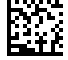

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HOOK™ Biotin Sulfhydryl Reactive

For Coupling of Biotin to Protein Sulfhydryl Groups

(Cat. # BS-11, BS-12, BS-13, BS-14)

INTRODUCTION 3
IODOACETYL REACTION CONDITIONS 3
MALEIMIDE REACTION CONDITIONS 3
PYRIDYLDITHIOL REACTION CONDITIONS 4
SPECIFICATIONS: HOOK™ BIOTIN REAGENT (SULFHYDRYL REACTIVE) 4
ITEMS SUPPLIED 5
STORAGE CONDITIONS 5
PRECAUTIONS 5
ADDITIONAL ITEM(S) REQUIRED 5
PREPARATION BEFORE USE 5
PROTOCOL 6
A. PROTEIN SAMPLE PREPARATION 6
B. CALCULATION OF QUANTITY OF BIOTIN AGENT NEEDED FOR CONJUGATION 6
C. PREPARATION OF BIOTIN REAGENT 7
D. BIOTIN CONJUGATION REACTION 7
E. REMOVAL OF UNCONJUGATED BIOTIN REAGENT 7
F. ESTIMATION OF BIOTIN INCORPORATION EFFICIENCY 8
IMPORTANT INFORMATION 8
PROTOCOL 1: CUVETTE PROTOCOL 8
PROTOCOL 2: MICROPLATE PROTOCOL 9
CALCULATIONS 9
TROUBLESHOOTING 11
APPENDIX 1: SAMPLE EQUILIBRATION WITH TUBE-O-DIALYZER™ 12
APPENDIX 2: BIOTIN REAGENT STRUCTURES 13
RELATED PRODUCTS 14

INTRODUCTION

This protocol is for use with the HOOK™ Biotin reagents that react with protein free sulfhydryl groups (Table 1). Sulfhydryl reactive reagents are more specific and react only with free sulfhydryl residues (-SH or thiol groups). The side chain of the amino acid cysteine is the most common source of free sulfhydryl groups. If free sulfhydryl residues are not available, they can be generated by either the reduction of disulfides (-S-S-) with reducing agents such as mercaptoethylamine, or by modifying lysine ε-amines with Traut's reagent or SATA. After reduction, excess reducing agent must be removed before coupling. In addition a metal chelating agent (EDTA) (an anti-oxidant) should be used to reduce the chances of reoxidation of sulfhydryls to disulfides.
There are three different reactions employed to couple biotin reagents to sulfhydryl residues and involve either iodoacetyl, maleimide or pyridylthiol groups.

Iodoacetyl Reaction Conditions

HOOK™-PEG_2-Iodoacetyl-biotin and HOOK™-Iodoacetyl-LC-biotin are both sulfhydryl reactive biotinylation reagents that react with thiol groups at pH7.5-8.5 and form stable thioether bonds. HOOK™- PEG_2-Iodoacetyl-biotin is water soluble, due to its polyethylene glycol (PEG) spacer arm, while HOOK™-Iodoacetyl-LC-biotin must be dissolved in an organic solvent prior to use. Both may react with imidazoles at pH 6.9-7.0. For specific reaction with sulfhydryls, limit the reaction to pH 7.5-8.5 and the molar ratio of iodoacetyl-biotin to protein such that the concentration of biotin is only slightly higher than the sulfhydryl concentration. Iodoacetyl reaction should be performed in dark to limit the formation of free iodine, which has the potential to react with tyrosine, tryptophan, and histidine residues. *For optimal iodoacetyl conjugation, we recommend Optimizer Buffer™-II*.

Maleimide Reaction Conditions

HOOK™-Biotin-BMMCC is a sulfhydryl reactive reagent that contains a maleimide functional group. The maleimide group is more specific for sulfhydryl residues than iodoacetyl groups, at pH7 maleimide groups are 1000 fold more reactive toward free sulfhydryls than amines. At pH > 8.5, maleimide groups favors primary amines. Conjugation is carried out at pH 6.5-7.5 for minimizing the reaction toward primary amine. At higher pH >8.0, hydrolysis of maleimide to maleamic acid also increases, which can compete with thiol modification. *Optimizer Buffer™-III provides ideal conditions for maleimide coupling reactions.*

