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A Geno Technology, Inc. (USA) brand name

HOOK™ Biotin
Amine Reactive

For the coupling of biotin to protein amine groups

(Cat. # BS-01 to BS-04, BS-06 to BS-10)

INTRODUCTION

This protocol is for use with the HOOK™ Biotin reagents that react with protein primary amines. Amines, lysine ε-amines and N-terminal α-amines, are the most abundant group in protein molecules and represent the most common target for biotinylation. For example, BSA contains 59 primary amines, of which up to 35 are available on the surface of the molecules and can be reacted with amine reactive esters.

Biotin, a 244 Dalton molecule, exhibits an extraordinary binding affinity for avidin and streptavidin (K_a=10^{15} M^{-1}). The biotinylated molecules are efficiently probed with avidin or streptavidin conjugated to reporter molecules, such as peroxidases or phosphatases. The use of biotin labeled proteins in ELISA and Western blotting is a popular technique.

The most widely used amine reactive biotinylation reagents are the water insoluble *N*-hydroxysuccinimide (NHS) esters or the water soluble *N*-hydroxysulfosuccinimide (sulfo-NHS) esters. The addition of a charged sulfonate (SO_3^-) on the *N*-hydroxysuccinimide ring of the sulfo-NHS esters results in their solubility in water (~10mM), but not permeable to plasma membranes. The solubility and impermeability to plasma membranes makes them ideal for studying cell surface proteins as they will only react with the protein molecules on the outer surface of plasma membranes. The reaction of the NHS and sulfo-NHS esters with amines are virtually identical leading to the formation of an amide bond and release of NHS or sulfo-NHS.

Both HOOK™-NHS-Biotin and HOOK™-sulfo-NHS-Biotin are available with various spacer arms (See Table 1). Also available is a cleavable form of HOOK™-sulfo-NHS-Biotin, HOOK™-sulfo-NHS-SS-Biotin, which has a disulfide bond in the spacer arm. The disulfide bond permits the cleavage of the biotin moiety from the protein, making its interaction with avidin/ streptavidin reversible. Disulfide bonds are cleaved under reducing conditions with 100mM mercaptoethanol, 30-50mM DTT, or 1% sodium borohydride.

HOOK™-PFP-Biotin is another reagent that reacts with amines and forms stable amide bonds. HOOK™-PFP-Biotin is more reactive than other NHS esters and can react with both primary and secondary amines at pH 7-9.

Each kit is designed for the conjugation of biotin to protein primary amine 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.

ITEM(S) SUPPLIED

Description	Size
HOOK™ Biotin Reagent (Table 1)	25mg
Optimizer Buffer™ I [5X]	2 x 25ml
Spin-*OUT*™ GT-600, 5ml	10 columns
OneQuant™ HABA/Avidin	24 vials
BiotinQuant™ Assay Buffer	25ml
Biotin Standard	1ml

SPECIFICATIONS: HOOK™ BIOTIN REAGENT (AMINE REACTIVE)

Cat. #	HOOK™ Biotin Reagent	Molecular Weight	Spacer Arm (Å)	Reactive Group	Membrane Permeable	Water Soluble*	Cleavable/ Reversible	Reaction pH
BS-01	**HOOK™-NHS-Biotin**	341.38	13.5	NHS-ester	Yes	No	No	7-9
BS-02	**HOOK™-NHS-LC-Biotin**	454.54	22.4	NHS-ester	Yes	No	No	7-9
BS-03	**HOOK™-NHS-LC-LC-Biotin**	567.70	30.5	NHS-ester	Yes	No	No	7-9
BS-04	**HOOK™-NHS-SS-Biotin**	440.52	24.3	NHS-ester	Yes	No	Yes	7-9
BS-06	**HOOK™-sulfo-NHS-Biotin**	443.43	13	sulfo-NHS ester	No	Yes	No	7-9
BS-07	**HOOK™-sulfo-NHS-LC-Biotin**	556.59	22.4	sulfo-NHS ester	No	Yes	No	7-9
BS-08	**HOOK™-sulfo-NHS-LC-LC-Biotin**	669.75	30.5	sulfo-NHS ester	No	Yes	No	7-9
BS-09	**HOOK™-sulfo-NHS-SS-Biotin**	606.69	24.3	sulfo-NHS ester	No	Yes	Yes	7-9
BS-10	**HOOK™-PFP-Biotin**	410.36	9.6	Pentafluorophenyl ester	Yes	No	No	7-9

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

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

- NHS esters are soluble in organic solvents and DMSO or DMF are the most commonly used, which are compatible with most proteins in a 20% solution. Sulfo-NHS ester is soluble in water, up to ~10mM and should only be dissolved immediately prior to use.
- Reactive pH is neutral pH and above. Competing hydrolysis of the NHS esters and Sulfo-NHS esters in aqueous solution is a major concern as the rate of hydrolysis increases with increasing pH. Half-life of 2-4 hours at pH7.0 increasing to a few minutes at pH 9.0.
- Reaction incubation time is a few minutes to a few hours at 4-35°C.
- Avoid buffers containing amines such as Tris or Glycine.

