Protocol

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Biotin-NHS ester with Biotrace™ Kit Technical Information and Protocol

Introduction

Gold Bio's Biotin-NHS ester with Biotrace™ kit provides all the necessary reagents for the controlled biotinylation of antibodies, proteins, or other primary amine-containing biomolecules. This advanced biotin labeling reagent contains a UV-traceable benzophenone moiety (350 nm) surrounded by two hydrophilic PEG4 spacer arms (Figure 1). The two hydrophilic PEG4 spacers enhance water solubility while reducing the tendency of some proteins to aggregate/precipitate during biotinylation. Extended PEG spacers provide increased conjugate yield while reducing non-specific assay interactions at higher biotin loads for enhanced assay performance. The traceable chromophore permits rapid determination of incorporated biotin by a simple measurement at two wavelengths (280 nm/350 nm). Biotin-NHS ester with Biotrace™ reagent is guaranteed to increase labeling reproducibility and yield for maximum assay robustness.

Biotin-NHS ester with Biotrace™ Kit (GoldBio Catalog # B-220-kit), provides sufficient reagents for 3 labeling reactions each containing 50-500 µg of antibody or other lysine-containing protein in a 100 µL reaction volume. This convenient UV traceable biotin labeling reagent permits rapid quantification of incorporated biotin without the need for a HABA biotin assay.

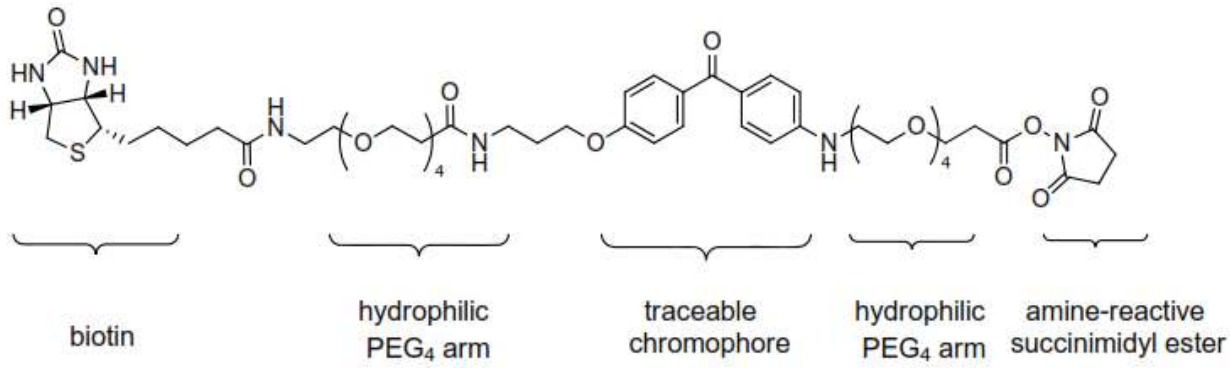

Figure 1. Chemical structure of Biotin-NHS ester with Biotrace™

Protein Requirements

- This kit requires 50-500 µg protein in a fixed volume of 100 µl (e.g. 0.5-5 mg/ml).

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- The protein to be biotinylated must be highly purified and its molecular weight known (e.g. 20-200 kDa).
- The protein must have available primary amines (e.g. N-terminus or lysine amino acid residues).
- Proteins must be free of exogenous primary amines (e.g. glycine or Tris) prior to labeling with NHS esters, if present these compounds must be removed with the desalting spin column provided (See Material Preparation).
- Buffer exchange columns provided are designed to process 50-500 µg of protein in a volume of 100 ± 10 µl.

Materials

Kit Contents

Biotin-NHS ester with Biotrace™ (MW: 1089.25 g/mol)	3 x 4 mg
Anhydrous DMSO	1 x 10 ml
BupH Saline Buffer Pack (BupH™ registered trademark ThermoScientific)	1 packet
Zeba™ Spin Columns (Zeba™ registered trademark ThermoScientific)	6 x 0.5 ml

Other Materials

- UV-VIS Spectrophotometer
- Pipettes and tips (P-10, P-100, P-1000)
- 1 L beaker stir bar with magnetic stir bar
- 6N NaOH
- Ultrapure water (e.g. 18 MΩ-cm)
- 1.5 ml conical tubes
- Semi-micro quartz cuvette (500-1000 µl)

Method

Preparation of BupH Buffer (pH 7.5)

1. Dissolve the dry-blend BupH buffer pack provided into 500 ml ultrapure water. Adjust the pH of the solution to 7.5±0.05 by drop wise addition of 6N NaOH. Adjust the final volume to 500 ml with ultrapure water. For long-term storage sterile-filter the solution. Do not add sodium azide or Proclin preservatives as these reagents interfere with protein A_{280} measurements.

