Sulfo-NHS-SS-Biotin Kit Protocol

1. Introduction

Sulfo-NHS-SS-Biotin Kit (Cat. No. K1006) contains all reagents that can perform 10 biotinylated labeling reactions. Sulfo-NHS-SS-Biotin is a water-soluble biotin reagent for labeling proteins, antibodies or other molecules with primary amines (NH2-). The biotin provided in the kit is sufficient and has been activated for immediate use. Each kit contains 10 desalting spin columns, which can desalinate labeled proteins without dialysis. The speed is faster than dialysis and traditional gel filtration. The protein recovery and desalting efficiency are better than ultrafiltration. And the whole process is simple and fast. Subsequently, the HABA solution and streptavidin contained in the kit can be used to test the biotin labeling efficiency.

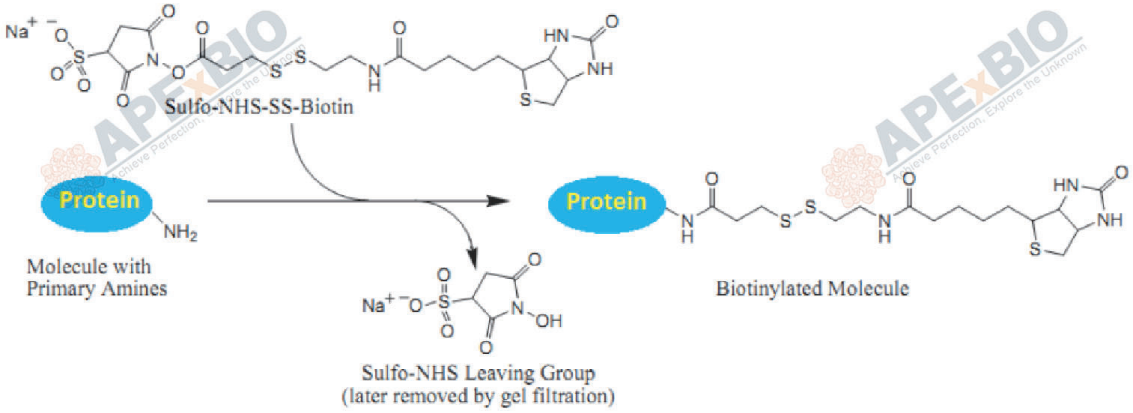

Figure 1. Reaction of Sulfo-NHS-SS-Biotin with primary amine.

2. Components

Sulfo-NHS-SS-Biotin	25 mg
Streptavidin	10 mg
HABA Solution	1 mL
PBS Pack (makes 1 L)	1 pack
Sephadex G-25 in PD-10 Desalting Columns	8.3 mL, 10 columns
Sufficient for: 10 labeling reactions, each with 1 to 10 mg of antibody	

3. Storage

Upon receipt store vials of Sulfo-NHS-SS-Biotin and Streptavidin at -20°C. Store remaining kit components kit at 4°C. Kit is shipped at room temperature. Store Sephadex G-25 in PD-10 Desalting Columns at 4°C to 30°C.

4. Important Product Information

- Sulfo-NHS-SS-Biotin is moisture-sensitive. Store the vial of biotin reagent and desiccant at -20°C. To avoid moisture condensation onto the product, fully equilibrate the reagent bottles to room temperature before opening.
- Prepare Biotin reagents according to the instructions before use. The NHS-ester moiety readily hydrolyzes and becomes inactive; so only a small amount of reagent can be weighed and dissolved at a time, and a storage solution cannot be prepared. Discard any unused reconstituted reagent.
- Avoid using primary amine-containing buffers (such as Tris or Glycine), as such buffers compete with the reaction. If necessary, protein samples can be exchanged for amine-free buffers by dialysis or concentration in PBS.
- Avoid reducing buffers during the labeling reaction to prevent the Sulfo-NHS-SS-Biotin disulfide bond from breaking.

