

CELLULAR IMAGING & ANALYSIS

SNAP-Cell[®] Starter Kit

Instruction Manual

NEB #E9100S
10 reactions

 NEW ENGLAND
BioLabs[®] Inc.
enabling technologies in the life sciences



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Kit Components:

All kit components should be stored at -20°C except where noted.

pSNAP _f Vector	20 µg
Supplied in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), 0.5 µg/µl	
pSNAP _f -Cox8A Control Plasmid	20 µg
Supplied in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), 0.5 µg/µl	
SNAP-Cell 505-Star	10 nmol
Supplied dry. Prepare a stock solution in DMSO as described on page 10.	
SNAP-Cell TMR-Star	6 nmol
Supplied dry. Prepare a stock solution in DMSO as described on page 10.	
SNAP-Cell Block	20 nmol
Supplied dry. Prepare a stock solution in DMSO as described on page 13.	

Note: For long-term storage, all kit components should be stored at -20°C. Plasmid solutions can be stored at 4°C for up to one week. Undissolved dye and blocking substrates can be stored at 4°C for up to 4 weeks protected from light and moisture. With proper storage at -20°C the substrates should be stable for at least three years dry or 3 months dissolved in DMSO.

Required Materials Not Included

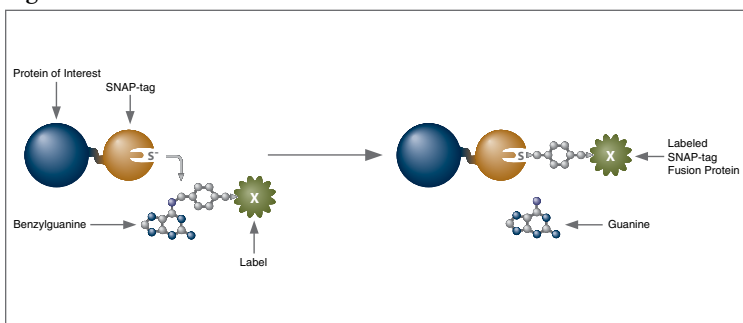
- Mammalian Cell Lines
- DNA Transfection Reagents
- Standard Tissue Culture Media and Plasticware
- DMSO
- Hoechst 33342 for Nuclear Staining (optional)

Introduction:

The SNAP-tag[®] is a novel tool for the specific, covalent attachment of virtually any molecule to a protein of interest, providing simplicity and extraordinary versatility to the imaging of proteins in live and fixed cells, and to the study of proteins *in vitro*. The creation of a single gene construct yields a tagged fusion protein capable of forming a covalent linkage to a variety of functional groups, including fluorophores, biotin, or beads. This system provides a powerful and unique tool to study the role of proteins in a variety of highly dynamic processes, including protein trafficking, turnover, and complex formation.

The SNAP-tag is a 20 kDa mutant of the human DNA repair protein O⁶-alkylguanine-DNA alkyltransferase (hAGT) that reacts specifically and rapidly with benzylguanine (BG) and benzylchloropyrimidine (CP) derivatives, leading to covalent labeling of the SNAP-tag with a synthetic probe (Figure 1). The SNAP-tag has a number of features that make it ideal for a variety of protein labeling applications. The rate of the reaction of the SNAP-tag with these derivatives is largely independent of the nature of the synthetic probe attached to BG, permitting the labeling of SNAP fusion proteins with a wide variety of functional groups. Many of these SNAP-tag substrates are cell-permeable, allowing live-cell imaging of protein expression and localization (Figure 2). The ability to turn on the signal at will, together with the availability of a cell-permeable nonfluorescent blocking agent (SNAP-Cell Block), allows time-resolved pulse-chase analysis of protein trafficking. Finally, the availability of orthogonal protein labeling systems from NEB permits simultaneous labeling of multiple proteins in a single cell (CLIP-tag, a SNAP-tag variant that reacts exclusively with O²-benzylcytosine substrates, and the ACP/MCP tags, small protein tags which can be enzymatically labeled on the cell surface with Coenzyme A derivatives).

