UV-Tracer™ Biotin-NHS Labeling Kit
Labeling of primary Amine-groups with UV-detectable Biotin

<table>
<thead>
<tr>
<th>Cat. No.</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLK-B104-K</td>
<td>3 labeling reactions</td>
</tr>
</tbody>
</table>

For in vitro use only!
Quality guaranteed for 12 months
Store at -20 °C

Kit contents
UV-Tracer™ NHS (C\textsubscript{52}H\textsubscript{76}N\textsubscript{6}O\textsubscript{17}S; MW: 1089.25 g/mol): 3 x 4 mg
Anhydrous DMSO: 1 x 10 ml
BupH Saline Buffer Pack (BupH™ registered trademark ThermoScientific): 1 packet
Zeba™ Spin Columns (Zeba™ registered trademark ThermoScientific): 6 x 0.5 ml

To be provided by you
UV-VIS Spectrophotometer
Table Top Centrifuge (e.g. Eppendorf 5810)
1 l beaker stir bar with magnetic stir bar
15 ml conical tubes
Ultrapure water (e.g. 18 MΩ-cm)
6 N NaOH

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1. Description
The UV-Tracer™ Biotin-NHS Labeling Kit provides all necessary reagents for 3 biotinylation reactions of primary amine (-NH\textsubscript{2})-containing proteins e.g. IgGs (50-500 µg in 500 µl).

It is based on an advanced biotin labeling reagent -UV-Tracer™ Biotin NHS - with a unique linker design that facilitates biotinylation and rapid quantification of the exact numbers of incorporated biotin molecules in a one-step procedure.

Figure 1 Chemical structure of UV-Tracer™ Biotin NHS. Red: UV-traceable benzophenone moiety.

The linker contains a UV-traceable benzophenone moiety (Abs.: 350 nm) surrounded by two hydrophilic PEG4 spacer arms (Fig. 1). The two hydrophilic PEG4 spacer arms enhance water solubility thereby reducing the tendency of some proteins to aggregate during biotinylation. The traceable chromophore permits rapid quantification of incorporated biotin molecules by a simple absorbance measurement at 280 nm and 350 nm. The resulting absorbance values are subsequently used to calculate the final protein concentration and the exact number of incorporated biotin molecules (Fig. 2).

Figure 2 UV scan (230-450 nm) of Goat IgG (unlabeled control) [A], and UV-Tracer™ Biotin NHS labeled Goat IgG (B). Samples were scanned (1:9 dilution, c=4 mg/ml) in BupH (pH 7.5). The degree of labeling was determined to be 9.6 biotins/goat IgG.
2. Protein requirements

a) This kit requires 50-500 µg protein in a fixed volume of 100 µl (e.g. 0.5-5 mg/ml).

b) The molecular weight needs to be known (e.g. 20-200 kDa).

c) Free primary amino groups are required (e.g. lysine or N-terminus of protein).

d) The protein preparation needs to be highly purified and has to be free of exogenous primary amines (e.g. glycine or Tris) prior to labeling with NHS esters. If they are present, these compounds must be removed (see 4.2b).

e) The provided buffer exchange columns are designed to process 50-500 µg of protein in a volume of 100 ± 10 µl.

3. Other important considerations

a) Use the UV-Tracer™ Biotin-NHS reagent immediately after reconstitution in DMSO. NHS-esters readily hydrolyze and become non-reactive. Use only freshly prepared reagent and discard any unused reconstituted reagent.

b) In every labeling reaction, the simplified protocol uses a fixed volume (5 µl) of UV Tracer™ Biotin NHS solution to label a fixed volume of protein (~ 100 µl). Consequently, the volume of anhydrous DMSO required to dissolve the UV Tracer™ Biotin NHS varies for each labeling reaction (see Appendix A, Part II for the calculation of the DMSO volume).

c) For maximum reproducibility, it is preferable to buffer exchange proteins into BupH Buffer (pH 7.5) with the spin columns provided prior to biotinylation. This simple procedure guarantees maximum consistency during the labeling reaction.

4. Biotinylation experiment

4.1 Preparation of BupH Buffer (pH 7.5)

a) Dissolve the dry-blend BupH buffer pack provided with 450 ml ultrapure water.

b) Adjust the pH of the solution to 7.5 ± 0.05 by drop wise addition of 6 N NaOH.

c) Adjust the final volume to 500 ml (ultrapure water).

Please note: For long-term storage sterile-filter the solution. Do not add sodium azide or proclin preservatives as these reagents interfere with protein A280 measurements.