5. Procedure for Biotin Proteins

5.1. Calculations

A) Calculate millimoles of Sulfo-NHS-SS-Biotin to add to the reaction for a 20-fold molar excess:

$$\text{mL protein} \times \frac{\text{mg protein}}{\text{mL protein}} \times \frac{\text{mmol protein}}{\text{mg protein}} \times \frac{20\ \text{mmol Biotin}}{\text{mmol protein}} = \text{mmol Biotin}$$

* 20 = Recommended molar fold excess of biotin per 1-10 mg/mL protein sample

B) Calculate microliters of 10 mM Sulfo-NHS-SS-Biotin (prepared in Step 5.2.c) to add to the reaction:

$$\text{mmol Biotin} \times \frac{607\ \text{mg}}{\text{mmol Biotin}} \times \frac{360\ \mu\text{L}}{2.2\ \text{mg}} = \mu\text{L Biotin Solution}\ ^{*}$$

* 607 = Molecular weight of Sulfo-NHS-SS-Biotin

* 360 = Microliters of water in which 2.2 mg of Sulfo-NHS-SS-Biotin is dissolved to make a 10 mM solution

C) For example：

For 1mL of a 2 mg/mL IgG (150000 MW) solution, 26.4 µL of 10 mM Sulfo-NHS-SS-Biotin will be added.

$$1\ \text{mL IgG} \times \frac{2\ \text{mg IgG}}{1\ \text{mL IgG}} \times \frac{1\ \text{mmol IgG}}{150000\ \text{mg IgG}} \times \frac{20\ \text{mmol Biotin}}{1\ \text{mmol IgG}} = 0.000266\ \text{mmol Biotin}$$

$$0.000266\ \text{mmol Biotin} \times \frac{607\ \text{mg}}{\text{mmol Biotin}} \times \frac{360\ \mu\text{L}}{2.2\ \text{mg}} = 26.4\ \mu\text{L Biotin Solution}$$

5.2. Biotin Labeling Reaction

A) Remove Sulfo-NHS-SS-Biotin from the freezer and allow it to equilibrate to room temperature.

B) Dissolve 1-10 mg of protein in 1.75-2.5 mL of phosphate buffered saline (PBS) and calculate the millimolar

amount of protein according to step 5.1.

*Note：Proteins that have been dissolved in amine-free buffers at pH 7.2-8.0 can be used without changing buffers. Proteins in Tris or other amine-containing buffers must be exchanged with PBS (either by dialysis or by using the desalting column included in the kit).

C) 2.2 mg Sulfo-NHS-SS-Biotin equilibrated to room temperature was dissolved in 360 µL of ultrapure water to prepare a 10 mM solution.

D) Add an appropriate amount of Sulfo-NHS-SS-Biotin solution to the protein solution (refer to the calculation in step 5.1).

E) Incubate reaction on ice for two hours or at room temperature for 30-60 minutes.

*Note：Although excess non-reacted and hydrolyzed biotin reagent remains in the solution, it is often possible to perform preliminary tests of the labeled protein by ELISA or Western blot. After confirming the function, perform buffer exchange using the desalting column through step 5.3 to obtain the best performance and stability. If a HABA assay is to be performed to determine the effect of biotin labeling, the protein must first be purified by buffer exchange.

5.3. Buffer Exchange and Remove Excess Biotin Reagent Using a Desalting Column

A) PD-10 Desalting column preparation. •Remove the top cap and pour off the column storage solution. •Remove the top filter using forceps. •Cut the sealed end of the column at notch. •Put the PD-10 Desalting column into a 50 mL collection tube by using the column adapter.

B) Column equilibration. •Fill up the column with equilibration buffer (PBS) and allow the equilibration buffer to enter the packed bed completely. •Repeat 3 times and discard the flow-through. •Fill up the column a fifth time with equilibration buffer and spin down at 1000 × g for 2 minutes. •Discard the flow-through. Note: About 25 mL equilibration buffer should be used in total for all three steps.

C) Sample application. •Add sample (1.75-2.5 mL) slowly in the middle of the packed bed.

D) Elution. •Place the PD-10 Desalting column into a new 50 mL collection tube. •Elute by centrifugation 1000 × g for 2 minutes. •Collect the eluate.

