SFP Synthase

1-800-632-7799
info@neb.com
www.neb.com

P9302S 005160318031

P9302S

25 nmol	**40 µM**	**Lot: 0051603**
Store at –20°C		**Exp: 3/18**

Introduction

SFP Synthase (4´-phosphopantetheinyl transferase) catalyzes the covalent transfer of substituents from derivatized coenzyme A (CoA) to ACP- or MCP-tagged fusion proteins exposed on the surface of living cells. The 25 nmoles of SFP Synthase provided is sufficient to make 25 ml of a 1 µM ACP-tag or MCP-tag fusion protein labeling solution.

The ACP-tag and MCP-tag are small protein tags (8 kDa) based on the acyl carrier protein (ACP). MCP-tag contains two mutations (D36T and D39G). Both allow the specific, covalent attachment of virtually any molecule to a protein of interest. Substrates for labeling are derivatives of coenzyme A (CoA). In the labeling reaction, the substituted phosphopantetheine group of CoA is covalently attached to a conserved serine residue on the ACP-tag or the MCP-tag by a phosphopantetheinyl transferase (SFP Synthase or ACP Synthase). Having no cysteines, the ACP-tag and the MCP-tag are particularly suited for specifically labeling cell-surface proteins, and should be useful for labeling secreted proteins with disulfide bridges such as antibodies.

While ACP Synthase (NEB #P9301) will preferentially modify the ACP-tag, SFP Synthase will modify both ACP-tag and MCP-tag. This principle can be employed for sequential dual labeling of two different proteins that localize to the cell surface. Cells co-expressing one ACP-tag fusion protein and one MCP-tag fusion protein can be incubated with ACP Synthase and one CoA substrate followed by labeling with SFP Synthase and a different CoA substrate.

There are two steps to using this system: Cloning and expression of the protein of interest as an ACP-tag or MCP-tag fusion, and labeling of the fusion protein using the SFP Synthase with the CoA substrate of choice. In this document, the labeling of fusion proteins with CoA substrates is described. The cloning of ACP-tag or MCP-tag protein fusions is described in the documentation supplied with the ACP-tag or the MCP-tag plasmids.

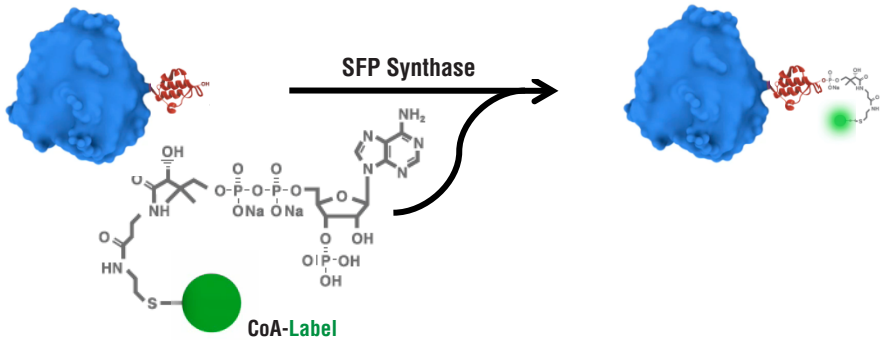

Figure 1. *SFP labeling reaction*

Source

An *E. coli* strain carrying the cloned *B.subtilis* gene for 4´–phosphopantetheinyl transferase (SFP).

Storage

SFP Synthase is stable for at least 2 years when stored at –20°C.

Supplied In: 50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM DTT and 50% glycerol.

Reagents Supplied

0.5 ml of 1 M $MgCl_2$

Materials Required but not Supplied

CoA substrates for labeling ACP-tag or MCP-tag fusion proteins.

Cells expressing ACP-tag or MCP-tag fusion proteins. Proteins of interest can be expressed with the ACP-tag or MCP-tag as either an N- or a C-terminal fusion, but note that the tag needs to be exposed to the extracellular surface of the plasma membrane for labeling with CoA substrates.

Tissue culture materials and media

Transfection reagents

Fluorescence microscope with suitable filter set

Quality Controls

Purity: Purity of SFP Synthase was determined by SDS-PAGE to be greater than 95% .

***In vitro* Labeling:** Reaction of CoA 488 (10 µM) with purified ACP-MBP (Maltose Binding Protein, 5 µM) and SFP Synthase (1 µM), *in vitro,* followed by mass spec analysis indicated an efficiency of labeling of ≥ 95%.

Cellular Protein Labeling: Cells transfected with pACP-ADRβ2 expressing ACP-ADRβ2 (cell surface) were labeled with 5 µM CoA 488 and 1 µM SFP Synthase for 60 minutes. Surface target was efficiently labeled.

Instructions for Use

ACP-tag or MCP-tag fusion proteins can be expressed by transient transfection. For expression of fusion proteins with the ACP-tag or the MCP-tag, refer to instructions provided with pACP-tag(m)-2 Vector (NEB #N9322) or pMCP-tag(m) Vector (NEB #N9317) cloning plasmids. For cell culture and transfection methods, refer to established protocols.

Dissolve one vial of CoA substrate (50 nmol) in 50 µl of DMSO to give a solution of 1 mM CoA substrate. Mix by vortexing for 10 minutes until all the CoA 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 the requirements. The substrate is soluble up to at least 10 mM.