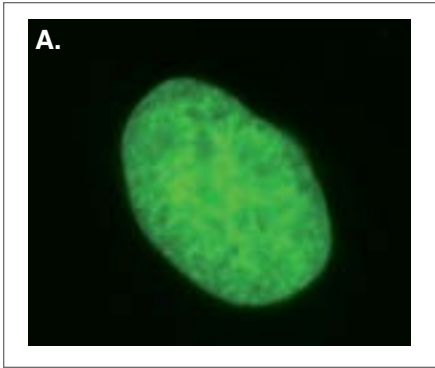
Figure 1.



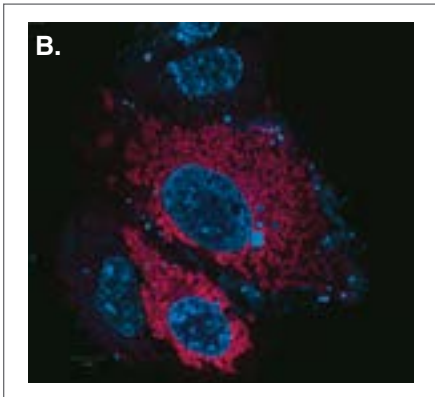
SNAP-tag reaction.

The SNAP-Cell Starter Kit contains a mammalian expression plasmid (pSNAP_f) encoding the SNAP-tag flanked by restriction sites for cloning a gene of interest, and two cell-permeable fluorescent SNAP-tag substrates. A positive control plasmid (pSNAP_f-Cox8A), encoding a SNAP-tagged protein (cytochrome c oxidase) with a well-characterized mitochondrial localization, is also included. Lastly, a negative control “blocking agent” (SNAP-Cell Block) is included that interacts with the SNAP-tag, but is not fluorescent. There are two steps to using this system: subcloning and expression of the protein of interest as a SNAP_f fusion, and labeling of the fusion with the SNAP-tag substrate of choice.

Figure 2. Live cell imaging of SNAP_f Fusion Proteins.



Live CHO-K1 cells transiently transfected with pSNAP_f-H2B. Cells were labeled with SNAP-Cell 505-Star (green) for 15 minutes at 37°C, 5% CO₂.



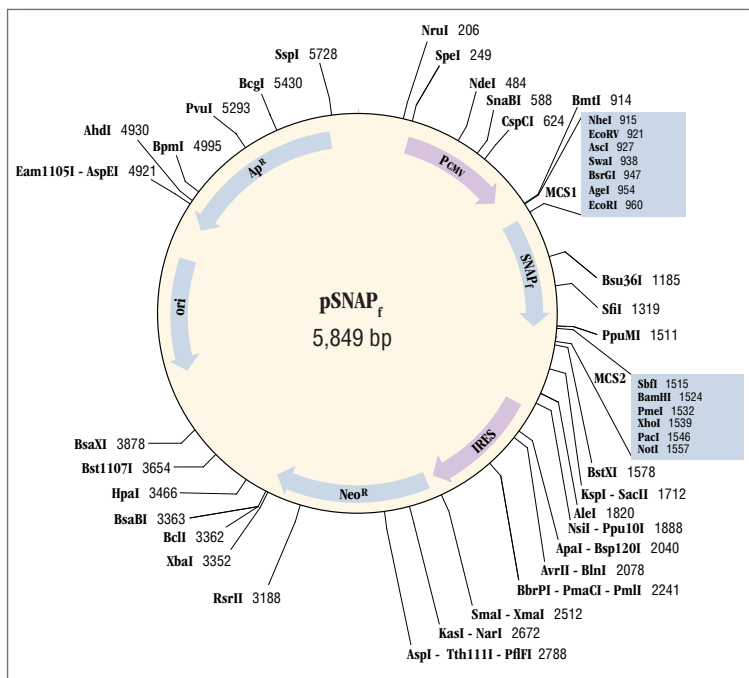
Live COS-7 cells expressing mitochondrial cytochrome oxidase 8A-SNAP_f (SNAP_f-Cox8A) were labeled with SNAP-Cell TMR-Star (red). Nuclei were counterstained with Hoechst 33342 (blue).