Pyridyldithiol Reaction Conditions

HOOK™-Biotin-PDA is a cleavable sulfhydryl reactive reagent. The reactive group is a pyridyldithiol that reacts with free sulfhydryl by disulfide exchange over a wide range of pH, forming a disulfide linkage. The optimal reaction pH is 6-9. Pyridine-2-thione is released, which absorbs light at 343nm. The coupling reaction can be monitored by measuring the absorbance of released pyridine-2-thione at 343nm. The disulfide bonds formed between HOOK™-Biotin-PDA and the protein can be cleaved with a reducing agent, generating the starting protein in its original form. This reagent is suitable for reversible applications. *Optimizer Buffer™-III provides the optimized conditions.*
Each kit is designed for the conjugation of biotin to protein free sulfhydryl groups and is supplied with the biotin reagent, a specific Optimizer Buffer™, for enhanced conjugation, Spin-*OUT*™ columns, for purification of labelled protein, and reagents to determine the amount of biotinylation. Each kit is designed for the coupling of 1-10mg protein in 1ml buffer, suitable for 10 couplings.

SPECIFICATIONS: HOOK™ BIOTIN REAGENT (SULFHYDRYL REACTIVE)

Cat. #	BS-11	BS-12	BS-13	BS-14
Name	**HOOK™-PEG$_2$-Iodoacetyl-Biotin**	**HOOK™-Iodoacetyl-LC-Biotin**	**HOOK™-Biotin-PDA**	**HOOK™-Biotin-BMMCC**
Molecular Weight	542.43	510.43	412.6	561.7
Spacer Arm (Å)	24.7	27.1	21.1	35.4
Reactive Group	Iodoacetyl	Iodoacetyl	Pyridyldithiol	Maleimide
Membrane Permeable	No	Yes	Yes	No
Water Soluble*	Yes	No	No	No
Cleavable/ Reversible	No	No	Yes	No
Reaction pH	7.5-8.5	7.5-8.5	9-Jun	6.5-7.5

Table 1: Properties of the Sulfhydryl Reactive Biotin Reagents. * For water insoluble reagents use DMSO or DMF.

ITEMS SUPPLIED

Description	Size
HOOK™ Biotin Reagent (See Table 1)	25mg
*Optimizer Buffer™ II [5X] *For Iodoacetyl Reactive Groups*	2 x 25ml
*Optimizer Buffer™ III [5X] *For Maleimide & Pyridyldithiol Reactive Groups*	2 x 25ml
SpinOUT™ GT-600, 5ml	10 columns
OneQuant™ HABA/Avidin	24 vials
BiotinQuant™ Assay Buffer	25ml
Biotin Standard	1ml

* Only one Optimizer Buffer is supplied depending on the HOOK™ Biotin Reagent supplied.

STORAGE CONDITIONS

The kits are shipped at ambient temperature. Upon arrival, store the kit components at -20˚C. Once the biotin reagent has been opened, store at -20°C with a desiccant as reagent is moisture sensitive. Allow it to warm to room temperature before opening.

PRECAUTIONS

- Add metal chelating agent EDTA as an anti-oxidant.
- Because these reagents only react with free sulfhydryl groups, the protein must be reduced using reducing reagents such as DTT, β-Mercaptoethanol or TECP, and free reducing reagents must be removed by dialysis against 1X Optimizer Buffer™ or the buffer of your choice before the biotinylation.
- For biotin reagents whose reactive group is iodoacetyl (Cat. # BS-11 and BS-12), the biotin conjugation reaction (Section D) should be protected from direct light. Place the reaction in the dark and cold.

ADDITIONAL ITEM(S) REQUIRED

- 15ml collection tubes
- DMSO or DMF, if required

PREPARATION BEFORE USE

1. Dilute and prepare 1X Optimizer Buffer™ (1ml 5X Optimizer Buffer™ per 4ml de-ionized water).
2. Warm the Biotin-Agent vial(s) to room temperature before opening to prevent the condensation and deterioration of the biotin agent.
3. Add 50µl ultra pure water to a vial of OneQuant™ HABA/Avidin. Incubate at room temperature for 5 minutes. Vortex to solubilize the HABA/Avidin.

PROTOCOL

A. Protein Sample Preparation

1a Dissolve 1-10mg protein in 0.5-2ml 1X Optimizer Buffer™ to a maximum concentration of 10mg/ml.

1b If your protein is in an aqueous buffer at a pH of 7.2-8.0, free from reducing agents then proceed to the next section.

