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 and pMCP-tag plasmids, respectively. The labeling of the fusion proteins with the CoA substrate 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

Storage
CoA 488 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 2 years dry or 3 months dissolved in DMSO.

Quality Controls
Purity and Characterization: Purity of CoA 488 was determined to be 99% by HPLC analysis. Molecular weight [M] was determined by MS to be 1477.2 (1477 expected).

In vitro Protein 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%.

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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- 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 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.

High Background
Background fluorescence may 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.

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

Photobleaching is generally not a problem as the CoA 488 substrate is 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 488 substrate (50 nmol) in 50 µl of 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:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized Water</td>
<td>28.25  µl</td>
<td>50 mM</td>
</tr>
<tr>
<td>1 M HEPES</td>
<td>2.5 µl</td>
<td>50 mM</td>
</tr>
<tr>
<td>50 mM DTT</td>
<td>1 µl</td>
<td>1 mM</td>
</tr>
<tr>
<td>50 mM MgCl$_2$</td>
<td>10 µl</td>
<td>10 mM</td>
</tr>
<tr>
<td>50 µM ACP-tag</td>
<td>5 µl</td>
<td>5 µM</td>
</tr>
<tr>
<td>Purified Protein</td>
<td>1.25 µl</td>
<td>1 µM</td>
</tr>
<tr>
<td>Synthase</td>
<td>1.25 µl</td>
<td>1 µM</td>
</tr>
<tr>
<td>250 µM CoA 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 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.

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