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A Geno Technology, Inc. (USA) brand name

HOOK™ Iodoacetyl-LC-Biotin

For the coupling of biotin to protein sulfhydryl groups

(Cat. # BS-12)

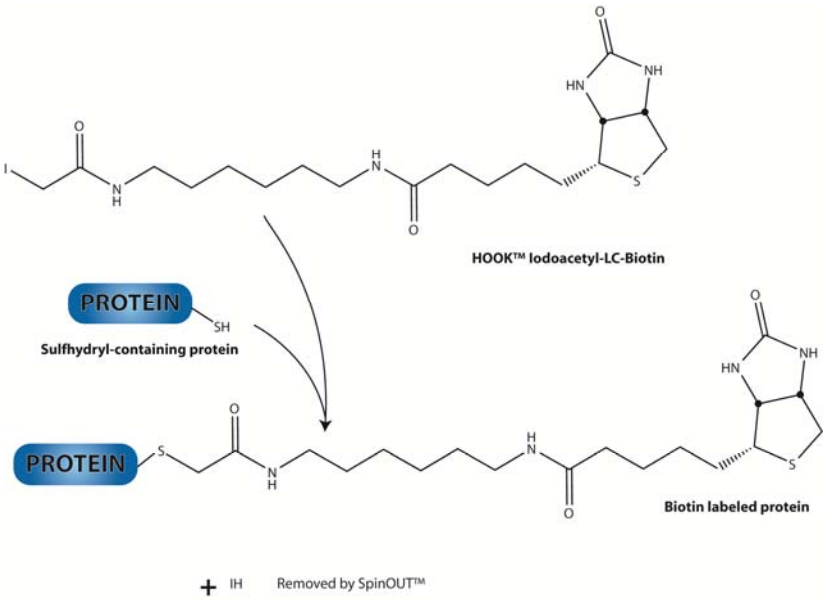
INTRODUCTION

This 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.

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, Western blotting and dot blotting is a popular technique.

HOOK™-Iodoacetyl-LC-biotin is a sulfhydryl reactive biotinylation reagent that reacts with thiol groups at pH7.5-8.5 and forms stable thioether bonds. 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.

HOOK™ Iodoacetyl-LC-Biotin kit is designed for the coupling of 1-10mg protein in 1ml buffer, suitable for 10 couplings.



ITEMS SUPPLIED (Cat. # BS-12)

Description	Size
HOOK™ Iodoacetyl-LC-Biotin Agent	25mg
Optimizer Buffer™ II [5X]	2 x 25ml
Spin-*OUT*™ GT-600, 5ml	10 columns
OneQuant™ HABA/Avidin	24 vials
BiotinQuant™ Assay Buffer	25ml
Biotin Standard	1ml

STORAGE CONDITON

The kit is shipped at Ambient Temp. 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 to warm to room temperature before opening.

SPECIFICATIONS

Molecular weight: 510.43
Spacer Arm (Å): 27.1
Membrane Permeable: Yes
Water Soluble: No
Reaction pH: 7.5-8.5

PRECAUTIONS

1. Allow reagent to warm to room temperature before opening as it is moisture sensitive.
2. Dissolve the HOOK™ Iodoacetyl-LC-Biotin immediately prior to use. Do not prepare stock solutions.
3. Avoid using buffers with free thiols or reducing agents.

ADDITIONAL ITEM(S) REQUIRED

DMF
15ml collection tubes

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.

A. PROTEIN SAMPLE PREPARATION

1. Dissolve 1-10mg protein in 0.5-2ml 1X Optimizer Buffer™ I to a maximum concentration of 10mg/ml.
2. The protein samples to be labelled require free (reduced) sulfhydryl groups, we suggest the following methods for protein reduction
   a. For complete reduction of large proteins, treat protein solutions with 1mM DTT or 5mM TCEP for 30 minutes and then remove reducing agent with a Spin-*OUT*™ column or by dialysis with Tube-O-DIALYZER™.
   b. For partial reduction (for antibodies), use 2-mercaptoethylamine (See Appendix 2)
   c. For the addition of sulfhydryls to proteins, use SATA (Cat. # BC96) (See Appendix 3) or Traut's reagent (Cat. # BC95).
3. For protein in buffer containing reducing agents or free thiols then a buffer exchange must be performed. The buffer exchange can be done by dialysis against Optimizer Buffer™ II, 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 D. 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

We recommend using a 4x molar excess of HOOK™ Iodoacetyl-LC-Biotin Agent to free sulfhydryls on proteins. To calculate the amount of free sulfhydryls use Ellman's reagent (See Appendix 4).

1. Dissolve 2mg HOOK™ Iodoacetyl-LC-Biotin in 1ml DMF for a 4mM solution
2. Calculate the amount of mM required for a 4 fold excess:

Free sulfhydryls (mM) (from Ellman's Reactio) X 4 = mM HOOK™ Iodoacetyl-LC-Biotin

3. Add the following volume to the protein solution

$$\frac{\text{mM HOOK™ Iodoacetyl-LC-Biotin}}{\text{4mM}} \times \text{Protein Sample Volume (µl)} = \text{µl HOOK™ Iodoacetyl-LC-Biotin solution}$$