1c For protein in buffers containing reducing agents a buffer exchange must be performed. The buffer exchange can be done by dialysis against Optimizer Buffer™, we recommend using our Tube-O-DIALYZER™ micro dialysis devices that ensure no loss of precious protein (See Appendix 1). Or one of the supplied Spin-*OUT*™ columns can be used for buffer exchange as described in Section E. Please note this kit is designed for 10 reactions and the Spin-*OUT*™ columns are for purification of the biotin labeled protein, using a column for buffer exchange will reduce the number of reactions that can be performed. Additional columns can be ordered at www.GBiosciences.com.

B. Calculation of Quantity of Biotin Agent Needed For Conjugation

To achieve approximately 4-6 biotin groups per antibody molecule, we recommend using a 20 molar excess of biotin to antibody. The extent of biotin labeling for other proteins is dependent on the distribution of amine groups and size of the protein, therefore the molar ratio can be adjusted to suit your needs.

1. Millimoles of HOOK™ Biotin Reagent to be added for a 20 mole excess:

$$\text{Protein Sample Volume (ml)} \times \frac{\text{Protein Sample Concentration (mg/ml)}}{\text{Protein Mol. Wt (Da)}} \times 20 = \text{mmol HOOK™ Biotin Reagent}$$

2. µl HOOK™ Biotin Reagent to add:

$$\text{mmol HOOK™ Biotin Reagent} \times \text{HOOK™ Biotin Reagent MW} \times \frac{500}{2} = \text{µl HOOK™ Biotin Reagent solution}$$

HOOK™ Biotin Reagent MW: See Table 1, column 3

500 = µl of water 2mg of HOOK™ Biotin reagent dissolved in

Example: For 0.5ml of a 5mg/ml IgG solution (150,000 Mol. Wt) solution.

$$0.5ml \times \frac{5mg/ml}{150{,}000Da} \times 20 = 0.000333mmol\ \text{HOOK™ Sulfo-NHS-Biotin}$$

$$0.000333mmol\ \text{HOOK™ Sulfo-NHS-Biotin} \times 443.43 \times \frac{500}{2} = 37\mu l\ \text{HOOK™ Sulfo-NHS-Biotin solution}$$

C. Preparation of Biotin Reagent

1. Warm the biotin-agent vials to room temperature before opening.
2. Immediately before using, add 500µl deionized water or solvent (DMSO or DMF), depending on water solubility (Table 1, column7) to every 2mg HOOK™ Biotin reagent.

   NOTE: *Make fresh each time and do not prepare stock solutions.*

D. Biotin Conjugation Reaction

1. Add the calculated volume (Section B) of freshly prepared HOOK™ Biotin Reagent Solution to the protein solution from Section A.
2. Incubate the reaction at room temperature for 30-60 minutes or on ice for 2 hours. Longer incubations can be performed, but these may be affected by protein degradation.

E. Removal of Unconjugated Biotin Reagent

1. Mark one side of the column and ensure in all centrifugations the mark is facing outwards during centrifugation.
2. Prepare the SpinOUT™ column by centrifuging the SpinOUT™ columns at 1,000g for 1 minute to compact the resin.
3. Remove the top and then bottom caps. Place into a 15ml collection tube.
4. Centrifuge the column at 1,000g for 2 minutes to remove the storage buffer.
5. Equilibrate the column with 2ml 1X Optimizer Buffer™, by adding slowly to the resin bed. Centrifuge at 1,000g for 2 minutes. Discard flow through and repeat this step a further 2 times.
6. Place the column in a new 15ml collection tube and remove the cap.
7. Slowly, apply the protein solution to the center of the SpinOUT™ resin.

   NOTE: *See the table above and Important Information for the recommended volumes to apply to the column.*

8. Centrifuge the column at 1,000g for 8 minutes to collect the desalted protein solution. Discard the column.
9. Store biotinylated protein in 0.1% sodium azide at 4°C until ready for use. Store at -20°C for long term storage.

F. Estimation of Biotin Incorporation Efficiency

The method of biotin incorporation estimation is based on the binding of avidin with HABA dye (2-(4-Hydroxyphenylazo)benzoic acid/ 2-(4'-Hydroxybenzeneazo)benzoic acid/ 4'-Hydroxyazobenzene-2-carboxylic acid), which produces a color that can be read at 500nm. The HABA-avidin complex can be displaced with free biotin or biotin conjugated with other molecules (proteins). Measuring the change in optical density of HABA-avidin complex with biotinylated proteins allows for accurate estimation of the molar ration of biotin conjugated to the protein/ antibody.