Construction and Expression of SNAP-tag Fusion Proteins:

The mammalian expression plasmid pSNAP_f is intended for the cloning and stable or transient expression of SNAP-tag protein fusions in mammalian cells. This plasmid encodes the SNAP_f gene, which is expressed under control of the CMV promoter. The expression vector has an IRES (internal ribosome entry site) and a neomycin resistance gene downstream of SNAP_f for the efficient selection of stable transfectants. Codon usage of the gene is optimized for expression in mammalian cells. pSNAP_f contains two multiple cloning sites to allow cloning of the fusion partner as a fusion to the N- or C-terminus of the SNAP-tag.

pSNAP_f contains an improved version of SNAP-tag, termed SNAP_f. SNAP_f displays faster kinetics in *in vitro* labeling and fast, specific and efficient labeling in live and fixed cell applications, thereby rendering it a desired research tool for analysis of protein dynamics.

Figure 3.



pSNAP_f plasmid map.

Detailed Description of pSNAP_f

The sequence of the cloning region can be found in the Appendix. The complete plasmid sequence can be downloaded at www.neb.com. This plasmid encodes the gene SNAP_f, which is a mutant form of the gene for human O⁶-alkylguanine-DNA-alkyltransferase (hAGT). The codon usage of the gene is optimized for expression in mammalian cells. In the plasmid sequence, the SNAP_f gene is encoded from bp 969 to 1514.

This plasmid is intended for the cloning and stable or transient expression of SNAP-tag protein fusions in mammalian cells. It is particularly suitable for the efficient production of stable cell lines expressing SNAP-tag gene fusions. The plasmid contains the CMV promoter followed by the genes for SNAP_f and neomycin resistance separated by the IRES of the encephalomyocarditis virus (ECMV), which permits the translation of two open reading frames from one messenger RNA. After selection of stable mammalian cells for neomycin resistance, nearly all surviving colonies should therefore stably express the SNAP_f fusion protein. An intron is also included because this is believed to improve expression levels. Unless your expression experiments require a pure population of cells, you can simply use the pool of resistant cells. Otherwise cell clones can be isolated and characterized using standard procedures. The plasmid also contains the beta-lactamase (Ampicillin resistance) gene for maintenance in bacteria. The gene of interest can be cloned upstream or downstream of the SNAP_f coding sequence, as a fusion to the N- or C-terminus of the SNAP_f. pSNAP_f can also be used as an expression control plasmid, expressing the SNAP-tag alone, in which case the SNAP-tag protein is distributed throughout the cell. The SNAP_f gene can be isolated from the plasmid using PCR or direct cloning in order to subclone it into a different vector system of choice.

Cloning of SNAP-tag Fusions in pSNAP_f Vector

Cloning by PCR

1. To subclone the gene of interest into pSNAP_f fused to the N-terminus of SNAP_f, use the available restriction sites: NheI, EcoRV (blunt), AscI, SmaI (blunt), BsrGI, AgeI or EcoRI, which are located upstream of the SNAP-tag.
2. To subclone the gene of interest into pSNAP_f fused to the C-terminus of SNAP_f, use the available restriction sites downstream of the SNAP-tag: SbfI, BamHI, PmeI (blunt), XhoI, PacI or NotI.

Note: When fusing the gene of interest to the C-terminus of SNAP_f, note that there is an in-frame stop codon between the PacI and NotI sites, so SbfI, BamHI, PmeI (blunt), XhoI or PacI must be used as the 5' cloning site for your insert.

PmeI and XhoI cannot be used together for cloning because they share a cytosine as part of their recognition sequences.

Primer Design and Cloning Considerations

1. Design your PCR primers to include a sufficient overlap with the sequence of the gene you want to amplify, adding 5–6 bases on the 5' side of the introduced restriction site to ensure efficient cleavage prior to cloning.
2. A stop codon can be included at the C-terminus of the fusion (in front of the downstream cloning site) in order to terminate translation at this position.
3. For fusions upstream of SNAP_f, ensure that a start codon is included. The addition of a Kozak sequence (e.g. GCCRCCATG, where the start codon is underlined) will increase the translation efficiency.
4. In general, any linker peptide between the proteins should be kept short to avoid degradation by proteases. If required, specific protease cleavage sites can be introduced into the linker peptide.
5. Care should be taken to design the cloning so that the fusion partners in the resulting construct are in frame.
6. Perform the PCR reaction and subsequent cloning steps according to established molecular biology protocols.
7. The ligated vector should be transformed into bacteria and the resulting plasmid isolated via a standard miniprep procedure.
8. After subcloning the gene of interest into pSNAP_f as a fusion with the SNAP_f gene, the resulting plasmid can be used for stable or transient expression of the SNAP_f fusion proteins in a suitable cell line.

