CoA Biotin

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S9351S 003160619061

S9351S

50 nmol **Lot:0031606**

Store at: –20°C **Exp: 6/19**

Introduction

CoA Biotin is a non cell-permeable substrate based on biotin with an amidocaproyl linker. It is suitable for applications such as biotinylation of ACP-tag or MCP-tag fusion proteins on the surface of 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 CoA Biotin substrate, sufficient to make 10 ml of a 5 µM solution for the labeling of ACP-tag or MCP-tag fusion proteins on cells.

The ACP-tag and MCP-tag are small protein tags (8 kDa) based on the acyl carrier protein. MCP-tag contains two mutations (D36T and D39G). Both allow the specific, covalent attachment of virtually any molecule to a protein of interest. Substrates are derivatives of coenzyme A (CoA). In the labeling reaction, the substituted phosphopantetheine group of CoA is covalently attached to a conserved serine residue of 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 label the ACP-tag, SFP Synthase (NEB #P9302) will modify both ACP-tag and MCP-tag.

There are two steps to using this system: subcloning and expression of the protein of interest as an ACP-tag or MCP-tag fusion, and labeling of the fusion protein using the appropriate synthase with the CoA substrate of choice. Expression of ACP- and MCP-tagged proteins is described in the documentation supplied with the pACP-tag(m)-2 (NEB #N9322) and pMCP-tag(m) (NEB #N9317) vectors, respectively. The labeling of the fusion proteins with the CoA Biotin is described below.

Materials required but not supplied:

ACP Synthase (NEB #P9301) for labeling ACP-tag

SFP Synthase (NEB #P9302) for labeling ACP-tag or MCP-tag

Cells expressing ACP-tag or MCP-tag fusion proteins

Tissue culture materials and media

Transfection reagents

Fluorescence microscope with suitable filter set

DMSO

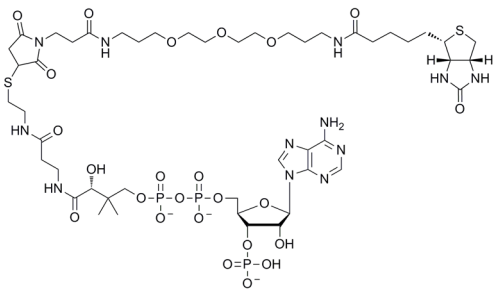

Figure 1. *Structure of CoA-Biotin (MW 1365.3 g/mol)*

Storage

CoA Biotin 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.

Quality Controls

Purity and Characterization: Purity of CoA-Biotin was determined to be 94% by HPLC analysis. Molecular weight $[M]^-$ was determined by MS to be 1363.4 (1363.4 expected).

***In vitro* protein labeling:** Reaction of CoA-Biotin (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%.

Instructions for Cellular Labeling

ACP-tag and MCP-tag fusion proteins can be expressed by transient transfection. For expression of fusion proteins with the ACP-tag or MCP-tag refer to instructions supplied with the pACP-tag(m)-2 (NEB #N9322) and pMCP-tag(m) (NEB #N9317) 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 labeling stock solution of 1 mM CoA substrate. Mix by vortexing for 10 minutes until all the 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.

Protocol for Labeling reaction:

1. Dilute the substrate stock solution 1:200 in medium to a final concentration of 5 µM. Mix substrate with medium thoroughly by pipetting up and down 10 times. For best performance, add the CoA substrate to complete medium, including serum (0.5% BSA can be used for experiments carried out in serum-free media). Add $MgCl_2$ to a final concentration of 10 mM. Finally, add ACP Synthase or SFP Synthase to a final concentration of 1 µM, a dilution of 1:40. Do not prepare more medium with substrate, $MgCl_2$, and synthase than you will consume 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.

Number of Wells in Plate	Recommended Volume for Cell Labeling
6	1 ml
12	500 µl
24	250 µl
48	100 µl
96	50 µl

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

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

Microscopy

Fixation

After labeling the ACP-tag or MCP-tag fusion proteins with CoA-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 ACP-tag or MCP-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.

Western Blotting

Biotinylated proteins from cell lysates can be visualized on Western blots using standard streptavidin-based detection reagents. For Western blotting experiments it may be more efficient to label the ACP-tag or MCP-tag fusion proteins after lysis of the cells.

Optimizing Labeling

Optimal substrate concentrations and reaction times range from 1–10 µM and 30 minutes to overnight, respectively, depending on experimental conditions and expression levels of the ACP-tag or MCP-tag fusion protein. Best results are usually obtained at concentrations between 1 and 5 µM substrate and 60 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 ACP-tag or MCP-tag fusion protein in live cells 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 processing the cells for imaging or blotting immediately after the labeling reaction.

Counterstaining

Cells can be counterstained with any live-cell dye that is compatible with the properties of the CoA substrate for simultaneous microscopic detection. We routinely add 5 µM Hoechst 33342 (nuclear stain) for 2 minutes after labeling with avidin/streptavidin followed by 2 short washing steps. Counterstaining of cells is also possible with dyes that do not enter live cells after fixation and permeabilization.

(see other side)

CERTIFICATE OF ANALYSIS

Immunocytochemistry

Antibody labeling of the fusion protein can be performed after ACP-tag or MCP-tag labeling and fixation of the cells according to standard protocols without loss of the 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.

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 ACP-tag or MCP-tag fusion protein. An ACP-tag or MCP-tag control plasmid may also be used as a positive control.

Weak Labeling

Weak labeling may be caused by insufficient exposure of the fusion protein to the substrate. Try increasing the concentration of CoA Biotin 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 CoA-Biotin substrate (50 nmol) in 50 µl of fresh DMSO to yield a 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 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.