Important Information

- Ensure that all free/ unconjugated biotin is removed from the labeled protein or other molecule before performing an estimation. We recommend desalting with our SpinOUT™ desalting spin columns or dialysis with our micro dialysis devices, Tube-O-DIALYZER™.
- During desalting or dialysis, we recommend exchanging the reaction buffer to BiotinQuant™ Assay Buffer to ensure accurate estimation. PBS or TBS may also be used, but avoid buffers containing potassium that may result in unwanted precipitation.
- A small variation in color between the OneQuant™ HABA/Avidin does not affect the performance of the reagents.
- The Biotin Standard is supplied as an optional positive control for the assay. Use 100µl in lieu of the biotinylated sample. See calculation for determining amount of biotin in the standard.

Protocol 1: Cuvette Protocol

1. Allow the reagents to warm to room temperature.
2. Pipette 850µl BiotinQuant™ Assay Buffer into a 1ml cuvette and zero the spectrophotometer at a 500nm wavelength.
3. Briefly centrifuge a OneQuant™ HABA/Avidin vial and then transfer entire contents to the cuvette and mix by gentle inversion.
4. Measure the absorbance of the HABA/Avidin complex at 500nm. This is your A_{500} HABA/Avidin reading.
5. Add 100µl biotinylated sample to the HABA/Avidin cuvette and mix well by inversion.
   NOTE: *If using optional Biotin Standard, replace the 100µl biotinylated sample with 100µl Biotin Standard.*
6. Measure the absorbance of the solution at 500nm. Record the absorbance once it has stabilized for 10-15 seconds. This is your A_{500} HABA/Avidin/Biotin Sample reading.
   NOTE: *If the absorbance is <0.3, dilute the biotin sample and repeat the assay.*
7. Go to the calculation section to determine the moles of biotin per mole of protein.

Protocol 2: Microplate Protocol

1. Allow the reagents to warm to room temperature.
2. Pipette 170µl BiotinQuant™ Assay Buffer into each microplate well. Blank the plate reader with a well containing only BiotinQuant™ Assay Buffer.
3. Briefly centrifuge a OneQuant™ HABA/Avidin vial and then add 10µl OneQuant™ HABA/Avidin to the cuvette and mix on an orbital shaker or equivalent.
4. Measure the absorbance of the HABA/Avidin complex at 500nm. This is your A_{500} HABA/Avidin reading.
5. Add 20µl biotinylated sample to the HABA/Avidin well and mix well as before.
   NOTE: *If using optional Biotin Standard, replace the 20µl biotinylated sample with 20µl Biotin Standard.*
6. Measure the absorbance of the solution at 500nm. Record the absorbance once it has stabilized for 10-15 seconds. This is your A_{500} HABA/Avidin/Biotin Sample reading.
   NOTE: *If the absorbance is <0.3, dilute the biotin sample and repeat the assay.*
7. Go to the calculation section to determine the moles of biotin per mole of protein.

Calculations

Based on Beer Lambert (Beer's) Law: $\mathbf{A_\lambda = \varepsilon_\lambda bC}$*, where*

- **A** is the absorbance at a particular wavelength (λ). HOOK™ BiotinQuant™ assay is performed at 500nm.
- **ε** is the extinction coefficient at the wavelength (λ). For HABA/Avidin samples at 500nm, pH7.0 this is 34,000$M^{-1}cm^{-1}$.
- **b** is the path length in centimeters. Cuvettes (10x10mm) have a pathlength of 1cm. The pathlength for microplates, using the indicated volumes, is normally 0.5cm.
- **C** is the molarity concentration of the sample (= mol/L = mmol/ml)

For calculating the number of moles of biotin per mole of protein or sample the following values are required:

- Concentration of protein/sample used (mg/ml)
- Molecular weight of protein, expressed as grams per mole (e.g. IgG = 150,000)
- A_{500} HABA/Avidin reading
- A_{500} HABA/Avidin/Biotin Sample
- Dilution factor (DF), if sample was diluted before adding to HABA/avidin solution.

