Protocol

TD-P Revision 2.0

Creation Date: 1/22/2015
Revision Date: 2/20/2019

Biotin-Maleimide with Biotrace™ Kit Technical Information and Protocol

Introduction

Gold Bio's Biotin-Maleimide with Biotrace™ kit provides all the necessary reagents for the controlled biotinylation of antibodies and other thiol-containing proteins. This advanced labeling reagent contains a UV-traceable benzophenone moiety (350 nm) surrounded by two hydrophilic PEG4 spacer arms (Figure 1). The maleimide functional group targets reduced thiols (-SH) while avoiding modification of susceptable lysine residues. The two hydrophilic PEG4 spacer arms 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 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-Maleimide with Biotrace™ reagent is guaranteed to increase labeling reproducibility and yield for maximum assay robustness.

Biotin-Maleimide with Biotrace™ Kit (GoldBio Catalog # B-420-Kit), provides sufficient reagents for 3 labeling reactions each containing 100-1000 µg of antibody or other thiol-containing protein in a 500 µl reaction volume. This convenient UV traceable biotin labeling reagent permits rapid quantification of incorporated biotin without the need for a HABA biotin assay.

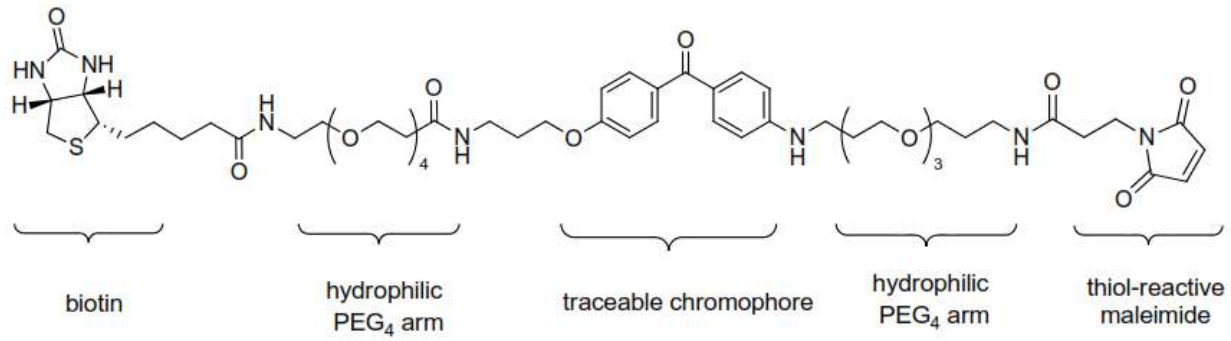

Figure 1. Chemical structure of Biotin-Maleimide with Biotrace™.

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Protein Requirements

This kit requires 100-1000 µg protein in a fixed volume of 500 µl (e.g. 0.2-2 mg/ml)

- The protein to be biotinylated must be highly purified and its molecular weight known (e.g. 20-200 kDa)
- The protein must have available thiols (e.g. cysteine). If absent sulfhydryls can be added to biomolecules using N-succinimidyl S-acetylthioacetate (e.g. SATA available from ThermoScientific)
- Proteins must be free of reducing agents (e.g. β-ME, DTT or TCEP) prior to labeling with maleimide esters, if present these compounds must be removed (See Material Preparation)
- Buffer exchange columns provided are designed to process 100-1000 µg of protein in a volume 500±50 µl.

Materials

Kit Contents

Biotin-Maleimide with Biotrace™ (Molecular Weight: 1098.30)	3 x 4 mg
Anhydrous DMSO	1 x 10 ml
0.5 M EDTA	1 x 0.1 ml
TCEP-HCL Reducing Agent (M.W. 286.65)	3 x 15 mg
BupH Saline Buffer Pack (BupH™ registered trademark ThermoScientific)	1 packet
Zeba™ Spin Columns (Zeba™ registered trademark ThermoScientific)	6 x 2 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 HCl
- Table top centrifuge
- Ultrapure water (e.g. 18 MΩ-cm)
- 15 ml conical tubes
- Quartz cuvette (500-1000 µl)

Method

Preparation of BupH Buffer (pH 6.5)

1. Dissolve the dry-blend BupH buffer pack provided into 500 ml ultrapure water. Adjust the pH of the solution to 6.5±0.05 by drop wise addition of 6N HCL. 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 (100-1000 µg) to be biotinylated is pure and free of exogenous thiols (e.g. DTT or β-ME), resuspend in 500 µl BupH buffer (pH 6.5) to obtain a 0.2-2 mg/ml solution, proceed to Protein Reduction.

