



BIOSCIENCES®

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HOOK™ -Biotin Kit

INTRODUCTION

Based on the protein target reactive groups, biotin reagents can be divided into amines reactive (such as N-hydroxysuccinimide (NHS) esters), sulfhydryl reactive (such as maleimide and iodoacetyl), carbohydrate reactive (such as hydrazides), and carboxyl reactive biotin reagents (such as amines). Photo-reactive biotin reagents react non-specifically upon exposure to UV light and are used when no appropriate reactive group is available on the target molecules. The HOOK™ - Biotin kit is provided with a reactive biotin reagent, ready-to-use buffers (Optimizer Buffers) for performing biotinylation reaction, Tube-O-Reactor™, and biotinylation estimation reagents. The entire labeling procedure is performed in a single Tube-O-Dialyzer™ - sample equilibration and optimization, biotin coupling reaction, and finally removal of the un-reacted biotin and storage of the reaction conjugated products for later use.

ITEM(S) SUPPLIED

HOOK™ - Biotin Agent (See Table 2)	25mg for BK-01 to BK-19 5mg for BK-20 (HOOK™-Psoralen-PEO-Biotin)
Optimizer Buffer™ I to V [5X] (Table 1)	2 x 25ml
<i>Tube-O-Reactor™</i>	5 x Tube-O-Dialyzers - <u>8kDa MWCO, Micro</u> 5 x Tube-O-Dialyzers - <u>8kDa MWCO, Medi</u> 5 x Micro Caps 5 x Micro Floats 5 x Medi Caps 5 x Medi Floats 5 x Micro Dialysis Cups 60 x Glass Balls
Avidin	2 x 5mg
HABA Dye Reagent	1ml
BiotinQuant™ Assay Buffer	25ml

Table 1: Optimizer Buffer™ Selection Guide

	Reaction Type	HOOK™ Biotin Kit
Optimizer Buffer™ I [5X]	Suitable for Amine Reactive & Photoreactive Reagents	BK-01 to BK-10, BK-20
Optimizer Buffer™ II [5X]	Suitable for Sulfhydryl Reactive Reagents	BK-11, BK-12
Optimizer Buffer™ III [5X]	Suitable for Sulfhydryl Reactive Reagents	BK-13, BK-14
Optimizer Buffer™ IV [5X]	For Carboxyl Reactive Reagents	BK-15, BK-16, BK-17
Optimizer Buffer™ V [5X]	For Carbohydrate Reactive Reagents	BK-18, BK-19

STORAGE CONDITION

The kit is shipped at Ambient Temp. Upon arrival, store the kit components at 4°C, except Avidin should be stored at -20°C.

ITEMS NEEDED BUT NOT SUPPLIED WITH THE KIT

DMSO or DMF for water insoluble biotin agent (see Table 2 for solvent requirement)
Shaker or Stir plate and stir bar.



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Table 2: HOOK™-Biotin Kits and specification of HOOK™-Biotin reagents supplied with each kit:

