SNAP-Cell Fluoroescein

Figure 1. Structure of SNAP-Cell Fluorescein (MW 712.7 g/mol)

Figure 2. Excitation (dotted line) and emission spectra of SNAP-Cell Fluorescein (fluorescent product of SNAP-Cell Fluorescein in cells) coupled to SNAP-tag in buffer at pH 7.5

Introduction
SNAP-Cell Fluorescein is a green fluorescent substrate that can be used to label SNAP-tag fusion proteins inside living cells or in vitro. This cell-permeable substrate (BG-Fluorescein) is based on diacytfluorescein and is suitable for standard fluorescein filter sets. Diacyetyfluorescein is essentially non-fluorescent, but it becomes fluorescent inside the cell when it is hydrolyzed by non-specific esterases, yielding fluorescein. It has an excitation maximum at 500 nm and an emission maximum at 532 nm. This substrate has limited photostability. If this presents a problem, we recommend using SNAP-Cell 505-Star, which has similar spectral characteristics but much greater photostability. This package contains 50 nmol of SNAP-Cell Fluorescein substrate, sufficient to make 10 ml of a 5 µM SNAP-tag fusion protein labeling solution.

SNAP-Cell Fluorescein substrate is soluble up to at least 10 mM. The substrate should be stable for at least three years dry or 3 months moisture. With proper storage at –20°C the substrate is stable for at least three years dry or 3 months moisture. With proper storage at –20°C the substrate is stored at –20°C.

Materials Required but not Supplied:
- Cells expressing SNAP-tag fusion proteins
- Tissue culture materials and media
- Transfection reagents
- Fluorescence microscope with suitable filter set
- DMSO

Storage
SNAP-Cell Fluorescein should be stored at –20°C (long term) or at 4°C in the dark (short term, less than 4 weeks). Protect the substrate from light and moisture. With proper storage at –20°C the substrate should be stable for at least three years dry or 3 months dissolved in DMSO.

Materials
- SNAP-Cell Fluorescein
- Transfection reagents
- Tissue culture materials and media
- Fluorescence microscope with suitable filter set
- DMSO

SNAP-Tag Fusion Proteins
SNAP-tag fusion proteins are the result of the expression of SNAP-tag fusion genes. The expression of SNAP-tag fusion proteins is described in detail in the documentation supplied with SNAP-tag plasmids. The labeling of fusion proteins with the SNAP-tag substrate is described below.

Optimizing Labeling
Optimal substrate concentrations and reaction times range from 1–10 µM and 15–60 minutes, respectively, depending on experimental conditions. 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 pre-stay more medium with SNAP-tag substrate than you will consume within one hour.

Quality Controls
Purity and Characterization: Purity of SNAP-Cell Fluorescein was determined to be 71% by HPLC analysis. Molecular weight [M+H]+ was determined by MS to be 713.2 (713.2 expected).

In vitro protein labeling: Reaction of SNAP-Cell Fluorescein (10 µM) with purified SNAP-tag protein (5 µM) in vitro for 30 minutes at 37°C, followed by mass spec analysis, indicated an efficiency of labeling of 95%.

Cellular Protein Labeling: Cells transiently transfected with SNAP-tag vectors expressing Histone H2B (intracellular) were labeled with 5 µM SNAP-Cell Fluorescein for 30 minutes, and visualized by fluorescent microscopy. The intracellular target was efficiently labeled.

Instructions for Cellular Labeling
SNAP-tag fusion proteins can be expressed by transient or by stable transfection. For expression of fusion proteins with the SNAP-tag refer to instructions supplied with the SNAP-tag plasmids. For cell culture and transfection methods, refer to established protocols.

1. Dilute the labeling stock solution 1:200 in 50 µl of DMSO to give a solution of 1 mM SNAP-tag substrate. Mix by vortexing for 10 minutes until all the SNAP-tag substrate is dissolved. Store this stock solution in the dark at 4°C, or for extended storage at –20°C. Different stock concentrations can be made, depending on your requirements. The substrate is soluble up to at least 10 mM.