4.2 Preparation of protein

a) If the protein is lyophilized (50-500 µg), pure and free of primary amines resuspend in 100 µl BupH buffer (pH 7.5) to obtain a 0.5-5 mg/ml solution. Then proceed to the Biotin labeling reaction (4.5).

b) If the protein is lyophilized and known to contain primary amines (e.g. Tris, Glycine) resuspend in 100 µl BupH (pH 7.5) and then proceed with buffer exchange (4.3 and 4.4). Proceed afterwards to the Biotin labeling reaction (4.5).

c) If the protein (500-5000 µg) is supplied in 100 µl of a suitable primary amine-free buffer at a concentration range from 0.5-5 mg/ml, proceed to the Biotin labeling step (4.5).

d) If the protein is supplied in a buffer known to contain free primary amines (e.g. Tris, Glycine) proceed with buffer exchange (4.3 and 4.4). Proceed afterwards to the Biotin labeling reaction step (4.5).

4.3 Equilibration of Spin Column

a) Twist off the column’s bottom closure and loosen the cap. Place each column into a clean 15 ml conical tube.

b) Centrifuge the column at 1,500 x g for 2 minutes to remove storage solution. Place a pen mark on the side of the column where the compacted resin is slanted upward. Place the column in the centrifuge with the mark facing away from the center of the rotor in all subsequent centrifugation steps.
Please note: The resin will appear white in color and compacted after centrifugation.

Please note: Concentrated protein solutions (e.g., 5 mg/ml) will require dilution prior to absorbance measurements. A micro-volume spectrophotometer can be used on small aliquots (1-2 μl) without dilution (e.g., Nanodrop®).

**4.4 Buffer exchange of protein**

a) Slowly apply 100 μl protein solution to the center of the equilibrated spin column (4.3).
b) Centrifuge at 1,500 x g for 2 minutes.
c) The eluate at the bottom of the 15 ml collection tube contains the buffer exchanged protein. Discard the used spin column.

d) Buffer exchange the biotinylated protein as described in section 4.4 thereby removing excess (= unbound) UV-Tracer™ Biotin NHS. You may use the buffer of your choice instead of BupH buffer, pH 7.5.

**5. Calculation of Labeling degree (DOL) and protein concentration**

a) Measure the conjugate absorbance at 280 nm and 350 nm with a UV-VIS-Spectrophotometer.

Please note: The resin will appear white in color and compacted after centrifugation.

c) Add 0.3 ml BupH buffer (pH 7.5) to the top of each spin column and remove the cap.
d) Centrifuge at 1500 x g for 2 minutes to remove buffer.
e) Repeat steps 3 and 4 two additional times, discarding buffer from the collection tube after each spin.
f) Transfer the equilibrated spin column (resin appears white and dry) into a clean 15 ml conical tube and proceed immediately with the buffer exchange of the protein.

d) Prepare a buffer exchange spin column as described in section 4.3.
e) Buffer exchange the biotinylated protein as described in section 4.4 thereby removing excess (= unbound) UV-Tracer™ Biotin NHS. You may use the buffer of your choice instead of BupH buffer, pH 7.5.

b) Add the required volume of DMSO to dissolve the required amount of UV-Tracer™ Biotin NHS (refer to Appendix A, Part II).
c) Add 5 μl UV-Tracer™ Biotin NHS solution to the reduced protein solution (~ 100 μl) and pipette the mixture several times up and down.
e) Incubate the reaction mixture for 1 hour at room temperature.
6. Troubleshooting

Problem 1: Poor or lower biotinylation efficiency than expected

<table>
<thead>
<tr>
<th>Possible Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incorrect protein concentration and/or possible contaminants in protein sample.</td>
<td>Buffer exchange the protein into BupH buffer (pH 7.5) using spin columns provided and confirm the concentration of the protein prior to labeling.</td>
</tr>
<tr>
<td>NHS-ester hydrolyzed</td>
<td>Store UV-Tracer™ Biotin NHS reagent at -20 °C. Allow product to equilibrate to room temperature before opening.</td>
</tr>
<tr>
<td>Protein has few or no available primary amino groups</td>
<td>Check primary structure of protein for the presence of lysine residues on NCBI protein database.</td>
</tr>
<tr>
<td>Low A350 absorbance of the biotinylated conjugate</td>
<td>Check spectrophotometer lamp for proper functioning.</td>
</tr>
</tbody>
</table>

Problem 2: Low conjugate yield

<table>
<thead>
<tr>
<th>Possible Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein may have aggregated/precipitated during biotinylation</td>
<td>Lower the amount of labeling reagent during the labeling reaction. Use 10% or lower volume of DMSO solvent during labeling reaction. Although rare, some proteins become unstable on biotinylation and cannot be labeled.</td>
</tr>
</tbody>
</table>

Appendix A

Part I

Determination of the required UV-Tracer™ Biotin NHS amount

a) Select the desired molar excess of UV-Tracer™ Biotin NHS (refer to Table 1 as a reference for the selection process). Typical labeling reactions use 10x to 20x reagent molar excess depending on the initial protein concentration and the number of available primary amine groups.