4= mmol HOOK™ Iodoacetyl-LC-Biotin molecular weight

C. PREPARATION OF BIOTIN AGENTS

1. Warm the biotin-agent vials to room temperature before opening.
2. Immediately before using, add 1000µl DMF to every 2mg HOOK™ Iodoacetyl-LC-Biotin for a 4mM working solution.
   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 10mM HOOK™ Iodoacetyl-LC-Biotin to the protein solution from Section A.
2. Incubate the reaction at room temperature in the dark for 90 minutes. Longer incubations can be performed, but these may be affected by protein degradation. ***NOTE:*** *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.*

E. REMOVAL OF UNCONJUGATED BIOTIN AGENTS

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™ II, 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 at 4°C in 0.1% sodium azide 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 $34{,}000M^{-1}cm^{-1}$.
- **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): $(0.9 \times A_{500}\text{ HABA/Avidin}) - (A_{500}\text{ HABA/Avidin/Biotin Sample}) = \Delta A_{500}$

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

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™ (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: PARTIAL REDUCTION OF ANTIBODIES WITH 2-MERCAPTOETHYLAMINE

1. Weigh out 1.5mg 2-mercaptoethylamine and add to 1ml of 1mg/ml IgG solution. Dissolve with gentle pipetting. Incubate at 37°C for 90 minutes.
2. The reducing agent can be removed from the IgG solution by dialysis, we recommend Tube-O-DIALYZER™. Transfer the solution to the Tube-O-Dialyzer™ and replace the cap, ensuring that the cap is not over tightened.
3. Follow the procedure in Appendix 1 for Tube-O-DIALYZER™ and dialyze against Optimizer Buffer™ II.
   NOTE: *Optimizer Buffer™ II contains EDTA to prevent oxidation of the reduced IgG. If another buffer is used ensure it contains 1-10mM EDTA.*
4. After dialysis, use the reduced IgG as soon as possible to prevent reoxidation.

APPENDIX 3: ADDITION OF SULFHYDRYL GROUPS TO PROTEINS

If your protein or peptide lacks free sulfhydryls or have very few, additional free sulfhydryl groups can be added with the use of SATA.

1. Weigh out 2mg SATA (Cat. # BC96) into a clean tube and immediately before use dissolve in 200µl DMF.
2. Add 4µl SATA solution to 1ml (1mg/ml) IgG solution to give a 25 molar excess of SATA. Mix and incubate at room temperature for 30 minutes.
3. The modified IgG solution is stable and can be stored at -20°C.
4. Deacetylation is required to complete the reaction. Weigh out 2mg hydroxylamine into a clean tube and immediately before use add 100µl 1X Optimizer Buffer™ II to make the deacetylation solution.
5. Add 50µl deacetylation solution to the IgG solution and incubate for 2 hours at room temperature.
6. The byproducts of the reaction can be removed from the modified IgG by dialysis using Tube-O-DIALYZER™. Follow the procedure in Appendix 1 for Tube-O-DIALYZER™ and dialyze against Optimizer Buffer™ II.
   NOTE: *Optimizer Buffer™ II contains EDTA to prevent oxidation of the reduced IgG. If another buffer is used ensure it contains 1-10mM EDTA.*
7. After dialysis, use the reduced IgG as soon as possible to prevent reoxidation.

APPENDIX 4: CALCULATION OF FREE SULFHYDRYLS WITH ELLMAN'S REAGENT

Ellman's reagent, 5, 5'-dithio-*bis*-(2-nitrobenzoic acid) (Cat. # BC87, also known as DTNB, is a versatile water-soluble compound for quantitating free sulfhydryl groups in solution. DTNB reacts with a free sulfhydryl group to yield a mixed disulfide and 2-nitro-5-thiobenzoic acid (NTB), a measurable yellow-colored product with molar extinction coefficient of 14.15 mM^{-1} cm^{-1}at 412 nm. DTNB is very useful as a sulfhydryl assay reagent because of its specificity for -SH groups at neutral pH, high molar extinction coefficient and short reaction time.

1. Make 10mM DTNB stock solution by dissolving 40mg DTNB in 10ml 0.1M Tris-HCl pH 7.5. The stock solution can be stored at 4°C for 3 months. Dilute the stock solution 100 fold with 0.1M Tris-HCl pH 7.5 to make 0.1mM DTNB working solution.
2. Aliquot 950µl of 0.1mM DTNB work solution to each 1.5ml centrifuge tube. Add 50□l test sample and mix by brief vortexing. Set a blank by adding 50µl of 0.1M Tris-HCl pH 7.5 to 950µl of 0.1mM DTNB work solution.
   ***NOTE**: The test sample may need to be diluted before applied to the assay and the dilution factor should be recorded. The 50µl test sample applied to the assay reaction should have a sulfhydryl concentration less than 0.5mM. Concentrations exceeding 0.5mM free sulfhydryl will result in high absorbance values and less accurate estimation of the concentration based on the extinction coefficient.*
3. Incubate 2 minutes at room temperature.
4. Measure the absorbance of the test sample with a spectrophotometer against blank at 412nm.
5. Calculate the concentration of free sulfhydryls in the sample from the molar extinction coefficient of NTB (14.15 mM^{-1} cm^{-1}) as follow:
   mM free sulfhydryls = Absorbance / (path length x 14.15 x 2) x 20 x dilution factor
   ***NOTE:** Path length is the cuvette path length in centimeter (cm); 1 mole free sulfhydryls reduce 1 mole DTNB to yield 2 moles NTB; 20 is the dilution factor of 50µl sample to 1ml assay volume.*

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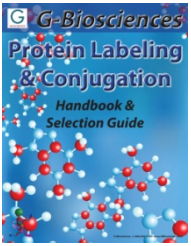

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