Protein Preparation

1. If the lyophilized protein (50-500 µg) to be biotinylated is pure and free of exogenous

amines, resuspend in 100 µl BupH buffer (pH 7.5) to obtain a 0.5-5 mg/ml solution. Proceed to Biotin Labeling Reaction (Step E).

2. If the lyophilized protein is known to contain exogenous amines (e.g. Tris, glycine) resuspend in 100 µl BupH buffer (pH 7.5) then proceed with buffer exchange (Step C and Step D below) with the spin columns provided.

3. If the protein to be biotinylated (50-500 µg) is already in 100 µl buffer solution (e.g. 0.5-5 mg/ml PBS), proceed to buffer exchange into BupH buffer (pH 7.5) (Step C and Step D below) with the spin columns provided.

Equilibration of Spin Column into BupH (pH 7.5)

1. Twist off the column's bottom closure and loosen the red cap. Place each column into a clean 1.5 ml microfuge tube.

2. Centrifuge column at 1,500 x g for 2 minutes to remove storage solution. Place a pen mark on the side of the column where the compacted resin is slanted upward. Place column in centrifuge with the mark facing away from the center of the rotor in all subsequent centrifugation steps.

Note: Resin will appear white in color and compacted after centrifugation.

3. Add 0.3 ml BupH buffer (pH 7.5) to the top of each spin column, replace the cap and loosen.

4. Centrifuge at 1500 x g for 2 minutes to remove buffer.

5. Repeat steps 3 and 4 two additional times, discarding buffer from the collection tube after each spin.

6. Transfer equilibrated spin column (resin appears white and dry) into a clean 1.5 ml microfuge tube and immediately proceed with buffer exchange of protein.

Buffer Exchange of Protein

1. Buffer exchange protein into BupH (pH 7.5) equilibrated spin column by slowly applying 100 µl protein solution to the center of the equilibrated resin bed.

2. Centrifuge at 1,500 x g for 2 minutes. Retain the eluate at bottom of 1.5 ml collection tube. Discard the used spin column.

3. Protein is now buffer exchanged.

Biotin Labeling Reaction

1. Select how much excess Biotin-NHS Ester with Biotrace™ to use during the labeling reaction (e.g. 10-fold excess). Refer to Appendix A, Part I as a reference guide.

2. Calculate the volume DMSO required to dissolve Biotin-NHS Ester with Biotrace™ reagent using calculations in Appendix A, Part II.

3. Add required volume DMSO to Biotin-NHS Ester with Biotrace™ reagent, vortex vigorously for 2 minutes to fully dissolve.

4. Add 5 µl biotin/DMSO reagent to protein solution (100 µl), pipette the mixture up and down several times to mix.

5. Allow labeling reaction to proceed for 1 hour at room temperature.

Removal of Excess Biotin Reagent

1. Prepare a buffer exchange spin column as described in Section C.

2. Buffer exchange biotin labeled protein as described in Section D (or buffer of choice).

3. Determine degree of labeling (DOL) and conjugate protein concentration (mg/ml)

Degree of Labeling (DOL) and Conjugate Protein Concentration (mg/ml)

1. Measure conjugate's absorbance at 280 nm and 350 nm in a semi-micro quartz cuvette. Note - Concentrated protein solutions (e.g. 5 mg/ml) will require dilution (e.g. 1:20) of a small aliquot prior to measurement to achieve desired range (e.g. 0.1 to 1.5 AU). A micro-volume spectrophotometer can be used on small aliquots (1-2 µl) without dilution (e.g. Nanodrop®).

2. Calculate the degree of labeling (DOL) and protein concentration with the calculations found in Appendix B, Part I.

Tips

- After reconstitution of Biotin-NHS ester with Biotrace™ reagent in DMSO, use it

immediately. NHS- esters readily hydrolyze and become non-reactive. Use only freshly prepared reagent and discard any unused reconstituted reagent.

- In every labeling reaction, the simplified labeling protocol uses a fixed volume (5 µl) of Biotin-NHS ester with Biotrace™/DMSO reagent to label a fixed volume of protein (100 µl). Consequently, the volume of anhydrous DMSO required to dissolve the biotin reagent varies for each labeling reaction (see DMSO volume calculations in Appendix A, Part II)
- For maximum reproducibility, it is preferable to buffer exchange proteins into BupH Buffer (pH 7.5) with the spin columns provided prior to biotinylation. This simple procedure guarantees maximum consistency during the labeling reaction.