Direct Cloning

1. Direct cloning can also be used to make fusions with the SNAP-tag. This is only possible if the fusion partner has compatible sites adjacent to the gene of interest.
2. Care should be taken to design the cloning strategy so that the fusion partners in the resulting construct are in frame.

Note: When fusing the gene of interest to the C-terminus of SNAP_f, note that there is a stop codon between the PaeI and NotI sites, so SbfI, BamHI, PmeI (blunt), XhoI or PacI must be used as the 5' cloning site for your insert.

PmeI and XhoI cannot be used together for cloning because they share a cytosine as part of their recognition sequences.

Expression of SNAP-tag Fusions

Transient Expression

Expression of the fusion protein cloned in pSNAP_f can be achieved by transiently transfecting cells in culture with standard transfection protocols. The appropriate reagent and transfection time to permit adequate expression must be empirically determined, using guidelines provided by the manufacturer of the transfection reagent as a starting point. We recommend using pSNAP_f or pSNAP_f-Cox8A as expression control plasmids. Figure 2B shows that the COX8-2-SNAP_f fusion protein (from pSNAP_f-Cox8A) gives a mitochondrial localized signal when labeled with SNAP-Cell substrates. If the empty pSNAP_f plasmid is used as a control vector for transfection, a uniform distribution of the SNAP-tag between nucleus and cytoplasm should be seen. Both pSNAP_f and the localization control plasmids have performed well in stable and transient transfection of CHO-K1, COS-7, U-2 OS and NIH 3T3 cells. Note that the intensity of the fluorescence may vary depending on cell line and labeling substrate used.

Stable Expression

pSNAP_f and pSNAP_f-Cox8A can be transfected as described above for transient transfection or by other standard transfection methods. Twenty four to 48 hours after transfection begin selecting mammalian cultures in 600–1,200 µg/ml G418 (geneticin) depending on the cell line. It is recommended that you establish a kill curve for each cell line to determine optimal selection conditions. After 8–12 days of continuous selection, stable colonies will become visible. It is possible to use pools of stable cell populations for initial cell labeling to test for the presence of SNAP-tag expression. In addition, monoclonal cell lines can be isolated and characterized if desired.

Use of the SNAP_f Control Plasmid pSNAP_f-Cox8A:

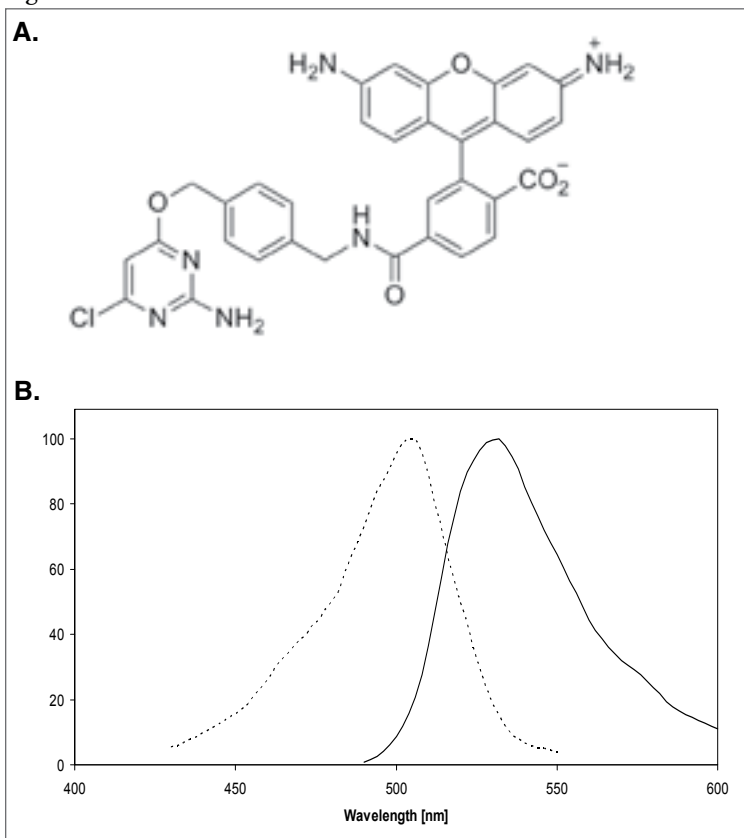
This control plasmid contains the gene encoding the Cytochrome c oxidase, subunit 8–2 (COX8-2) protein cloned upstream of the SNAP_f coding sequence in pSNAP_f, as a fusion to the N-terminus of SNAP_f. Cytochrome c oxidase is localized to the inner mitochondrial membrane and is the terminal enzyme of the respiratory chain. The COX8-2-SNAP_f fusion protein gives mitochondrial fluorescence when labeled with SNAP-Cell substrates (Figure 2B). The full sequence and map for pSNAP_f-Cox8A can be downloaded at www.neb.com. We strongly recommend carrying out parallel expression and labeling experiments with this plasmid as a positive control for your experiment, using the procedure described above.