Please note: Over modification of antibodies or other proteins with biotin can affect their function and stability.

Table 1 Typical labeling efficiencies achieved for goat IgG.
(DOL: Degree of Labeling)

<table>
<thead>
<tr>
<th>Goat IgG (150 kDa)</th>
<th>Molar Equivalents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5x</td>
</tr>
<tr>
<td>(mg/mL)</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>1.1</td>
</tr>
<tr>
<td>1</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
</tr>
<tr>
<td>4</td>
<td>3.1</td>
</tr>
</tbody>
</table>

b) Calculate millimole (mmol) UV-Tracer™ Biotin-NHS (M=1089.25 g/mol) required to label a protein with a desired molar excess:

\[
\text{mmol UV} = \frac{N_b \times C_p \times V_p}{MW_p} 
\]

Where:
- \( N_b \): molecular excess of UV-Tracer™ Biotin NHS
- \( C_p \): protein concentration [mg/ml]
- \( V_p \): volume of protein sample [ml] (fixed: 0.1 ml)
- \( MW_p \): molecular weight of protein [Dalton]

Example:

0.0000133 mmol of UV-Tracer™ Biotin NHS are required to label 0.1 ml of a 1 mg/ml goat IgG solution (MW_p=150 kDa) with a 20-fold molar excess.
Part II

Determination of the DMSO volume (µl) required to dissolve a desired amount of UV-Tracer™ Biotin NHS (Part I)

a) Calculate microliters anhydrous DMSO required to dissolve UV-Tracer™ Biotin NHS reagent:

\[
\mu L \text{ DMSO} = \frac{\text{mmol UV-Tracer biotin (vial)} \times V_{\text{UV-Tracer}}}{\text{mmol UV-Tracer biotin (Part I)}}
\]

\[
= \frac{0.0184}{\text{mmol UV-Tracer biotin}}
\]

mmol UV-Tracer™ Biotin (vial) = 0.00367 mmol (=4 mg)

V_{UV-Tracer} : Required volume of UV-Tracer™ solution = 5 µl

**Example:**

Dissolve 1 vial of UV-Tracer™ Biotin NHS with 1383.5 µl anhydrous DMSO and mix 5 µl of this solution with the protein to achieve a 20x molar excess of UV-Tracer™ Biotin NHS in the final solution (4.6).

**Please note:** Each vial of UV-Tracer™ Biotin NHS can accommodate 1,600 µl DMSO. If the required volume of DMSO is greater than this nominal volume, transfer the dissolved reagent to a larger vial and add DMSO to achieve the required volume.

Appendix B

Part I

a) Calculation of the Degree of Labeling (DOL) according to the law of Lambert-Beer:

(1) \( c_{\text{Biotin}} [\mu M] = \frac{A_{350}}{e_{350} \times d} \)

(2) \( c_{\text{Protein}} [\mu M] = \frac{A_{280C}}{e_{280} \times d} \)

(3) \( \text{DOL} = \frac{c_{\text{Biotin}}}{c_{\text{Protein}}} \)

d = layer thickness of cuvette (e.g. 1 cm)

\( A_{350} = \text{UV-Tracer™ Biotin absorbance at 350 nm} \)

\( e_{350} = \text{molar extinction coefficient of UV-Tracer™ Biotin} = 19474 \text{ M}^{-1} \text{cm}^{-1} \)

\( A_{280} = \text{protein conjugate absorbance at 280 nm} \)

\( A_{280C} = \text{corrected protein conjugate absorbance at 280 nm} = A_{280} - (A_{350} \times 0.4475) \)

\( e_{280} = \text{molar extinction coefficient of protein} \)

b) Calculate conjugate protein concentration (mg/ml)

(4) \( c_{\text{Protein}} \text{ [mg/ml]} = \frac{c_{\text{Protein}} [\mu M]}{MW_p} \times \text{dilution factor} \)

\( c_{\text{Protein}} [\mu M] = \text{determined with Eq. (2)} \)

\( MW_p = \text{molecular weight of protein (Dalton)} \)

**Example:**

A Goat IgG antibody (MW_p = 150 kDa) was labeled using a 20x molar excess of UV-Tracer™ Biotin NHS. The conjugates absorbance at 280 nm and 350 nm were determined with a quartz cuvette (d = 1 cm):

\( A_{280} = 1.48 \text{ (no dilution)} \)

\( A_{350} = 0.92 \text{ (no dilution)} \)

\( e_{280} \text{ (IgG)} = 204000 \text{ M}^{-1} \text{cm}^{-1} \)

Results according to (1) – (4):

(1) \( c_{\text{Biotin}} = 47.24 \mu M \)

(2) \( c_{\text{Protein}} = 5.24 \mu M \)

(3) \( \text{DOL} = 9.02 \)

(4) \( c_{\text{Protein}} = 0.78 \text{ mg/ml} \)