Troubleshooting

Problem	Possible Cause	Solution
Poor or lower than expected biotinylation of proteins	Incorrect protein concentration and/or possible contaminants in protein sample.	- Buffer exchange protein into reaction buffer using spin columns and confirm concentration of protein prior to labeling.
	NHS-ester hydrolyzed	- Store Biotin-NHS Ester with Biotrace™ reagent at -20°C. - Allow product to equilibrate to room temperature before opening.
		- Avoid buffers that contain primary amines such as Tris and glycine. Buffer exchange proteins before labeling whenever possible.
	Protein has few or no lysine residues	- Check the primary structure of the protein for the presence of lysine residues using the NCBI protein database
	Low A_{350} absorbance of the biotinylated conjugate	- Check spectrophotometer lamp for proper functioning
Low conjugate yield	Protein may have aggregated /precipitated during biotinylation	- Lower the amount of labeling reagent during the labeling reaction. - Use 10% or lower volume DMSO solvent during labeling reaction. Though rare, some proteins become unstable on biotinylation and cannot be labeled.

Calculations

Excess Biotin-NHS Ester with Biotrace™ Reagent to Use in Labeling Reaction.
Select the molar excess of Biotin-NHS Ester with Biotrace™ reagent you wish to use in the labeling reaction. Refer to Table 1 as a reference guide in the selection process. Typical labeling reactions use a 10 to 20- fold molar excess. Over modification of antibodies or other proteins with biotin can affect their function and stability.

Goat IgG (150 kDa)	Molar Equivalents		
	5x	10x	20x
(mg/mL)	DOL	DOL	DOL
0.5	1.1	2.1	4.5
1	1.9	3.7	8.7
2	2.5	6.2	9.7
4	3.1	5.2	9.6

BSA (66.4 kDa)	Molar Equivalents		
	5x	10x	20x
(mg/mL)	DOL	DOL	DOL
0.5	2.2	3.5	4.9
1	2.3	4.6	6.1
2	2.2	4.9	7.3
4	2.4	4.7	6.7

Determine Required DMSO Volume (µl) to Dissolve Biotin-NHS Ester with Biotrace™ Reagent.

1. Calculate millimoles Biotrace™ Biotin-NHS required to a label a protein at a desired molar excess (N_b), concentration (C_p and volume (V_p) with MW_p:

$$nmol\ reagent\ required = N_b\ x\ C_p\ x\ V_p\ x\ \frac{1}{MW_P}$$

N_b = molar excess Biotrace™ Biotin-NHS
C_p = protein concentration (mg/ml)
V_p = volume of protein to be labeled (ml)
MW_p = protein molecular weight (Daltons)

2. Calculate microliters anhydrous DMSO required to dissolve Biotrace™ Biotin NHS reagent:

$$\mu l\ DMSO\ required = \frac{0.01836}{mmole\ biotin\ reagent\ required}$$

Example 1: Determine DMSO Volume Required (µl) to Use for the Following Labeling Reaction
To label 0.1 ml of a 1 mg/ml IgG solution (M.W. 150 kDa) with a 20-fold molar excess Biotrace™ Biotin NHS, dissolve reagent (4 mg) into 1383.5 µl anhydrous DMSO, then add 5 µl of this stock to antibody solution.

1. Calculate millimoles Biotrace™ Biotin-NHS (M.W. 1098.25) required to label protein at a desired molar excess (N_b), concentration (C_p) and volume (V_p) with MW_p:

$$mmol\ reagent\ required = N_b \times C_b \times C_b \times \frac{1}{MW_p}$$

$$mmol\ reagent\ required = 20 \times 2.0\ mg/ml \times 0.1\ ml \times \frac{1}{150,000\ dalton} = 0.000133\ mmol$$

$$mmol\ reagent\ required = 0.000133\ mmol$$

N_b = molar excess Biotrace™ Biotin-NHS
C_p = protein concentration (mg/ml)
V_p = volume of protein to be labeled (ml)
MW_p = protein molecular weight (Daltons)

2. Calculate the volume anhydrous DMSO (µl) required to dissolve Biotrace™ Biotin NHS (4 mg) as follows:

$$\mu l\ DMSO\ required = \frac{mmol\ reagent\ per\ vial \times 5\ \mu l}{mmol\ reagent\ required} = \frac{0.00367 \times 5\ \mu l}{mmol\ reagent\ required}$$

$$\mu l\ DMSO\ required = \frac{0.0184}{0.0000133} = 1383.5\ \mu l$$

$$\mu l\ DMSO\ required = 1383.5\ \mu l$$

Calculate conjugate's DOL (# biotin/protein) and protein concentration (mg/ml) using Equations 1, 2, 3 and 4 below:

Eq. 1 $$number\ of\ biotin\ per\ protein = \frac{molarity\ biotin}{molarity\ protein}$$