Labeling SNAP-tag Fusion Proteins:

The kit includes two cell-permeable fluorescent SNAP-tag substrates, SNAP-Cell 505-Star and SNAP-Cell TMR-Star. Both can be used to label SNAP_f fusion proteins inside living or fixed cells, on cell surfaces, or in solution.

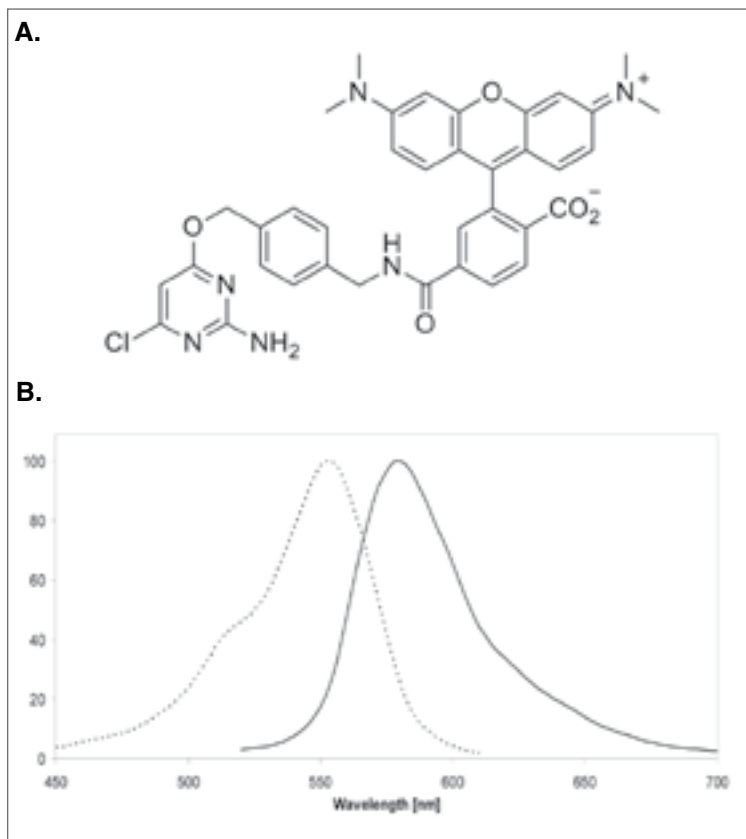
SNAP-Cell 505-Star (CP-6-505) is a photostable green fluorescent substrate that is based on the single isomer 6-carboxyrhodamine 110 fluorescent dye and is suitable for standard fluorescein filter sets. It has an excitation maximum at 504 nm and an emission maximum at 532 nm (Figure 4). This kit contains 10 nmol of SNAP-Cell 505-Star substrate, sufficient to make 2 ml of a 5 μ M SNAP_f fusion protein labeling solution.

Figure 4.



(A) Structure of SNAP-Cell 505-Star (MW 621.0 g/mol), (B) Excitation (dotted line) and emission spectra of SNAP-Cell 505-Star after coupling to SNAP-tag in buffer at pH 7.5

Figure 5.



(A) Structure of SNAP-Cell TMR-Star (MW 677.1 g/mol) (B) Excitation (dotted line) and emission spectra of SNAP-Cell TMR-Star after coupling to SNAP-tag in buffer at pH 7.5.