6. HABA Assay for Measuring the Level of Biotin Incorporation

6.1. Reagent Preparation

A) Phosphate-buffered Saline (PBS): 100 mM sodium phosphate, 150 mM sodium chloride; pH 7.2

B) HABA/Streptavidin Solution: Add 10mg of Streptavidin and 600 µL of 10 mM HABA to 19.4 mL of PBS. If prepared correctly, the A_{500} of this solution will be 0.9-1.3 in a 1 cm cuvette. The solution is stable for two weeks at 4°C. If a precipitate forms in the HABA solution, it can be filtered and then used

6.2. Procedure for Estimating Biotin Incorporation

A) Procedure Option 1 –Cuvette Format

a. Pipette 900 µL of HABA/Streptavidin solution into a 1 mL cuvette.
b. Measure the absorbance of the solution in the cuvette at 500 nm and record the value as "A_{500} HABA/Streptavidin".
c. Pipette 100 µL of the biotin-labeled protein sample into a cuvette containing HABA/streptavidin and mix thoroughly.
d. Measure the absorbance of the solution in the cuvette at 500 nm. Once the value remains constant for more than 15 seconds, record the value as "A_{500} HABA/Streptavidin/Biotin". If A_{500} HABA/Streptavidin/Biotin <0.3, dilute the biotinylated protein sample and repeat the assay, but remember to account for the dilution in during calculations.
e. Proceed to Step 6.3: Calculation of moles of Biotin per mole of protein.

B) Procedure Option 2 –Microplate Format

a. Pipette 180 µL of HABA/Streptavidin solution into a microplate well.
b. Measure the absorbance of the solution in the well at 500 nm and record the value as "A_{500} HABA/Streptavidin".
c. Add 20 µL of the biotin-labeled protein sample to the well containing HABA/streptavidin and mix thoroughly.
d. Measure the absorbance of the solution in the well at 500 nm. Once the value remains constant for more than 15 seconds, record the value as "A_{500} HABA/Streptavidin/Biotin".
e. Proceed to Step 6.3: Calculation of moles of Biotin per mole of protein.

6.3. Calculations for Moles of Biotin per Mole of Protein

A) These calculations are based on the Beer Lambert Law (Beer's Law): $\mathbf{A_\lambda = \varepsilon_\lambda bC}$

Where:

A is the absorbance of the sample at a particular wavelength (λ). The wavelength for the HABA assay is 500nm. There are no units for absorbance.

ε is the absorptivity or extinction coefficient at the wavelength (λ). For HABA/Streptavidin samples at 500 nm, pH 7.0 extinction coefficient is equal to 34000 $M^{-1}cm^{-1}$.

b is the cell path length expressed in centimeters (cm). A 10 mm square cuvette has a path length of 1.0 cm. Using the recommended microplate format volumes, the path length is typically 0.5 cm.

C is the concentration of the sample expressed in molarity (= mol/L = mmol/mL).

B) The values needed for calculating the number of moles of biotin per mole of protein or sample are as follows:

a. Concentration of the protein or sample used, expressed as mg/mL
b. Molecular weight (MW) of the protein, expressed as grams per mole (e.g., HRP = 40000; IgG =150000)
c. Absorbance at 500 nm for HABA/Streptavidin Solution (A_{500} H\S)

d. Absorbance at 500 nm for HABA/Streptavidin/Biotin reaction mixture (A_{500} H\S\B)

e. Dilution factor, if the sample is diluted before adding it to the HABA/Streptavidin Solution

C) Calculations for Moles of Biotin per Mole of Protein

a. Calculation #1 is for the concentration of biotinylated protein in mmol/mL (before any dilution for the assay procedure):

$$\text{mmol protein/mL} = \frac{\text{concentrationprotein (mg/mL)}}{\text{MWof protein (mg/mmol)}} = \text{Calc\#1}$$

b. Calculation #2 is for the change in absorbance at 500 nm:

$$\text{Cuvette: } \Delta A_{500} = (0.9^* \times A_{500}\ \text{H\S}) - (A_{500}\ \text{H\S\B}) = \text{Calc\#2}$$

$$\text{Microplate: } \Delta A_{500} = (A_{500}\ \text{H\S}) - (A_{500}\ \text{H\S\B}) = \text{Calc\#2}$$