2. If the lyophilized protein is known to contain exogenous thiols (e.g. DTT, β-ME) resuspend in 500 µl BupH (pH 6.5) then proceed with buffer exchange (Step C and Step D below), then proceed to Protein Reduction.

3. If the purified protein to be biotinylated (100-1000 µg) is already in 500 µl of a suitable thiol- free buffer (e.g. MOPS, Tris-HCL, or PBS) at a concentration range from 0.2-2 mg/ml, proceed to Protein Reduction.

Equilibration of Spin Column into BupH (pH 6.5)

1. Twist off the column's bottom closure and loosen the cap. Place each column into a clean 15 ml conical tube.

2. Centrifuge column at 1,000 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 1 ml BupH buffer (pH 6.5) to the top of each spin column, replace the cap and loosen.

4. Centrifuge at 1000 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 the equilibrated spin column (resin appears white and dry) into a clean 15 ml conical tube and immediately proceed with buffer exchange of protein.

Buffer Exchange of Protein

1. Buffer exchange protein into BupH (pH 6.5) equilibrated spin column by slowly applying 500 µl protein solution to the center of the equilibrated resin bed.

2. Centrifuge at 1,000 x g for 2 minutes. Retain the eluate at bottom of 15 ml collection tube. Discard the used spin column.

3. Protein is now buffer exchanged.

Reduction of Protein

1. Prior to biotinylation, proteins containing disulfide bonds or free cysteines must be reduced with TCEP to insure proper labeling.

2. Add 500 µl ultrapure water to a vial containing 15 mg TCEP-HCL (105mM), vortex to dissolve crystals completely.

3. Add 5 µl 500mM EDTA (provided) to 500 µl protein solution (0.2-2 mg/ml), pipette the mixture up and down several times to mix.

4. Add 25 µl dissolved TCEP (105mM) to protein solution, pipette up and down several times to mix.

5. Allow the reaction to incubate for 30 minutes.

6. Prepare a buffer exchange spin column as described in the Equilibration of Spin Column into Buph (pH 6.5) section.

7. Buffer exchange TCEP reduced protein as described in the Buffer Exchange of Protein section.

8. Immediately after buffer exchange, add 5 µl 500mM EDTA (provided) to the protein solution, pipette up and down several times to mix.

Biotin Labeling Reaction

1. Select how much excess Biotin-Maleimide with Biotrace™ to use during the labeling reaction (e.g. 20-fold excess). Refer to the Calculations section.

2. Determine the volume DMSO required to dissolve Biotin-Maleimide with Biotrace™ reagent using calculations in Calculations section.

3. Add required volume DMSO to Biotrace™ Biotin reagent, vortex vigorously for 2 minutes until reagent is fully dissolved.

4. Add 10 µl biotin/DMSO reagent to protein solution (~525 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 the Equilibration of Spin Column into Buph (pH 6.5) section.

2. Buffer exchange biotin labeled protein as described in the Buffer Exchange of Protein section (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 quartz cuvette.
   Note- concentrated protein solutions (e.g. 2 mg/ml) will require dilution of an small aliquot prior to absorbance measurements, while very dilute solutions (e.g. < 0.2 mg/ml) may need 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 the Calculations section.

