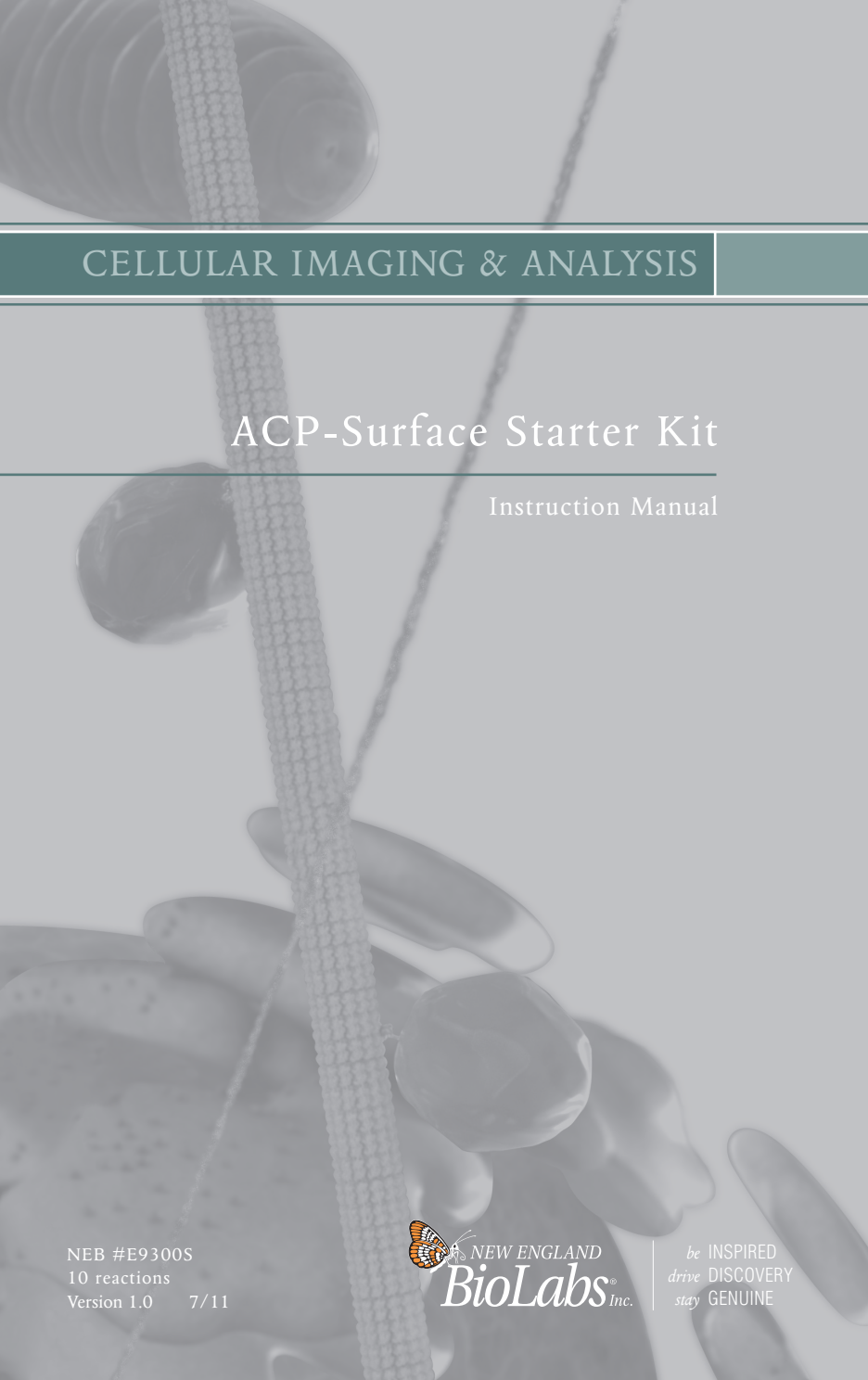


CELLULAR IMAGING & ANALYSIS

ACP-Surface Starter Kit

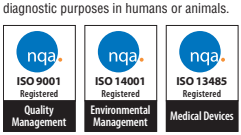
Instruction Manual

NEB #E9300S
10 reactions
Version 1.0 7/11

NEW ENGLAND BioLabs Inc.

be INSPIRED
drive DISCOVERY
stay GENUINE

This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.



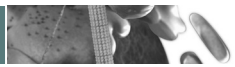


Table of Contents:

Kit Components:

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

pACP-tag(m)-2 Vector ..... 20 µg
Supplied in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), 0.5 µg/µl

pACP-ADRβ2 Control Plasmid ..... 20 µg
Supplied in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), 0.5 µg/µl

CoA 488 ..... 10 nmol
Supplied dry. Prepare a stock solution in DMSO as described on page 10.