SNAP-Cell TMR-Star (CP-TMR-Star) is a red fluorescent substrate that is based on tetramethylrhodamine and is suitable for standard rhodamine filter sets. It has an excitation maximum at 554 nm and an emission maximum at 580 nm (Figure 5). This kit contains 6 nmol of SNAP-Cell TMR-Star substrate, sufficient to make 2 ml of a 3 μ M SNAP₁ fusion protein labeling solution.

Instructions for Cellular Labeling

Preparation of Labeling Stock Solution

Dissolve one vial of SNAP-tag substrate in 10 μ l of fresh DMSO to yield a labeling stock solution of 1 mM SNAP-Cell 505-Star or 0.6 mM SNAP-Cell TMR-Star. 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 substrates are soluble up to at least 10 mM.

Protocol for Intracellular Labeling Reaction

1. Dilute the labeling stock solution 1:200 in medium to yield a labeling medium of 5 μ M SNAP-Cell-505-Star or 3 μ M SNAP-Cell TMR-Star. Mix dye 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.

NUMBER OF WELLS IN A PLATE	RECOMMENDED VOLUME FOR CELL LABELING
6	1 ml
12	500 μ l
24	250 μ l
48	100 μ l
96	50 μ l

These recommendations are for culturing cells in polystyrene plates. For confocal imaging, we recommend using chambered coverglass such as Lab-Tek II Chambered Coverglass which is available in a 1, 2, 4 or 8 well format from Nunc (www.nuncbrand.com).

2. Replace the medium on the cells expressing a SNAP_i fusion protein with the SNAP-tag labeling medium and incubate at 37°C, 5% CO₂ for 10–15 minutes.
3. Wash the cells three times with tissue culture medium containing 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.
4. Image the cells using an appropriate filter set. SNAP_i fusion proteins labeled with SNAP-Cell 505-Star should have an excitation maximum at 504 nm and an emission maximum at 532 nm, and can be imaged with standard fluorescein filter sets. SNAP_i fusion proteins labeled with SNAP-Cell TMR-Star should have an excitation maximum at 554 nm and an emission maximum at 580 nm, and can be imaged with standard rhodamine filter sets.

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

Notes for Cellular Labeling

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_f fusion protein to generate a control. This can be achieved using the included nonfluorescent SNAP-tag substrate, SNAP-Cell Block (bromothienylpteridine, 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 with SNAP-Cell Block is on page 13.

Optimizing Labeling

Optimal substrate concentrations and reaction times range from 1–20 μM and 5–30 minutes, respectively, depending on experimental conditions and expression levels of the SNAP_f fusion protein. Best results are usually obtained at concentrations between 1 and 5 μM substrate and 10–15 minutes reaction time. Increasing substrate concentration and reaction time usually results in a higher background without necessarily increasing the signal to background ratio.

Stability of Signal

The turnover rates of the SNAP_f 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. As an alternative to visualize proteins with fast turnover rates, SNAP_f fusion proteins can be labeled at lower temperatures (4 or 16°C). Labeling times may need to be optimized.

Fixation of Cells

After labeling the SNAP_f fusion proteins, the cells can be fixed with standard fixation methods such as para-formaldehyde, ethanol, methanol, methanol/acetone 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 final 30 minutes incubation (Step 3 above) as a DNA counterstain for nuclear visualization. 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 experience with the protein of interest. For example some fixation methods destroy epitopes of certain proteins and therefore do not allow antibody staining afterwards.

Instructions for Labeling of Proteins *in vitro*

1. Dissolve the vial of SNAP-Cell 505-Star (10 nmol) in 10 μ l of fresh DMSO or the vial of SNAP-Cell TMR-Star (6 nmol) in 6 μ l 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 μ M stock for labeling proteins *in vitro*.
2. Set up the reactions, in order, as follows:

COMPONENT	VOLUME	FINAL CONCENTRATION
Phosphate Buffered Saline (PBS)	42 μ l	1X
50 mM DTT	1 μ l	1 mM
50 μ M SNAP-tag Purified Protein	5 μ l	5 μ M
250 μ M SNAP-tag Substrate	2 μ l	10 μ M
Total Volume	50 μ l	

3. Incubate in the dark for 15 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 you may wish to separate the nonreacted substrate from the labeled SNAP_f fusion protein. You can use gel filtration or dialysis. Please refer to the vendor's instructions for the separation tools you are using.