Tips

- After reconstitution of Biotin-Maleimide with Biotrace™ reagent in DMSO, use it immediately. Although the maleimide group is more stable than other functional groups, it will hydrolyze to form a nonreactive maleimic acid. Maintain unused stock solution under moisture-free condition (e.g., capped under an inert gas such as argon or nitrogen) at 4°C. Equilibrate reagent vial at room temperature before opening to avoid moisture condensation inside the container.
- Access to a table top centrifuge (e.g. Eppendorf 5810 or similar) capable of handling 15 ml conical tubes is required for the spin columns provided.
- In every labeling reaction, the simplified protocol uses a fixed volume (10 µl) of Biotin-Maleimide with Biotrace™/DMSO reagent to label a fixed volume of protein (~525 µl). Consequently, the volume of anhydrous DMSO required to dissolve the biotin reagent varies for each labeling reaction (see DMSO volume calculations in the Calculations section).
- The amount of biotin incorporated during the labeling reaction depends primarily on the number and availability of reduced thiols. Generally, a 10 to 20-fold molar excess of reagent is sufficient for most proteins. Over modification of the protein with biotin can affect both function and aqueous stability.

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 BupH buffer (pH 6.5) using spin columns provided and confirm concentration of protein prior to labeling.
	Maleimide-ester hydrolyzed	Store Biotin-Maleimide with Biotrace™ reagent at -20C. Allow product to equilibrate to room temperature before opening.
		Avoid buffers that may contain free thiols (β-ME or DTT). Buffer exchange proteins before labeling whenever possible.
	Protein has few or no thiol residues	Check primary structure of protein for the presence of cysteine residues on NCBI protein database. Some proteins may require modification with SATA (or similar reagent) to introduce thiol functional groups.
	Low A_{350} absorbance of the biotinylated conjugate	Check spectrophotometer lamp for proper functioning
Low conjugate yield	Protein may have aggregated/ precipitated during biotinylation	Use appropriate relative centrifugal force (e.g. 1000 x g) and recommended spin time to buffer exchange protein. Although rare, some proteins become unstable in aqueous solution on biotinylation and cannot be labeled.

Calculations

Excess Biotin-Maleimide with Biotrace™ Reagent to Use in Labeling Reaction.
Select the molar excess of Biotin-Maleimide with Biotrace™ reagent you wish to use in the labeling reaction. Typical labeling reactions use 5 to 20-fold reagent molar excess depending on initial protein concentration and the number of available thiols. Over modification of antibodies or other proteins with biotin can affect their function and stability.

Table 1. Goat IgG and molecular equivalents.

Goat IgG (150 kDa)	Molar Equivalents		
	5x	10x	20x
(mg/ml)	DOL	DOL	DOL
0.2	0.6	1.6	3.5
1	1.9	3.6	6.5
5	3.4	5.5	9.5

Note: Goat IgG possess 32 cysteine residue.

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

1. Calculate millimoles Biotrace™ Biotin-Maleimide (M.W. 1098.31) 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-Maleimide
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 Maleimide reagent:

$$\mu l\ DMSO\ required = \frac{0.0911}{mmole\ biotin\ reagent\ required}$$

Example 1: Determine DMSO Volume Required (µl) to Use for the Following Labeling Reaction To label 0.5 ml of a 2 mg/ml IgG solution (M.W. 150 kDa) with a 20-fold molar excess Biotrace™ Biotin Maleimide, dissolve reagent (4 mg) into 684.8 µl anhydrous DMSO, then add 25 µl of this stock to antibody solution.

1. Calculate millimoles Biotrace™ Biotin-Maleimide (M.W. 1098.30) required to label protein at a desired molar excess (*Nb*), concentration (*Cp*) and volume (*Vp*) with *MWp*:

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

$$mmol\ reagent\ required = 20\ \times 2.0\ mg/ml\ \times 0.5\ ml\ \times \frac{1}{150,000\ dalton} = 0.000133\ mmol$$

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

N_b = molar excess Biotrace™ Biotin-Maleimide

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:

$$\mu l\ DMSO\ required\ = \frac{mmol\ reagent\ per\ vial\ \times 25\ \mu l}{mmol\ reagent\ required} = \frac{0.00364\ \times 25\ \mu l}{mmol\ reagent\ required}$$

$$\mu l\ DMSO\ required\ = \frac{0.0911}{0.000133} = 684.8\ \mu l$$

$$\mu l\ DMSO\ required\ = 684.8\ \mu l$$

Note: Each vial of Biotrace™ Biotin Maleimdie can accommodate 1,600 µl DMSO, if the required volume of DMSO is greater than this nominal volume, transfer the dissolved reagent to a alrger vial and add DMSO to achieve the requisite volume.