Cat. #	HOOK™ Biotin Reagent	Molecular Weight	Spacer Arm (Å)	Reactive Group	Membrane Permeable	Water Soluble*	Cleavable/ Reversible	Reaction pH
BK-00	d-Biotin (vitamin H)	244.32	0					-
AMINE REACTIVE REAGENTS								
BK-01	HOOK™-NHS-Biotin	341.38	13.5	NHS-ester	Yes	No	No	7-9
BK-02	HOOK™-NHS-LC-Biotin	454.54	22.4	NHS-ester	Yes	No	No	7-9
BK-03	HOOK™-NHS-LC-LC-Biotin	567.70	30.5	NHS-ester	Yes	No	No	7-9
BK-04	HOOK™-NHS-SS-Biotin	440.52	24.3	NHS-ester	Yes	No	Yes	7-9
BK-06	HOOK™-sulfo-NHS-Biotin	443.43	13	sulfo-NHS ester	No	Yes	No	7-9
BK-07	HOOK™-sulfo-NHS-LC-Biotin	556.59	22.4	sulfo-NHS ester	No	Yes	No	7-9
BK-08	HOOK™-sulfo-NHS-LC-LC-Biotin	669.75	30.5	sulfo-NHS ester	No	Yes	No	7-9
BK-09	HOOK™-sulfo-NHS-SS-Biotin	606.69	24.3	sulfo-NHS ester	No	Yes	Yes	7-9
BK-10	HOOK™-PFP-Biotin	410.36	9.6	Pentafluorophenyl ester	Yes	No	No	7-9
SULFHYDRYL REACTIVE REAGENTS								
BK-11	HOOK™-PEO-Iodoacetyl-Biotin	542.43	24.7	Iodoacetyl	No	Yes	No	7.5-8.5
BK-12	HOOK™-Iodoacetyl-LC-Biotin	510.43	27.1	Iodoacetyl	Yes	No	No	7.5-8.5
BK-13	HOOK™-Biotin-PDA	412.60	21.1	Pyridyldithiol	Yes	No	Yes	6-9
BK-14	HOOK™-Biotin-BMCC	533.68	32.6	Maleimide	No	No	No	6.5-7.5
CARBOXYL REACTIVE REAGENTS								
BK-15	HOOK™-Biotin-Pentylamine	328.47	18.9	Amine	No	No	No	4-6
BK-16	HOOK™-Biotin-PEO-Amine	374.50	20.4	Amine	No	Yes	No	4-6
BK-17	HOOK™-Biotin-PEO-LC-Amine	418.55	22.9	Amine	No	Yes	No	4-6
CARBOHYDRATE (OXIDIZED) REACTIVE REAGENTS								
BK-18	HOOK™-Biotin-Hydrazide	258.34	15.7	Hydrazide	Yes	No	No	4-6
BK-19	HOOK™-Biotin-LC-Hydrazide	371.50	24.7	Hydrazide	Yes	No	No	4-6
PHOTOREACTIVE REAGENTS FOR DNA, RNA & PROTEIN								
BK-20	HOOK™-Psoralen-PEO-Biotin	688.79	36.9	Psoralen	No	Yes	No	4-6

* For water insoluble reagents use DMSO or DMF

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. Tube-O-DIALYZER™ are supplied in a preservative to maintain quality. Prior to use discard the preservative from the tube and place the dialysis cap upside down in a beaker or other suitable container and add 1-2ml DI water or dialysis buffer to rinse. Keep the Tube-O-DIALYZER™ membrane wet until required.

SAMPLE EQUILIBRATION

Prepare protein sample in an appropriate 1X Optimizer Buffer™ for optimal reaction (See Table 1:Optimizer Buffer™ Selection Guide).

For dry sample, dissolve 2-10mg of protein in 1ml of 1X Optimizer Buffer™.

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: The kit is supplied with 8kDa MWCO Tube-O-Dialyzer™, however 1, 4, 15 and 50kDa MWCO are also available. Visit our website for further information.

2. Pipette 3-5ml appropriate 1X Optimizer Buffer™ into a Micro Dialysis Cup. If a small magnetic stir bar is available add to the Micro Dialysis Cup, if not add 3-5 glass balls.
3. Remove the Tube-O-DIALYZER™ dialysis cap from the rinse water/buffer and carefully remove excess liquid with a pipette tip.
4. Screw the dialysis cap on to the Tube-O-DIALYZER™ tube until finger tight. 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). We recommend inverting the Tube-O-DIALYZER™ in the Tube-O-DIALYZER™ centrifuge adaptor (Cat. # 786-145) or a 50ml centrifuge tube and centrifuging for 5 seconds at 500-1,000g. Do not spin longer as this may cause the membrane to rupture.

5. 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™.
6. 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 4.
7. Dialyze at room temperature, or 4°C if required, for 1-2 hours.
8. After dialysis, remove the Tube-O-DIALYZER™ from the float and immediately spin the Tube-O-DIALYZER™ (in upright position) for 5-6 seconds at 500-1,000xg.

NOTE: Do not spin longer as this may cause the membrane to rupture.

OPTIONAL: Depending on the nature of the sample, the sample may be dialyzed a second time for 1-2 h in fresh 3-5ml 1X Optimizer Buffer™ to ensure complete exchange of the buffer and equilibration of the sample.

PREPARATION OF BIOTIN AGENTS

1. Warm the biotin-agent vials to room temperature before opening.
2. Immediately before using, add appropriate solvent (200µl/ 2 mg biotin) to prepare 10 mg biotin/ml solution.