Eq. 2 $$molarity\ of\ biotin = \frac{A_{350}}{\epsilon_{350}}$$

Eq. 3 $$molarity\ of\ protein = \frac{A_{280C}}{\epsilon_{280}}$$

Eq. 4 $$mg/ml = \frac{A_{280} - (A_{350} \times 0.4475)}{\left(\frac{E1\%}{10}\right)} \times dilution\ factor$$

A_{350} = *conjugate absorbance at* 350 ± 5 *nm*
ε_{350} = *molar extinction coefficient Biotrace™ Biotin* = 19,474$M^{-1}cm^{-1}$

A_{280} = conjugate absorbance at 280 nm

A_{280c} = corrected conjugate absorbance at 280 nm = A_{280} - (A_{350} x (0.4475))

ϵ_{280} = molar extinction coefficient protein ($M^{-1}cm^{-1}$) = $\frac{MW_p \ x \ E1\%}{10}$

Example 1. Determine DOL and Conjugate Protein Concentration for the Following Labeling Reaction.

A Goat IgG antibody 0.1 ml at 1.0 mg/ml was labeled using a 20-fold molar excess Biotrace™ Biotin reagent. The (undiluted) conjugate's A_{280} and A_{350} were 1.48 and 0.922, respectively. Goat IgG E1% = 13.6 (i.e. 204, 000 M^{-1} cm^{-1}).

Calculate DOL (# biotin/protein) as follows:

By Equation 2 $$molarity\ of\ biotin = \frac{0.922}{19{,}474\ M^{-1}\ cm^{-1}} = 47.35\ \mu M$$

By Equation 3 $$molarity\ of\ IgG = \frac{1.48 - (0.922 \times 0.4475)}{204{,}000\ M^{-1}\ cm^{-1}} = 5.23\ \mu M$$

By Equation 1 $$Number\ of\ biotin\ per\ IgG = \frac{47.35\ \mu M}{5.23\ \mu M} = 9.05$$

Calculate conjugate protein concentration (mg/ml)

By Equation 4 $$mg/ml = \frac{1.48 - (0.922 \times 0.4475)}{1.36} \times 1 = 0.78\ mg/ml$$

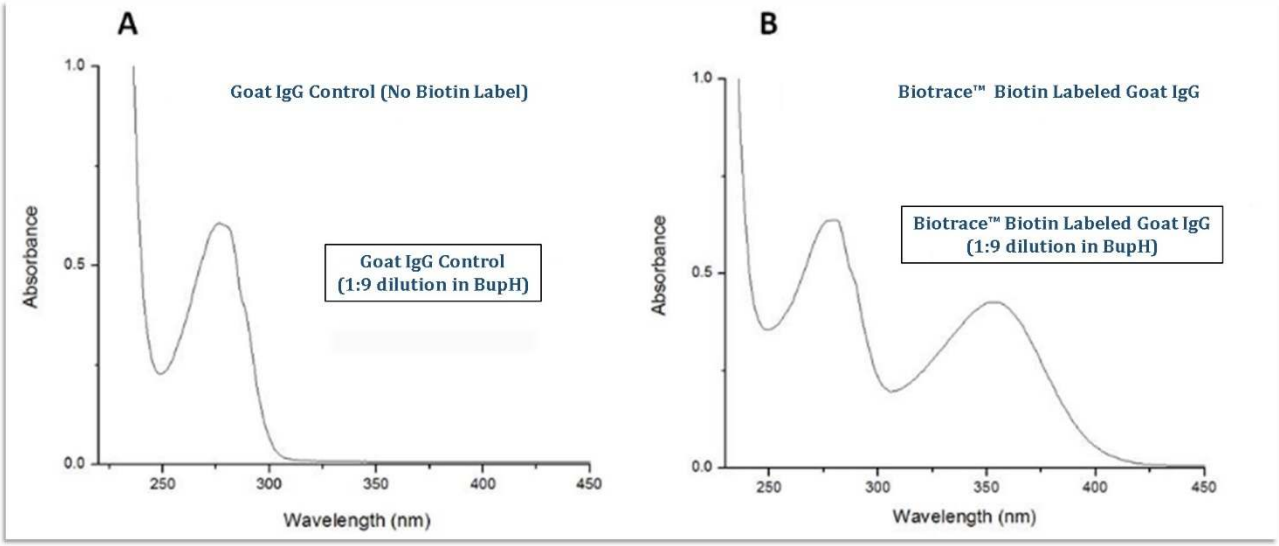

Figure 2. UV scan (230-450 nm) of Goat IgG (unlabeled control) (A), and Biotrace™ Biotin labeled Goat IgG (B). A small aliquot was diluted into BupH (1:9) from a 4 mg/ml stock prior to the scan. The degree of labeling was determined to be 9.6 biotins/ IgG.