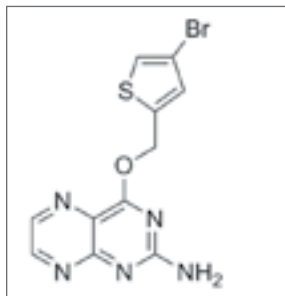
Note for Labeling *in vitro*

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 (e.g. for a redox-sensitive protein) if handling at temperatures above 4°C is minimized. SNAP_f fusion proteins can be purified before labeling, but the labeling reaction also works in non-purified protein solutions (including cell lysates).

Use of SNAP-Cell Block to Block Reactivity of SNAP_f Fusion Proteins:

SNAP-Cell Block (bromothienylpteridine, BTP) is a non-fluorescent compound that blocks the reactivity of the SNAP-tag in live cells (Figure 6). It can be used to generate inactive controls in live and fixed cell labeling experiments performed with SNAP_f fusion proteins. SNAP-Cell Block is highly membrane permeable and once in the cell reacts with the SNAP-tag irreversibly inactivating it for subsequent labeling steps.

Figure 6.



Structure of SNAP-Cell Block (MW 338.2 g/mol).

Instructions for Use with SNAP-Cell Substrates

In many cases the labeling of a non-transfected cell sample or a mock-transfected cell sample will be completely sufficient as a negative control for cell labeling. In some cases, however, it may be desirable to block the SNAP-tag activity in a cell sample expressing the SNAP_f fusion protein to generate a control. This is done by a pre-incubation of the cells with SNAP-Cell Block, followed by the incubation with the labeling solution. 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.

Note that SNAP-Cell Block is a potent blocker of the SNAP-tag. Always take care to avoid carryover of SNAP-Cell Block to samples that you do not wish to block.

Preparation of Stock Solution

Dissolve one tube of SNAP-Cell Block (20 nmol) in 10 μ l of fresh DMSO to give a stock solution of 2 mM. Mix by vortexing for 10 minutes, until all the SNAP-Cell Block is dissolved. Store this stock solution in the dark at 4°C or for extended storage at -20°C. We recommend using a final concentration of 10 μ M, which is a 1:200 dilution of this stock solution.

Blocking SNAP-tag Activity with SNAP-Cell Block

The following steps describe the use of SNAP-Cell Block in a typical control labeling experiment:

1. Prepare two cell samples suitable for labeling, each expressing the SNAP_f fusion protein of interest.
2. Mix an appropriate amount of medium with SNAP-Cell Block stock solution in a ratio of 1:200 to give a blocking medium of 10 μ M SNAP-Cell Block. For best performance, add the dissolved SNAP-Cell Block to complete medium, including serum. Do not prepare more medium with SNAP-Cell Block than you will consume within one hour.
3. Mix an appropriate amount of medium with DMSO in a ratio of 1:200, to give a final concentration of 0.5% v/v DMSO.
4. Replace the medium on one sample of cells with the blocking medium. These are your blocked cells. Replace the medium on the other sample of cells with the medium containing DMSO. These are your test cells. Incubate both cell samples at 37°C, 5% CO₂ for 10–15 minutes.
5. Remove SNAP-Cell Block or DMSO-containing medium by washing both samples of cells twice with complete medium.
6. Label both cell samples with the SNAP-Cell substrate using the protocol on page 10.
7. Inspect both samples under the fluorescence microscope. The blocked cells should show no fluorescence, whereas the test cells should show fluorescence localized to where the SNAP_f fusion protein is present in the cell.

Note that there is a constant turnover and resynthesis of proteins in the cell. After having blocked all existing SNAP_f fusion proteins within the cell, new SNAP_f fusion protein molecules may be synthesized in the meantime and may get labeled during incubation with a fluorescent SNAP-tag substrate. This will give the impression that the blocking was ineffective. In order to minimize these effects of protein synthesis and protein transport, cells may have to be treated with cycloheximide and incubation with the fluorescent SNAP-tag substrate may have to be performed at 4°C.