BIOTIN CONJUGATION REACTION

1. Gently loosen the Tube-O-Dialyzer™ cap and add an appropriate volume of the concentrated and freshly prepared biotin solution to give a 20 fold molar excess of biotin over protein in the reaction solution (see the calculation below).

NOTE: For most cases, 20 molar excess of biotin agent to sample protein can be used to ensure sufficient biotinylation. For some proteins, the reacting molar ratio of biotin agent to protein and the reacting time may have to be adjusted. Read “Specific Reaction Considerations” below.

Biotin conjugation efficiency can be determined using the reagents and protocol supplied with this kit.

2. Replace the cap and vortex the Tube-O-Dialyzer™ for a brief 10 seconds.
3. Invert the Tube-O-Dialyzer™ and reposition into the Micro-Dialyzer tank without any buffer and incubate the Tube-O-Reactor™ at room temperature for 30-40 minutes.

NOTE: Reaction incubation conditions may be different for different biotin agents, see “Specific Reaction Considerations” below.

REMOVAL OF UNCONJUGATED BIOTIN AGENTS

1. Dialyze the reaction mixture in the Tube-O-Dialyzer™ against an appropriate 1X Optimizer Buffer™ (3-5ml) or any appropriate buffer of your choice for at least 2-4 hours and 1-2 changes of fresh dialysis buffer.
2. Discard the dialysis cap and replace with the supplied Storage Cap.
3. Store biotinylated protein at 4°C in 0.1% sodium azide until ready for use.

CALCULATION OF BIOTIN AGENT NEEDED FOR CONJUGATION

$\mu\text{moles of protein} = \mu\text{g protein} / \text{MW protein}$

$\mu\text{moles biotin reagent to add} = \mu\text{moles protein} \times 20$

$\mu\text{g biotin reagent to add} = \mu\text{moles biotin to add} \times \text{MW biotin}^*$

$\mu\text{g biotin reagent to add can be converted to } \mu\text{l to add from the concentration of } 10\mu\text{g}/\mu\text{l}$

*MW of appropriate biotin selected

SPECIFIC REACTION CONSIDERATIONS

For sulfhydryl reactive biotin reagents (Cat. # BK-11 to BK-14)

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. # BK-11 and BK-12), the biotin conjugation reaction (Step 1-3) should be protected from direct light. Place the Tube-O-Reactor™ assembly in dark and cold.

For carboxyl reactive biotin reagents (Cat. # BK-15 to BK-17)

The reaction of carboxyl and biotin needs carbodiimide to mediate. Immediately before the biotin conjugation reaction, prepare 1 mg/ml EDC (Cat. # BC25) in 1X Optimizer Buffer™ IV. Add 12ul EDC solution per ml biotin-protein reaction mixture and mix (after biotin conjugation reaction Step 1). If precipitate is formed, centrifuge for a brief 5-10 seconds at 1000xg and remove the precipitate.

For carbohydrate reactive biotin reagents (Cat. # BK-18 and BK-19)

In order to react with the hydrazide group of biotin reagent, glycoproteins must be oxidized to generate aldehyde group. Prepare 20mM sodium meta-periodate (NaIO₄) in 1X Optimizer Buffer™ V just before use. Cool the protein solution and the sodium meta-periodate solutions on ice. Add equal volume of ice-cold sodium meta-periodate solution to your protein solution and mix well. Incubate for 30 minutes on ice and in dark. Dialyze the protein solution against 1X Optimizer Buffer™ V before proceeding to the biotin conjugation reaction step.

For photoreactive biotin reagents (Cat. # BK-20)

Dissolve DNA or RNA to a concentration of 1ug/μl in 1X Optimizer Buffer™ I. Prepare 20mM HOOK™-Psoralen-PEO-Biotin in DI-water and protect from light. Add HOOK™-Psoralen-PEO-Biotin to the sample solution to final concentration ~200μM and mix well. Irradiate the open tube under a long wavelength UV light (~365nm) at least 10-30 minutes. After irradiation, free HOOK™-Psoralen-PEO-Biotin can be removed by dialysis against 1X Optimizer Buffer™ I or precipitation of DNA or RNA with ethanol.