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 0.5 ml at 2.0 mg/ml was labeled using a 20-fold molar excess Biotrace™ Biotin Maleimide reagent. The conjugate's A_{280} and A_{350} (1:4 dilution) was determined to be 0.6739 and 0.2112, respectively. Goat IgG E1% = 13.6 (i.e. 204, 000 M^{-1} cm^{-1}).

Calculate DOL (# biotin/protein) as follows:

By Equation 2 $$molarity\ of\ biotin = \frac{0.2112}{19{,}474\ M^{-1}\ cm^{-1}} = 10.85\ \mu M$$

By Equation 3 $$molarity\ of\ IgG = \frac{0.6739 - (0.2112 \times 0.4475)}{204{,}000\ M^{-1}\ cm^{-1}} = 2.84\ \mu M$$

By Equation 1 $$Number\ of\ biotin\ per\ IgG = \frac{10.85\ \mu M}{2.84\ \mu M} = 3.82$$

Calculate conjugate protein concentration (mg/ml)

By Equation 4 $$mg/ml = \frac{0.6739 - (0.2112 \times 0.4475)}{1.36} \times 4 = 1.7\ mg/ml$$

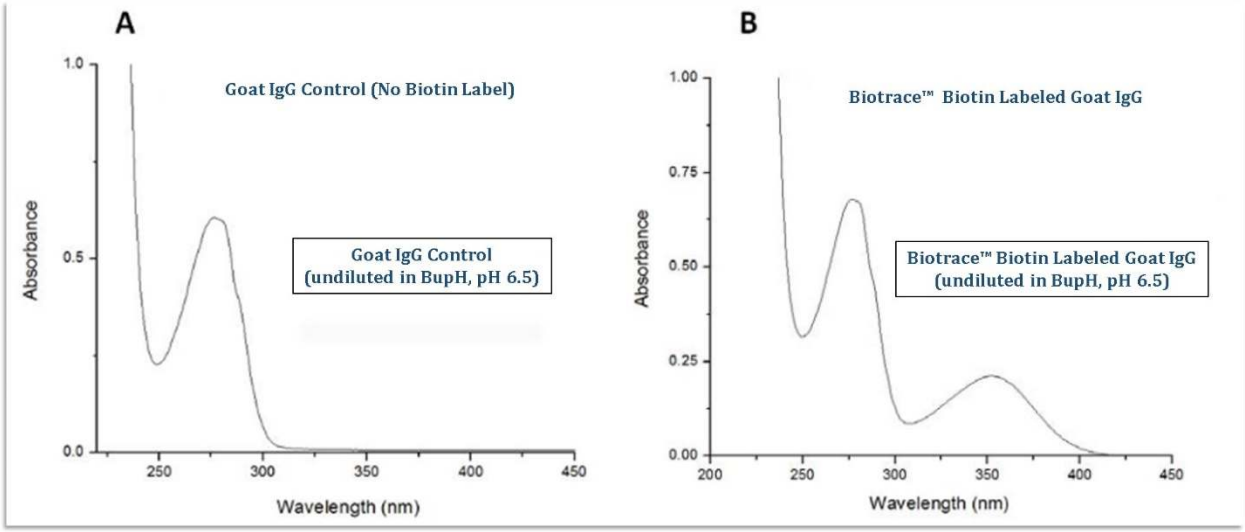

Figure 2. UV scan (230-450 nm) of Goat IgG (unlabeled control) (A), and Biotin-Maleimide with Biotrace™ labeled Goat IgG (B). Samples were scanned (1:4 dilution) at 0.425 mg/ml in BupH (pH 6.5). The degree of labeling

was determined to be 3.8 biotins/ IgG.