1. Calculate mmol biotinylated protein/ml:

Calculation #1: $$\frac{\text{protein concentration (mg/ml)}}{\text{MW of protein (mg/mmol)}} = \text{mmol protein/ml}$$

2. Calculate change in absorbance at 500nm:

Calculation #2 (Cuvette): $(0.9 \times A_{500}$ HABA/Avidin) –(A_{500} HABA/Avidin/Biotin Sample) $= \Delta A_{500}$

Calculation #2 (Microplate): (A_{500} HABA/Avidin) –(A_{500} HABA/Avidin/Biotin Sample) $= \Delta A_{500}$

NOTE: 0.9 is the correction factor for the dilution of the HABA/Avidin with the sample in the cuvettes. This is not necessary for microplates as the dilution is offset by the increase in volume and therefore the light path (b).

3. Calculate concentration of biotin in reaction (mmol/ml):

Calculation # 3: $$\frac{\Delta A_{500}}{34{,}000 \times b} = \frac{\text{Calculation \#2}}{34{,}000 \times b} = \frac{\text{mmol biotin}}{\text{ml reaction mixture}}$$

NOTE: b = lightpath, which is 1cm for cuvettes and 0.5cm for microplates.

4. Calculate mmol of biotin per mmol of protein:

Calculation # 4: $$\frac{\text{mmol biotin in original sample}}{\text{mmol protein in original sample}} = \frac{\text{mmol biotin in reaction} \times 10 \times \text{DF}}{\text{Calculation \#1}} = \frac{\text{Calculation \#3} \times 10 \times \text{DF}}{\text{Calculation \#1}}$$

NOTE: DF is the dilution factor. 10 is for the 10 fold dilution of the biotinylated protein sample in the reaction mixture.

5. Calculate concentration of biotin in Biotin Standard (mM):

Calculation # 5: $$\frac{\Delta A_{500} \times 10 \times 1000}{34{,}000 \times b} = \quad = \text{[Biotin Standard] (mM)}$$

NOTE: b = lightpath, which is 1cm for cuvettes and 0.5cm for microplates. 10 is for the 10 fold dilution of the Biotin Standard in the reaction mixture.

TROUBLESHOOTING

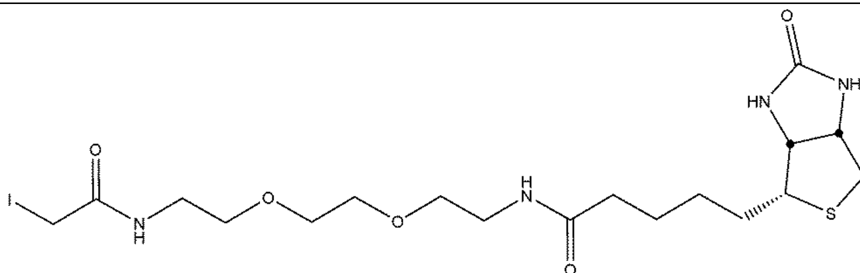
Issue	Suggested Reason	Possible Solution
ΔA_{500} is ≤ 0	Low or zero biotinylation of protein.	Lack of functional groups for biotinylation, use a different coupling chemistry.
	Incomplete reagent mixing	Ensure all the OneQuant™ HABA/Avidin is fully dissolved before using
	Particulates in protein solution interfering with absorbance	Filter protein solution before assaying
	Potassium ions present in sample	Ensure samples are in BiotinQuant™ Assay Buffer
Biotin levels are unexpectedly high	Free, Unconjugated biotin not removed	Desalt or dialyze biotinylated sample before use to remove free biotin.

APPENDIX 1: SAMPLE EQUILIBRATION WITH TUBE-O-DIALYZER™ (NOT SUPPLIED)

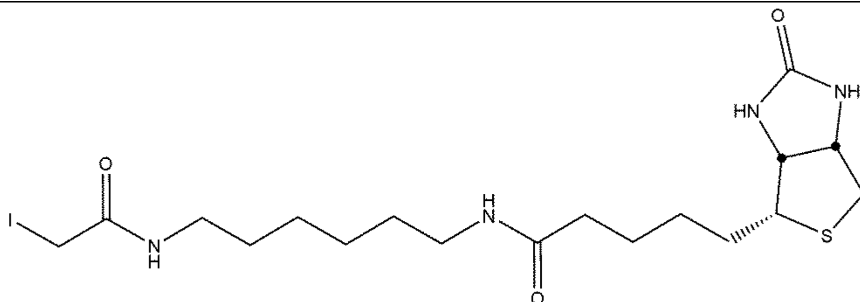
If protein solution is in an incompatible buffer, dialyze and equilibrate into 1X Optimizer Buffer™ as follows:

