Introduction
SNAP-Biotin is a cell-permeable substrate (BG-Biotin) based on biotin with an amidocaproyl linker. It is suitable for applications such as biotinylation of SNAP-tag® fusion proteins in living cells for detection with streptavidin fluorophore conjugates or labeling in solution for analysis by SDS-PAGE/Western blot or for capture with streptavidin for binding and interaction studies. This package contains 50 nmol of SNAP-Biotin substrate, sufficient to make 10 ml of a 5 μM solution for the labeling of SNAP-tag fusion proteins in cells.

The SNAP-tag is a novel tool for protein research, allowing the specific, covalent attachment of virtually any molecule to a protein of interest. The SNAP-tag is based on mammalian O-alkylguanine-DNA-alkyltransferase (AGT). SNAP-tag substrates are derivatives of benzylpurines and benzylchloropyrimidines. In the labeling reaction, the substituted benzyl group of the substrate becomes covalently attached to the SNAP-tag.

There are two steps to using this system: subcloning and expression of the protein of interest as a SNAP-tag fusion, and labeling of the fusion with the SNAP-tag substrate of choice. Expression of SNAP-tag fusion proteins is described in the documentation supplied with SNAP-tag plasmids. The labeling of fusion proteins with the SNAP-Biotin substrate is described below.

Materials required but not supplied:
- Cells expressing SNAP-tag proteins
- Tissue culture materials and media
- Transfection reagents
- DMSO

Protocol for Labeling Reaction:
1. Dilute the labeling stock solution 1:200 in medium to yield a labeling medium of 5 μM SNAP-Biotin substrate. Mix substrate with medium thoroughly by pipetting up and down 10 times (necessary for reducing backgrounds). For best performance, add the SNAP-tag substrate to complete medium, including serum (0.5% BSA can be used for experiments carried out in serum-free media). Do not prepare more medium with SNAP-tag substrate than you will consume within one hour.

2. Replace the medium on the cells expressing a SNAP-tag fusion protein with the SNAP-tag labeling medium and incubate at 37°C, 5% CO₂ for 30 minutes.

3. Wash the cells three times with tissue culture medium with serum and incubate in fresh medium for 30 minutes. Replace the medium one more time to remove unreacted SNAP-tag substrate that has diffused out of the cells.

   We recommend routinely labeling one well of non-transfected or mock-transfected cells as a negative control.

Microscopy
Fixation: After labeling the SNAP-tag fusion proteins with SNAP-Biotin, the cells can be fixed with 3.3% para-formaldehyde which does not result in the loss of signal. Avoid fixation using ethanol as this may lead to a high background staining of endogenous biotinylated proteins found preferentially in mitochondria.

Detection: To visualize the SNAP-tag fusion protein in situ, permeabilize the cells with 0.5% Triton in PBS and block the cells with 1% BSA in PBS containing 0.5% Triton. Incubate the fixed cells with an appropriate streptavidin/avidin conjugate (e.g. streptavidin-fluorophore) and image the cells according to the instructions supplied with the conjugate.
Troubleshooting for Cellular Labeling

No Labeling
If no labeling is seen, the most likely explanation is that the fusion protein is not expressed. Verify your transfection method to confirm that the cells contain the fusion gene of interest. If this is confirmed, check for expression of the SNAP-tag fusion protein. If no antibody against the fusion partner is available, Anti-SNAP-tag Antibody (NEB #P9310) can be used. Alternatively, SNAP-Vista Green (NEB #S9147) can be used to confirm presence of SNAP-tag fusion in cell extracts following SDS-PAGE, without the need for Western blotting.

Weak Labeling
Weak labeling may be caused by insufficient exposure of the fusion protein to the substrate. Try increasing the concentration of SNAP-tag substrate and/or the incubation time. Improving the protein expression may also improve the signal. If the protein has limited stability in the cell, it may help to analyze the samples immediately after labeling.