Protocol for Labeling Reaction

1. Dilute 1 mM CoA substrate stock solution 1:200 in culture medium (a final concentration of 5 µM). We obtain best performance by adding the substrate to complete medium, including serum. Add $MgCl_2$ (1:100) to a final concentration of 10 mM. Finally, add the SFP Synthase to a final concentration of 1 µM, a dilution of 1:40. Do not prepare more medium with CoA substrate, $MgCl_2$ and SFP Synthase than will be consumed within one hour.
2. Replace the medium on the cells expressing an ACP-tag or MCP-tag fusion protein with the labeling medium and incubate at 37°C, 5% CO_2 for 60 minutes.
3. Wash the cells three times with tissue culture medium with serum.
4. Image the cells using an appropriate filter set.
5. We recommend routinely labeling one well of non-transfected or mock-transfected cells for comparison.

Notes for Cellular Labeling

Optimizing Labeling

The substrate concentration can be varied between 1 and 10 µM depending on the experimental conditions, expression levels of the ACP-tag or MCP-tag fusion protein, and incubation time with the substrate. Best results are usually obtained at concentrations between 1 and 5 µM. An increase of the substrate concentration usually results in a higher background and does not necessarily increase the signal to background ratio.

The incubation time can be varied between 30 and 60 minutes depending on the experimental conditions, expression levels of the ACP-tag or MCP-tag fusion protein and substrate concentration. We recommend routine incubation times of 60 minutes. Longer incubation times tend to result in stronger background staining and do not necessarily increase the signal to background ratio.

Stability of Labeling

The turnover rates of the ACP-tag or MCP-tag fusion protein under investigation may vary widely depending on the fusion partner. Where protein turnover is rapid, we recommend analyzing the cells under the microscope immediately after the labeling reaction or, if possible, fixing the cells directly after labeling.

Fixation of Cells

After labeling the ACP-tag or MCP-tag fusion proteins, the cells can be fixed with standard fixation methods such as paraformaldehyde, ethanol, methanol, methanol/acetone, etc., without loss of signal. We are not aware of any incompatibility of the ACP-tag or MCP-tag label with any fixation method.

(see other side)

Counterstaining

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

Antibody Labeling

Antibody labeling at the surface of living cells after ACP-tag or MCP-tag labeling is possible. Antibody labeling after fixation of the cells is also possible using standard protocols without loss of the ACP-tag or MCP-tag signal (see fixation of cells). 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.

Experimental Conditions that do not Allow Fetal Calf Serum

If fetal calf serum must be omitted due to the experimental setup, the labeling can be done in medium without serum. Higher background levels might be observed as fetal calf serum in the labeling solution reduces the background staining. We recommend re-evaluating the dye concentration and incubation time if this is a problem. The addition of 0.5% BSA may be helpful in some cases to block non-specific background.

Troubleshooting for Cellular Labeling

No Labeling

If no labeling is seen, the most likely explanation is that the fusion protein is not expressed. Verify the transfection method to confirm that the cells contain the fusion gene of interest. If this is confirmed, check for expression of the ACP-tag or MCP-tag fusion protein via Western blot.

Weak Labeling

Weak labeling may be caused by insufficient exposure of the fusion protein to the substrate. Try increasing the concentration of CoA 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.

Weak labeling can also be caused by loss of activity of the SFP Synthase. Increasing the concentration of SFP Synthase may help.

High Background

Background fluorescence can be controlled by reducing the concentration of CoA 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. Addition of DNAse I (5 units/ml final concentration) can help reduce the background caused by non-transfected plasmid DNA aggregating at the surface of cells.

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 ACP-tag or MCP-tag from the N- to the C-terminus or vice versa.

Photobleaching is not generally a problem as the CoA substrates are very photostable. However, if problems with photobleaching are experienced, addition of a commercially available anti-fade reagent may be helpful.

Instructions for Labeling of Proteins *in vitro*:

1. Dissolve the vial of CoA substrate (50 nmol) in 50 µl of fresh DMSO to yield a labeling stock solution of 1 mM CoA substrate. Mix by vortexing for 10 minutes until all the CoA 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:

Component	Volume	Final Concentration
Deionized Water	28.25 µl	
1 M HEPES	2.5 µl	50 mM
50 mM DTT	1 µl	1 mM
50 mM $MgCl_2$	10 µl	10 mM
50 µM ACP-tag or MCP-tag Purified Protein	5 µl	5 µM
40 µM SFP Synthase	1.25 µl	1 µM
250 µM CoA Substrate	2 µl	10 µM
Total Volume	50 µl	

3. Incubate in the dark for 60 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 CoA fusion protein by gel filtration or dialysis. Please refer to the vendor's instructions for the separation tools used.

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 ACP- or MCP-tag. The stability of the ACP- or MCP-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.

ACP- or MCP-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 the ACP- or MCP-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 the 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 ACP-tag/MCP-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 the ACP- or MCP-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 ACP- or MCP-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 the fusion protein is particularly sensitive to degradation or to loss of activity, try reducing the labeling time or decreasing the labeling temperature. We recommend overnight incubation when labeling at 4°C.