CoA 547 ..... 10 nmol
Supplied dry. Prepare a stock solution in DMSO as described on page 10.

SFP Synthase (40 µM) ..... 125 µl
Supplied in 50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM DTT and 50% glycerol.

1M $MgCl_2$ ..... 0.5 ml

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 substrates can be stored at 4°C for up to 4 weeks protected from light and moisture. With proper storage at –20°C the SFP Synthase should be stable for at least two years, and the CoA substrates should be stable for at least 2 years dry or 3 months dissolved in DMSO.

Required Materials Not Included

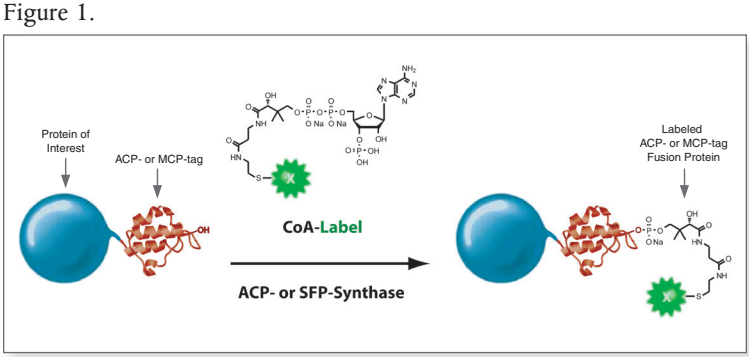
Mammalian Cell Lines
DNA Transfection Reagents
Standard Tissue Culture Media and Plasticware
DMSO
Hoechst 33342 for Nuclear Staining (optional)

Introduction:

The ACP-tag is a novel tool for the specific, covalent attachment of virtually any molecule to a cell surface 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 covalent derivatization with a variety of functional groups, including fluorophores and biotin. This system provides a powerful and unique tool to study the role of cell surface proteins in a variety of highly dynamic processes, including receptor internalization, turnover and complex formation.

The ACP-tag and the related MCP-tag are small protein tags (77 amino acids, 8 kDa) based on the acyl carrier protein from *E. coli*. Both can be enzymatically modified with fluorophores, biotin etc. using substrates that are derivatives of coenzyme A (CoA). In the labeling reaction, the substituted phosphopantetheine group of the CoA substrate is covalently attached to a conserved serine residue of the ACP-tag or the MCP-tag by a 4´-phosphopantetheinyl transferase (SFP Synthase from *B. subtilis*, or ACP Synthase from *E. coli*) (Figure 1). The ACP-tag has a number of features that make it ideal for a variety of protein labeling applications. The rate of the reaction of the synthase with CoA derivatives is largely independent of the nature of the synthetic probe attached to CoA, permitting the labeling of ACP and MCP fusion proteins with a wide variety of functional groups (Figure 2). The ability to turn on the signal at will allows time-resolved analysis of protein trafficking and receptor internalization. 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. Finally, the availability of orthogonal protein labeling systems from NEB permits simultaneous labeling of multiple proteins in a single cell (SNAP-tag, a variant of the human DNA repair enzyme hAGT that transfers a label onto itself from O^6-benzylguanine substrates, and CLIP-tag, a SNAP-tag variant that transfers a label onto itself from O^2-benzylcytosine substrates).

Figure 1.

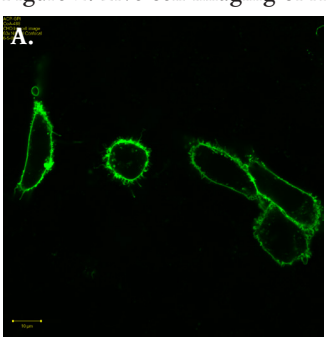


ACP-tag labeling reaction.

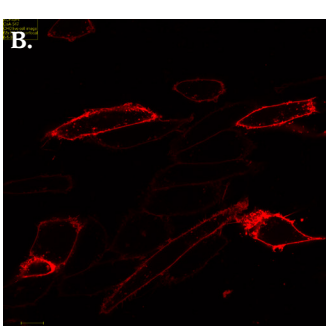
While the ACP Synthase (NEB #P9301) will modify predominantly the ACP-tag, the included SFP Synthase will efficiently label both the ACP-tag and MCP-tag (the MCP-tag contains two mutations relative to the ACP-tag, D36T and D39G, which abolish recognition by the ACP Synthase while preserving recognition by SFP Synthase). 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 are first incubated with ACP Synthase and one CoA substrate to label the ACP-tag, followed by incubation with the SFP Synthase and a different CoA substrate to label the MCP-tag.