Troubleshooting:

Cloning of the Gene of Interest

If subcloning of the gene of interest into the pSNAP_f vector does not work, reconfirm all the cloning steps (primer design, choice of restriction site, etc.). If all steps are confirmed as being correct, then try the cloning using different restriction sites. Be sure to include a positive and negative control for the ligation reaction. Alternatively try to subclone the SNAP_f gene into an expression vector already containing your gene of interest.

Expression

In general we have not experienced problems expressing SNAP_f protein fusions. However if your fusion protein does not appear to be expressed, try expressing the H2B-SNAP_f or COX8-2-SNAP_f protein fusions as positive controls using cells transiently transfected with pSNAP_f-H2B (NEB #N9186) or pSNAP_f-Cox8A (included). Labeling of such cells with a fluorescent SNAP-Cell substrate should show strong nuclear or mitochondrial localized fluorescence, respectively. The empty pSNAP_f plasmid can also be used as a control (cytosolic and nuclear fluorescence). Note that the intensity of this fluorescence may vary depending on cell line and substrate used. Expression of the localization controls but not your fusion protein can be due to a variety of causes. It is possible that this fusion protein may be toxic for your cell line. It is difficult to troubleshoot such instances, but the use of a different expression plasmid or cell line or tagging the opposite end of the protein may help. Signs of host cell toxicity could include slow proliferation or apoptosis. Counterstaining live cells with Hoechst 33342 or fixed cells with DAPI can be used to determine whether nuclei are healthy if toxicity is suspected.

Problems with 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_f 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_f 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 within the range of 1–20 μM and 5–30 minutes, respectively. 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

If the fluorescence signal decreases rapidly, it may 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. Photobleaching is generally not a problem as both SNAP-Cell 505-Star and SNAP-Cell TMR-Star are very photostable. However, if you experience problems with photobleaching, addition of a commercially available anti-fade reagent may be helpful.

Problems with Labeling in vitro

Solubility

If solubility problems occur with your SNAP_f fusion protein, we recommend testing a range of pH (pH 5.0–pH 10.0). 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 (NEB #S9147).

If the SNAP_f 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_f 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.

Appendix:

Sequence of SNAP-tag Region of pSNAP_f Vector

Unique restriction sites in the regions flanking the SNAP_f gene are displayed above the coding strand. The complete sequence of pSNAP_f and pSNAP_f-Cox8A can be downloaded at www.neb.com.

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      NheI   EcoRV   AscI           SwaI   BsrGI   AgeI   EcoRI
...GCTAGC GATATCGGCG CGCCAGCATT TAAATCTGTA CAGACCGGTG AATTC
      CGATCG CTATAGCCGC GCGGTCGTAA ATTTAGACAT GTCTGGCCAC TTAAG...
```

```

           SbfI   BamHI   PmeI   XhoI           PacI           NotI
...CCTGCA GCGCGATCCG CGTTTAAACT CGAGGTTAAT TAATGAGCGG CCGC
      GGACGT CCGCCTAGGC GCAAATTTGA GCTCCAATTA ATTACTCGCC GGCG...
```

Note

NEB 10-beta Competent *E. coli* (High Efficiency) (NEB #C3019) is recommended for propagating and subcloning of the vector and control plasmid.

Ordering Information

PRODUCT	NEB #	SIZE
SNAP-Cell Starter Kit	E9100S	10 reactions
COMPANION PRODUCTS		
SNAP-Vista® Green	S9147S	50 nmol
SNAP-Vista Blue	S9146S	50 nmol
SNAP-Biotin®	S9110S	50 nmol
pSNAP-tag(T7)-2 Vector	N9181S	20 µg
pSNAP _f -ADRβ2 Control Plasmid	N9184S	20 µg
pSNAP _f -H2B Control Plasmid	N9186S	20 µg
pSNAP _f -Cox8A Control Plasmid	N9185S	20 µg
SNAP-Cell 360	S9101S	50 nmol
SNAP-Cell 430	S9109S	50 nmol
SNAP-Cell 505-Star	S9103S	50 nmol
SNAP-Cell Oregon Green	S9104S	50 nmol
SNAP-Cell Fluorescein	S9107S	50 nmol
SNAP-Cell TMR-Star	S9105S	30 nmol
SNAP-Cell Block	S9106S	100 nmol
Anti-SNAP-tag Antibody (Polyclonal)	P9310S	100 µl
SNAP-tag Purified Protein	P9312S	50 µg

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