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

3. Wash the cells three times with tissue culture medium with serum and incubate them 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.

4. Image the cells using an appropriate filter set. SNAP-tag fusion proteins labeled with SNAP-Cell Fluorescein should have an excitation maximum at 500 nm and an emission maximum at 532 nm, and can be imaged with standard fluorescein filter sets.

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

Notes
Blocking Unreacted SNAP-tag with SNAP-Cell Block
In many cases the labeling of a non-transfected cell sample or a mock-transfected cell sample will be completely sufficient as a control. In some cases, however, it may be desirable to block the SNAP-tag activity in a cell sample expressing the SNAP-tag fusion protein to generate a control. This can be achieved using a nonfluorescent SNAP-Cell Block (bromothenylpteridine, BTP). SNAP-Cell Block may also be used in pulse-chase experiments to block the SNAP-tag reactivity during the chase between two pulse-labeling steps. A protocol for blocking is included with SNAP-Cell Block (NEB #S9106).

Optimizing Labeling
Optimal substrate concentrations and reaction times range from 1–10 µM and 15–60 minutes, respectively, depending on experimental conditions.
conditions and expression levels of the SNAP-tag fusion protein. Best results are usually obtained at concentrations between 1 and 5 µM substrate and 30 minutes reaction time. Increasing substrate concentration and reaction time usually results in a higher background and does not necessarily increase the signal to background ratio.

**Stability of Signal**
The turnover rates of the SNAP-tag fusion protein under investigation may vary widely depending on the fusion partner. We have seen half-life values ranging from less than one hour to more than 12 hours. Where protein turnover is rapid, we recommend analyzing the cells under the microscope immediately after the labeling reaction or, if the application allows it, fixing the cells directly after labeling.

**Fixation of Cells**
After labeling the SNAP-tag fusion proteins, the cells can be fixed with standard fixation methods such as para-formaldehyde, ethanol, methanol, methanol/aceton etc., without loss of signal. We are not aware of any incompatibility of the SNAP-tag label with any fixation method.

**Counterstaining**
Cells can be counterstained with any live-cell dye that is compatible with the fluorescent properties of the SNAP-tag substrate for simultaneous microscopic detection. We routinely add 5 µM Hoechst 33342 to the medium prior to the first wash step (Step 3) as a DNA counterstain for nuclear visualization and leave this on the cells for 2 minutes prior to completing the wash steps. Counterstaining of cells is also possible after fixation and permeabilization.

**Immunocytochemistry**
Antibody labeling can be performed after SNAP-tag labeling and fixation of the cells according to standard protocols without loss of the SNAP-tag signal. The fixation conditions should be selected based on the protein of interest. For example some fixation methods destroy epitopes of certain proteins and therefore do not allow antibody staining afterwards.

**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, following the guidelines described above. Alternatively the protein may be poorly expressed and/or turn over rapidly. If the protein has limited stability in the cell, it may help to analyze the samples immediately after labeling.

**High Background**
Background fluorescence may be controlled by reducing the concentration of SNAP-tag substrate used, and by shortening the incubation time. The presence of fetal calf serum or BSA during the labeling incubation should reduce non-specific binding of substrate to surfaces.

**Signal Strongly Reduced After Short Time**
Fluorescein has only limited photostability. Plan your experimental protocol accordingly. Minimize the cells’ exposure to light during and after labeling and to the excitation light. If you experience problems with photobleaching when working with labeled fixed cells, addition of a commercially available anti-fade reagent may be helpful.

If the fluorescence signal decreases rapidly, it could also be due to instability of the fusion protein. The signal may be stabilized by fixing the cells. Alternatively try switching the SNAP-tag from the N- to the C-terminus or vice versa.

**Instructions for Labeling of Proteins in vitro**

1. Dissolve the vial of SNAP-Cell Fluorescein (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>Final 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><strong>Total Volume</strong></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 a fluorescent gel scanner 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).

**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.