ADDITIONAL ITEMS 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™ I to a maximum concentration of 10mg/ml.

1b If your protein is in an amine–free buffer at a pH of 7.2-8.0 then proceed to the next section.

1c For protein in Tris or other amine containing buffers a buffer exchange must be performed. The buffer exchange can be done by dialysis against Optimizer Buffer™ I, 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.

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

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

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. Prepare the Spin-*OUT*™ column by removing the top and then bottom caps. Place into a 15ml collection tube.
2. Centrifuge the column at 1,000g for 2 minutes to remove the storage buffer. Discard storage buffer and return column to 15ml collection tube.
3. Equilibrate the column with 2ml 1X Optimizer Buffer™ I, 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.
4. Place the column in to a clean 15ml collection tube and apply the sample directly to the center of the resin bead. Allow the sample to migrate into the resin bed.
5. Centrifuge the column at 1,000g for 2 minutes. The flow through is the purified labeled protein sample.
6. Store biotinylated protein in 0.1% sodium azide at 4°C until ready for use. Store at -20°C for long term storage.

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
  $34{,}000M^{-1}cm^{-1}$.
- **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:

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

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):

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

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

4. Calculate mmol of biotin per mmol of protein:

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

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):

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

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

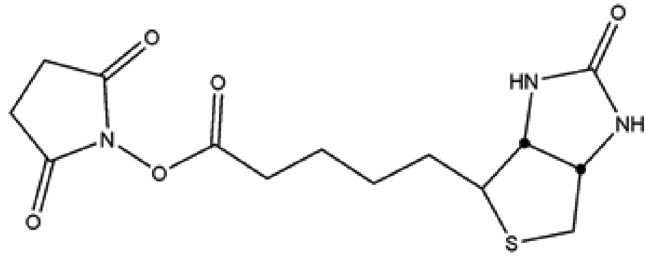
Issue	Suggested Reason	Possible Solution
ΔA_{500} is ≤0	Low or zero biotinylation of protein.	Lack of functional groups for biotinylation, use a different coupling chemistry.
	Incomplete reagent mixing	Ensure all the OneQuant™ HABA/Avidin is fully dissolved before using
	Particulates in protein solution 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™

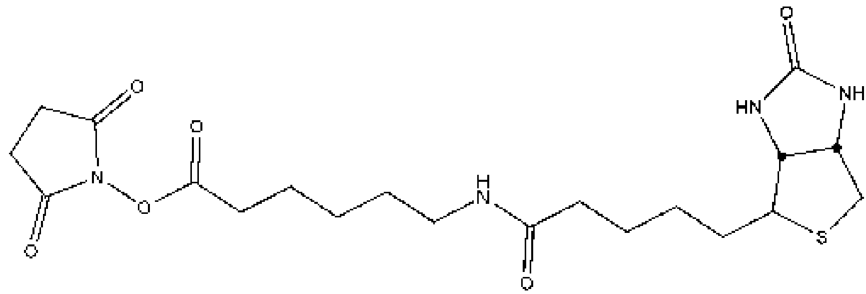
If protein solution is in an incompatible buffer, dialyze and equilibrate into 1X Optimizer Buffer™ as follows:

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.

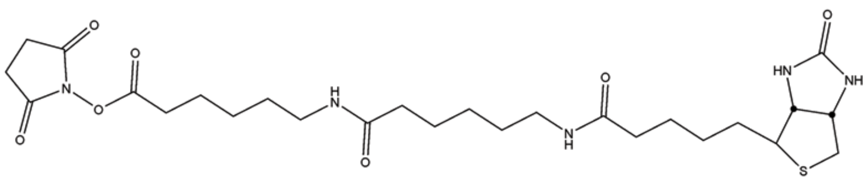
APPENDIX 2: BIOTIN REAGENT STRUCTURES



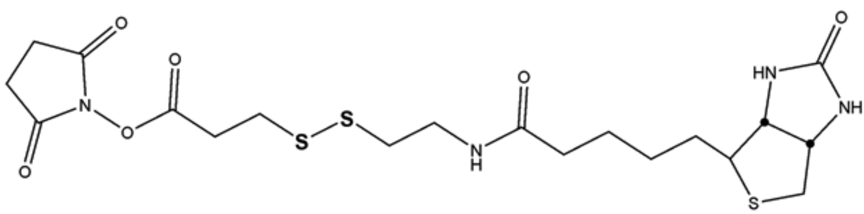
HOOK™-NHS-Biotin (Cat. # BS-01)