*Note: The cuvette format requires the 0.9 correction factor to adjust for dilution of the H\S Solution by the biotinylated protein sample.

c. Calculation #3 is for the concentration of biotin in mmol per mL of reaction mixture:

$$\frac{\text{mmol Biotin}}{\text{mL reaction mixture}} = \frac{\Delta A500}{(34000 \times \text{b})} = \frac{\text{Calc\#2}}{(34000 \times \text{b})} = \text{Calc\#3}^*$$

* Note: b is the light path length (cm) of the sample. Use b = 1 with the cuvette format. Use b = 0.5 with the microplate format when using a standard 96-well plate and the volumes specified in the procedure.

d. Calculation #4 is for the mmol of biotin per mmol of protein:

$$= \frac{\text{mmol Biotin in original sample}}{\text{mmol protein in original sample}} =$$

$$\frac{(\text{mmol per mL Biotin in reaction mixture})\ (10)\ (\text{dilution factor})}{\text{mmol per mL protein in original sample}} = \frac{(\text{Calc\#3}) \times 10 \times \text{dilution factor}}{\text{Calc\#1}}$$

*Note: The original biotinylated protein sample was diluted 10-fold in the reaction mixture. Therefore, a multiplier of 10 is used in this step to convert the biotin concentration in the reaction mixture to the biotin concentration in the original sample. Calculation #4 yields the biotin: protein molar ratio (average #4 of biotin molecules per protein molecule).

D) Example HABA Assay calculation: In this example, the labeled protein is IgG (MW 150000) at 0.69 mg/mL. The absorbance measurements were A_{500} H\S = 0.904 and A_{500} H\S\B = 0.771

#1: $$\text{mmol protein/mL} = \frac{0.69\ \text{mg/mL}}{150000\ \text{mg/mmol}} = 4.6 \times 10^{-6}$$

#2: $\Delta A_{500} = (0.9 \times 0.904) - 0.771 = 0.0426$

#3: $$\frac{\text{mmol Biotin}}{\text{mL reaction mixture}} = \frac{0.0426}{(34000 \times 1)} = 1.25 \times 10^{-6}$$

#4: $$\frac{\text{mmol Biotin}}{\text{mmol protein}} = \frac{(1.25 \times 10-6) \times (10)}{4.6 \times 10-6} = \frac{12.5 \times 10-6}{4.6 \times 10-6} = 2.72 \text{ Biotin molecules per IgG molecule}$$

7. Troubleshooting the HABA Assay

Problem	Cause	Solution
ΔA_{500} in HABA assay is ≤ 0	The protein sample has no or a low level of biotinylation because of limited accessible functional groups on the protein.	Repeat biotinylation with alternative chemistry (e.g., sulfhydryl-reactive) or use a higher molar ratio of biotinylation reagent.
	Incomplete mixing of reagent.	Completely solubilize and mix HABA/Streptavidin before diluting.
	Particles in sample contribute to absorbance.	Filter protein sample to remove particles.
High levels of biotin	Nonreacted biotin was not removed.	Dialyze or desalt sample before performing the assay.

8. References

[1]. Altin, J.G., et al. (1995). A one-step procedure for biotinylation and chemical crosslinking of lymphocyte surface and intracellular membrane-associated molecules. Anal Biochem 224:382-9.

[2]. Chaiet, I. and Wolf, F.J. (1964). The properties of streptavidin, a biotin-binding protein produced by *streptomycetes*. Arch Biochem Biophys 106:1-5.

[3]. Gitlin, G., et al. (1987). Studies of the biotin-binding site of avidin. Biochem J 242:923-6.

[4]. Gretch, D.R., et al. (1987). The use of biotinylated monoclonal antibody and streptavidin affinity chromatography to isolate herpes virus hydrophobic proteins or glycoproteins. Anal Biochem 163:270-7.

[5]. Hnatowich, D.J., et al. (1987). Investigations of avidin and biotin for imaging applications. J Nucl Med 28:1294-302.

[6]. Shimkus, M., et al. (1985). A chemically cleavable biotinylated nucleotide: Usefulness in the recovery of protein-DNA complexes from avidin affinity columns. Proc Natl Acad Sci82:2593-7.