Instructions for Labeling of Proteins in vitro

1. Dissolve the vial of SNAP-Biotin (50 nmol) in 50 μl of fresh DMSO to yield a labeling stock solution of 1 mM SNAP-tag substrate. Mix by vortexing for 10 minutes until all the SNAP-tag substrate is dissolved. Dilute this 1 mM stock solution 1:4 in fresh DMSO to yield a 250 μM stock for labeling proteins in vitro.

2. Set up the reactions, in order, as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate Buffered Saline (PBS)</td>
<td>42 μl</td>
<td>1X</td>
</tr>
<tr>
<td>50 mM DTT</td>
<td>1 μl</td>
<td>1 mM</td>
</tr>
<tr>
<td>50 μM SNAP-tag Purified Protein</td>
<td>5 μl</td>
<td>5 μM</td>
</tr>
<tr>
<td>250 μM SNAP-tag Substrate</td>
<td>2 μl</td>
<td>10 μM</td>
</tr>
<tr>
<td>Total Volume</td>
<td>50 μl</td>
<td></td>
</tr>
</tbody>
</table>

3. Incubate in the dark for 30 minutes at 37°C.

4. Run sample on an SDS-PAGE gel and detect using standard streptavidin-based detection reagents or store samples at −20°C or −80°C in the dark.

Removal of Unreacted Substrate (optional)
After the labeling reaction, the unreacted substrate can be separated from the labeled SNAP-tag fusion protein by gel filtration or dialysis. Please refer to the vendor’s instructions for the separation tools you are using.

Notes for Labeling in vitro
We recommend the routine addition of 1 mM DTT to all buffers used for handling, labeling and storage of the SNAP-tag. The stability of the SNAP-tag is improved in the presence of reducing agents; however it can also be labeled in their absence, if handling at temperatures above 4°C is minimized.

SNAP-tag fusion proteins can be purified before labeling, but the labeling reaction also works in non-purified protein solutions (including cell lysates).

Confimation of Labeling by Western blot Analysis
Labeled SNAP-tag fusion proteins can be easily analyzed on a SDS-PAGE gel/Western blot because the covalently bound label will remain attached to the protein. The biotin label can be detected on an SDS-PAGE gel followed by Western blot using a Horseradish Peroxidase or Alkaline Phosphatase labeled avidin/streptavidin (e.g. streptavidin-HRP) and the corresponding detection method as described by the supplier of the enzyme conjugate.

Troubleshooting for Labeling in vitro

Solubility
If solubility problems occur with your SNAP-tag fusion protein, we recommend testing a range of pH (pH 5.0–pH 10.0) and ionic strengths. The salt concentration may also need to be optimized for your particular fusion protein (50–250 mM).

Loss of Protein Due to Aggregation or Sticking to Tube
If stickiness of the fusion protein is a problem, we recommend adding Tween 20 at a final concentration of 0.05% to 0.1%. The SNAP-tag activity is not affected by this concentration of Tween 20.

Incomplete Labeling
If exhaustive labeling of a protein sample is not achieved using the recommended conditions, try the following protocol modifications: Increase the incubation time to two hours total at 25°C or to 24 hours at 4°C; or halve the volume of protein solution labeled. Both approaches may be combined. If you still have poor labeling results, we recommend checking the activity of the SNAP-tag using SNAP-Vista Green.

If the SNAP-tag fusion has been stored in the absence of DTT or other reducing agent, or has been stored at 4°C for a prolonged period, its activity may be compromised. Include 1 mM DTT in all solutions of the SNAP-tag fusion protein, and store the fusion protein at −20°C.

Using less than the recommended amount of substrate stock solution can significantly slow down the reaction rate.

Loss of Activity of Protein of Interest
If your fusion protein is particularly sensitive to degradation or to loss of activity, you can try reducing the labeling time or decreasing the labeling temperature. If you label at 4°C we recommend overnight incubation.