1. Pipette your sample directly into the Tube-O-DIALYZER™ tube. For Tube-O-DIALYZER™ Micro use 20-250µl and for Tube-O-DIALYZER™ Medi use 0.2-2.5ml.
   NOTE: *Tube-O-Dialyzer™ is available in 1, 4, 8, 15 and 50kDa MWCO. Visit our website for further information.*
2. Pipette 3-5ml appropriate 1X Optimizer Buffer™ into a Micro Dialysis Cup or small beaker. If a small magnetic stir bar is available add to the Micro Dialysis Cup, if not add 3-5 glass balls.
3. Screw the dialysis cap on to the Tube-O-DIALYZER™ tube. Invert the Tube-O-DIALYZER™, ensuring the entire sample rests upon the membrane.
   NOTE: *If sample is too viscous, centrifuge the Tube-O-DIALYZER™ in an inverted position (i.e. the dialysis membrane facing downward). Centrifuge for 5 seconds at 500-1,000g.*
4. Keeping the Tube-O-DIALYZER™ in an inverted position, slide the supplied float onto the Tube-O-DIALYZER™ tube. Place the Tube-O-DIALYZER™ in the Micro Dialysis Cup with the Optimizer Buffer™.
5. Ensure that the dialysis membrane contacts the dialysis buffer. If there are large air bubbles trapped underneath the dialysis membrane surface, tilt the tube or squirt buffer to remove the air bubbles. Gently, stir the dialysis buffer with a magnetic stir or place on an orbital shaker. For efficient and complete dialysis we recommend inverting or gently tapping the Tube-O-DIALYZER™ 1-2 times during dialysis to mix the sample. If necessary repeat the centrifugation in step 3.
6. Dialyze at room temperature, or 4°C if required, for 1-2 hours.
7. Repeat the dialysis with 1-2 changes of buffer.
8. After dialysis, remove the Tube-O-DIALYZER™ from the float and immediately spin the Tube-O-DIALYZER™ (in up-right position) for 5-6 seconds at 500-1,000xg.

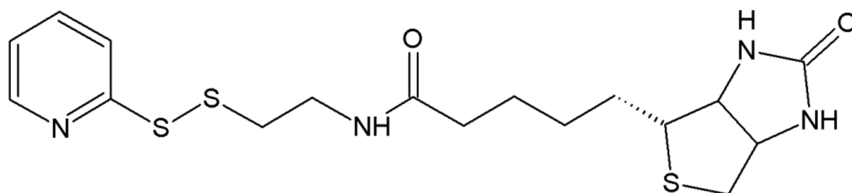
APPENDIX 2: BIOTIN REAGENT STRUCTURES



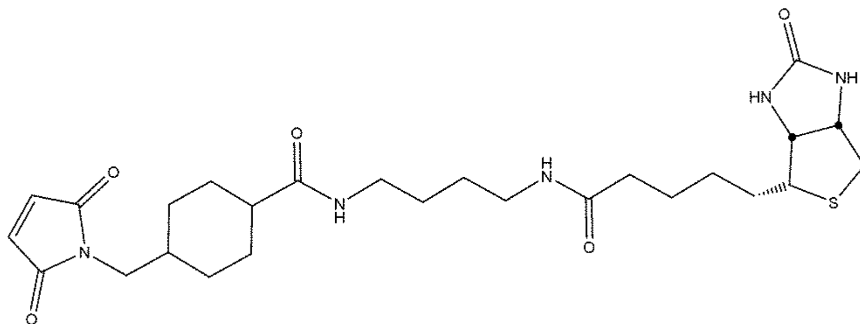
HOOK™-PEG$_2$-Iodoacetyl-Biotin (Cat. # BS-11)



HOOK™-Iodoacetyl-LC-Biotin (Cat. # BS-12)



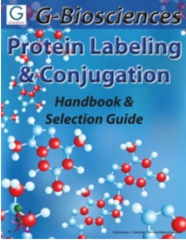
HOOK™-Biotin-PDA (Cat. # BS-13)



HOOK™-Biotin-BMMCC (Cat. # BS-14)

RELATED PRODUCTS

Download our Protein Labeling and Conjugation Handbook.



http://info.gbiosciences.com/complete-protein-labeling-conjugation-handbook/

For other related products, visit our website at www.GBiosciences.com or contact us.

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