INSTRUCTIONS FOR BIOTINYLACTION OF CELL SURFACE PROTEINS

NOTE: For cell surface protein labeling, we recommend our HOOK™ Cell Surface Protein Isolation kit that uses G-Biosciences HOOK™ biotin labeling and purification technology in conjunction with our Mammalian Cell PE LB™ lysis buffer to conveniently label cell surface proteins and isolate them for further analysis, including Western blotting.

Cell Sample Preparation:

1. Wash cells three times with ice-cold PBS buffer to remove any contaminating proteins.
2. Suspend the cells at a concentration of 25 x 10⁶ cells/ml in PBS.

NOTE: Other cell concentrations can be used based on cell size, type, etc. The concentration of biotinylation reagent can be scaled up or down accordingly.

Biotin Agent & Cell Surface Reaction

1. Prepare membrane Impermeable biotin agent (see Table 2) as described above.
2. Add 0.5mg Biotin Agent per ml of reaction volume (solid form of biotin agent can be directly added if it is water soluble).
3. Gently mix and incubate at room temperature for 30 minutes.
4. Wash cells three times with ice-cold PBS to remove any remaining biotinylation reagent. The cells surface proteins are now biotinylated.

ESTIMATION OF BIOTIN INCORPORATION EFFICIENCY

The method of biotin incorporation estimation is based on binding of avidin with HABA dye [2-(4-hydroxyazobenzene)-benzoic acid], which produces a color that can be read at 500nm. The HABA-avidin complex can be displaced with free biotin or biotin conjugated molecules (proteins). Measuring the change in optical density of HABA-avidin complex with biotinylated proteins allows estimation of biotin conjugated with the protein.

For the estimated biotin incorporation efficiency, the kit is supplied with the following components: Avidin, HABA Dye Reagent and BiotinQuant Assay Buffer.

Preparation of Avidin-HABA Reagent:

Avidin-HABA Reagent: Dissolve 5 mg avidin in 9.6 ml BiotinQuant™ Assay Buffer and mix with 0.4 ml HABA Solution. The solution can be used for two weeks if stored at 4°C.

Protocol For Estimation of Biotin Incorporation

1. Add 0.9 ml Avidin-HABA Reagent in 1ml cuvette and measure the absorbance at 500nm (OD avidin-HABA) against Assay Buffer. Add 0.1 ml biotinylated protein sample and mix.
2. Measure the absorbance of the mix solution at 500nm when the value is stable for at least 15 seconds (OD sample-mix). If the value is not greater than 0.4, dilute the sample and repeat the assay.

CALCULATION OF BIOTIN INCORPORATION

1. OD change = 0.9 x OD avidin-HABA - OD sample-mix
2. Concentration of bound biotin (μmoles biotin/ml) = OD change / 34
3. Concentration of sample protein (μmoles/ml)
= μg of protein / MW protein / volume of the biotinylated protein solution (ml)
4. Moles biotin / mole protein
= (Concentration of bound biotin x dilution factor) / Concentration of sample protein

Note: 34 is the apparent absorbance change at 500nm per μmole of biotin bound per ml.

The dilution factor is 10 in this assay protocol because 0.1 ml sample mixed with 0.9ml avidin-HABA solution. Any other dilution factor used must be counted also.

RELATED PRODUCTS:

1. **Avidin (Cat. #BKC-02)** - affinity purified for estimation of biotin incorporation and other applications.
2. **HABA (Cat. #BKC-03)** - biotin estimation dye reagent.
3. **DMSO (Cat. #BKC-17) & DMF (Cat. #BKC-16)** - Vials containing un-hydrous DMSO [Dimethyl sulfoxide (CH₃)₂SO] and DMF [N,N-Dimethylformamide HCON(CH₃)₂]. Suitable for biotinylation reaction applications.
4. **Sodium meta-periodate (Cat. #BKC-15)** - Vials containing 5g sodium meta-periodate(NaIO₄). Suitable for oxidizing glycoproteins
5. **EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide) (Cat. #BC25)** - Reactive group: carbodiimide, target group: amino and carboxyl groups, form amide bond. Supplied in 1g and 5g sizes.
6. **Tube-O-Array Dialyzer™ (Cat. #786-145A)** - A high throughput method for sample preparation and optimization. Specifically developed for dialysis-equilibration of samples prior to 2D-gel analysis or other applications. Optimize up to 12 samples at a time.

NOTE: For other related products, visit our web site at www.GBiosciences.com or contact us.

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