of 5 ml each. The gel filtration column can be regenerated at least 5 times.
9. For prolonged storage, wash the column with 50 ml of 0.01 M PBS, 10 washes of 5 ml each, containing 0.05% sodium azide and store capped, with 2 ml of PBS above the gel, at 2–8 °C.

#### Determination of the Biotin:Protein Ratio

In order to determine the level of protein biotinylation, it is necessary to measure the protein concentration ( $A_{280}$ ) and the biotin content (with the avidin-HABA assay).

1. Dilute 0.1 ml of the pooled biotinylated protein fraction to 1 ml with 0.01 M PBS (0.1 ml of protein + 0.9 ml of 0.01 M PBS). Place the diluted sample in a 1.0 cm pathlength quartz cuvette. Record the absorbance of the sample at 280 nm ( $A_{280}$ ). Label "Protein Sample".

- Incubate another 0.1 ml sample of the pooled biotinylated protein fraction with 10  $\mu$ l of the Pronase Solution for 1.5 hours at 37 °C or overnight at room temperature. Label "Biotin Sample".

**Note:** The biotinylated IgG is digested with 0.1% pronase to prevent steric hindrance of biotin binding to avidin and nonspecific binding of HABA to the biotinylated protein.

- Blank spectrophotometer at 500 nm with 0.01 M PBS.
- Add 0.1 ml of the HABA Solution (10 mM, Vial 5) to 3.2 ml of the Avidin Solution and mix gently. The absorbance of the Avidin-HABA Solution at 500 nm should be ~1.0 (1.0 cm pathlength).

**Note:** The Avidin Solution and HABA Solution remain active for 1 year. The Avidin-HABA Solution remains active for 2 weeks at 2–8 °C. If a precipitate forms, it can be filtered and the filtrate then used.

- Mix 0.9 ml of the Avidin-HABA Solution with 0.1 ml of the pronase treated sample (Biotin Sample).
- Mix 0.9 ml of the Avidin-HABA Solution with 0.1 ml of 0.01 M PBS (HABA Control).
- Record the absorbance of the Biotin Sample (step 5) and the HABA Control (step 6) at 500 nm in a 1.0 cm pathlength cuvette.
- The  $A_{500}$  value for the HABA Control should be ~0.9. If the  $A_{500}$  value for the Biotin Sample is  $\leq 0.2$ , the Biotin Sample should be diluted and the assay repeated.

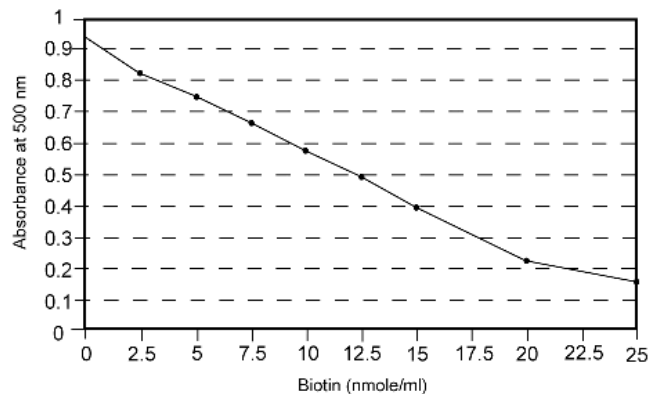
### Calculations

Methods for determination of the molar ratio of biotin to protein based on the avidin-HABA assay. In this reaction, biotin displaces the HABA bound to avidin.

#### Method A

This method uses a standard curve of known biotin concentrations. A typical standard curve for biotin is shown in Figure 3. Use the linear portion of the curve with biotin concentrations ranging from 2–20 nmole/ml.

**Figure 3.**  
Typical Standard Curve for Biotin



- Determine the protein concentration of the labeled protein using the Protein Sample. Protein concentration is calculated from the following formula:

$$\text{Protein (mg/ml)} = \frac{A_{280} \times 10 (\text{dilution factor})}{\epsilon_{280}}$$

where the extinction coefficient of IgG at 1 mg/ml ( $\epsilon_{280}$ ) = 1.4

As an example, if the  $A_{280}$  = 0.630, then:

$$\begin{aligned} \text{Protein (mg/ml)} &= \frac{0.630 \times 10}{1.4} \\ \text{Protein (mg/ml)} &= 4.5 \end{aligned}$$

- Determine the number of nmoles of IgG in the sample (IgG, molecular mass ~150,000)

$$\text{nmoles/ml} = \frac{\text{mg protein/ml}}{\text{mw of IgG}} \times 10^6$$

( $10^6$  is conversion from moles/L to nmoles/ml)

$$\text{nmoles/ml} = \frac{4.5 \text{ mg/ml}}{150,000} \times 10^6$$

$$\text{nmoles/ml} = 30$$

3. To determine the number of nmoles of biotin present in the biotinylated sample:
- Run a HABA assay standard curve substituting known concentrations of biotin (0–20 nmole/ml) for the Biotin Sample.
  - Determine the  $A_{500}$  of the Biotin Sample from step 5 of the avidin-HABA assay.
  - From the HABA assay standard curve, determine the number of nmoles of biotin present. For example, if the  $A_{500}$  of the biotin solution was 0.566, then from the standard curve (see Figure 2), this reading corresponds to 10.1 nmole of biotin/ml. Correcting for the original dilution (10-fold), yields a value of 101 nmole of biotin/ml.

4. The biotin to protein (IgG) ratio is calculated as follows:

$$\text{Biotin:Protein Ratio} = \frac{\text{nmoles/ml biotin}}{\text{nmoles/ml protein (IgG)}}$$

$$\text{Biotin:Protein Ratio} = \frac{101 \text{ nmoles/ml biotin}}{30 \text{ nmoles/ml protein (IgG)}}$$

$$\text{Biotin:Protein Ratio} = 3.4$$

#### Method B

With this method, the molar ratio of biotin to protein is calculated using the extinction coefficient for the avidin-HABA complex.

- Determine the mg and nmoles of protein present (see steps 1 and 2 of Method A).
- Measure the  $A_{500}$  of the HABA Control and the Biotin Sample per the instructions in the avidin-HABA assay.
- Calculate the corrected  $A_{500}$  by subtracting the  $A_{500}$  (Biotin Sample) from the  $A_{500}$  (HABA Control).
- The biotin concentration (in nmoles/ml) is calculated using the following formula:

$$\text{Biotin (nmoles/ml)} = \frac{\text{Corrected } A_{500} \times 10 \times 10^6}{\epsilon_{500}}$$

Where: (10 is dilution factor)

( $10^6$  conversion from moles/L to nmoles/ml)

HABA/Avidin molar extinction coefficient ( $\epsilon_{500}$ ) = 34,000 ( $A_{500}$  of a 1 M biotin solution)

As an example, if corrected  $A_{500}$  = 0.334, then:

$$\text{Biotin (nmoles/ml)} = \frac{0.334}{34,000} \times 10 \times 10^6$$

$$\text{Biotin (nmoles/ml)} = 98.2$$

5. The biotin to protein ratio is calculated as follows:

$$\text{Biotin:Protein Ratio} = \frac{\text{nmoles/ml biotin}}{\text{nmoles/ml protein (IgG)}}$$

$$\text{Biotin:Protein Ratio} = \frac{98.2 \text{ nmoles/ml biotin}}{30 \text{ nmoles/ml protein (IgG)}}$$

$$\text{Biotin:Protein Ratio} = 3.3$$

#### **References**

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