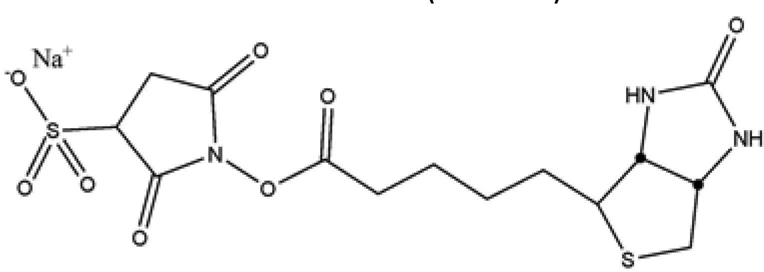
HOOK™-NHS-LC-Biotin (Cat. # BS-02)



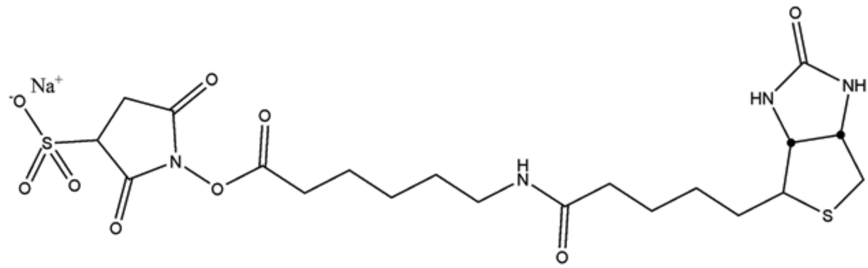
HOOK™-NHS-LC-LC-Biotin (Cat. # BS-03)



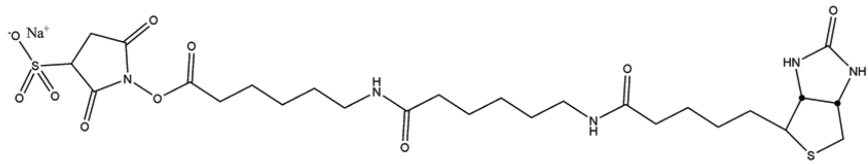
HOOK™-NHS-SS-Biotin (Cat. # BS-04)



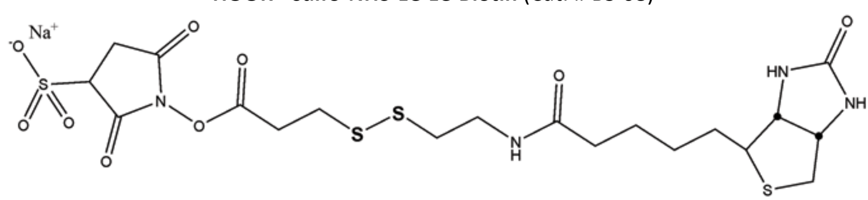
HOOK™-sulfo-NHS-Biotin (Cat. # BS-06)



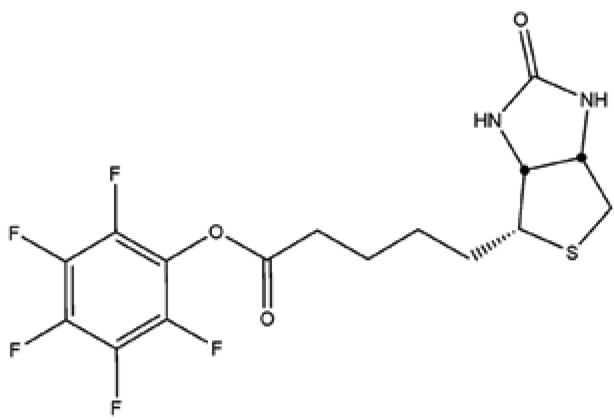
HOOK™-sulfo-NHS-LC-Biotin (Cat. # BS-07)



HOOK™-sulfo-NHS-LC-LC-Biotin (Cat. # BS-08)



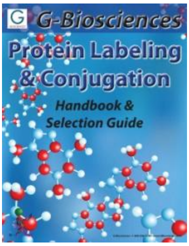
HOOK™-sulfo-NHS-SS-Biotin (Cat. # BS-09)



HOOK™-PFP-Biotin (Cat. # BS-10)

RELATED PRODUCTS

Download our Protein Labeling & Conjugation Handbook



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

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