The ACP-Surface Starter Kit contains a mammalian expression plasmid (pACP-tag(m)-2) encoding the ACP-tag flanked by restriction sites for cloning a gene of interest, SFP Synthase, 1 M $MgCl_2$ and two non-cell-permeable fluorescent CoA substrates. A positive control plasmid (pACP-ADRβ2), encoding an ACP-tagged protein (Beta-2 Adrenergic Receptor) with a well-characterized cell surface localization, is also included. There are two steps to using this system: subcloning and expression of the protein of interest as an ACP-tag fusion, and enzymatically labeling the fusion with the CoA substrate of choice.

Figure 2. Live cell imaging of ACP-tag fusion proteins.



Live CHO-K1 cells transiently transfected with pACP-GPI. Cells were labeled with CoA 488 (green) in the presence of ACP Synthase for 30 minutes.

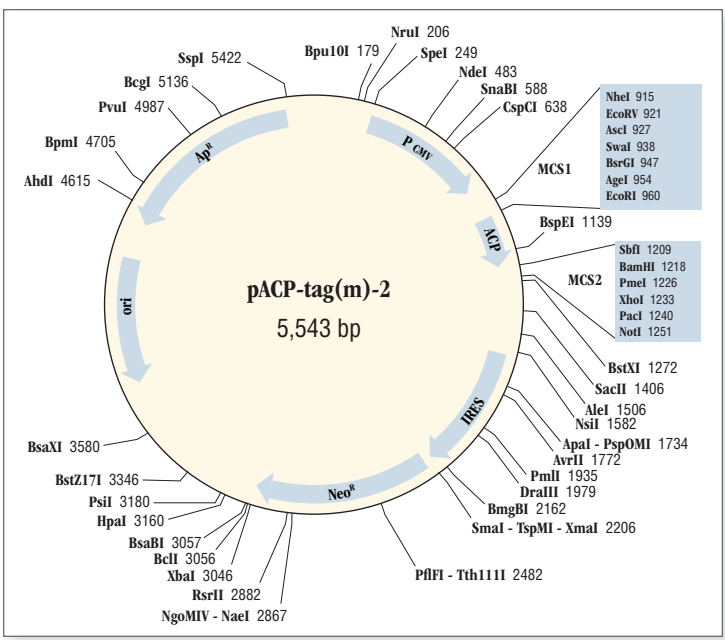


Live CHO-K1 cells transiently transfected with pACP-GPI. *Cells were labeled with CoA 547 (red) in the presence of SFP Synthase for 30 minutes.*

Construction and Expression of ACP-tag Fusion Proteins

The mammalian expression plasmid pACP-tag(m)-2 (Figure 3) is intended for the cloning and transient or stable expression of ACP-tag protein fusions in mammalian cells. The plasmid encodes ACPwt, an Acyl Carrier Protein from *E. coli*, expressed under control of the CMV promoter. The expression vector has an IRES (Internal Ribosome Entry Site) and a neomycin resistance gene downstream of the ACP-tag for the efficient selection of stable transfectants. Codon usage of the gene is optimized for expression in mammalian cells. pACP-tag(m)-2 contains two multiple cloning sites to allow cloning of the fusion partner as a fusion to the C-terminus of the ACP-tag and an appropriate signal peptide to the N-terminus of the ACP-tag.

Figure 3.



pACP-tag(m)-2 plasmid map.

Detailed Description of pACP-tag(m)-2

The sequence of the cloning region of pACP-tag(m)-2 can be found in the Appendix. The complete plasmid sequence can be downloaded at www.neb.com. This plasmid encodes the gene ACPwt which is the wild type Acyl Carrier Protein from *E. coli*. The codon usage of the gene is optimized for expression in mammalian cells. In the plasmid sequence, the ACPwt gene is encoded from bp 975 to 1208.

This plasmid is intended for the cloning and stable or transient expression of ACP-tag protein fusions in mammalian cells. It is suitable for the efficient production of stable cell lines expressing ACP-tag gene fusions. The plasmid contains the CMV promoter followed by the genes for the ACP-tag 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; therefore after selection of stable mammalian cells for neomycin resistance, nearly all surviving colonies should stably express the ACP-tag fusion protein. 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 contains the β-lactamase (Ampicillin resistance) gene for maintenance in bacteria. The gene of interest should be cloned downstream of the ACP-tag coding sequence, as a fusion to the C-terminus of the ACP-tag. An appropriate cell surface signal peptide should be cloned upstream of the ACP-tag as an N-terminal fusion. A Kozak sequence is located upstream of the ACPwt gene to increase the translation efficiency of the fusion protein. The ACPwt 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 ACP-tag Fusions in pACP-tag(m)-2 Vector

Cloning by PCR

1. To subclone a gene of interest or an appropriate cell surface signal peptide into pACP-tag(m)-2 as an N-terminal fusion to the ACP-tag, use the available restriction sites: NheI, EcoRV(blunt), AscI, SwaI (blunt), BsrGI, AgeI or EcoRI which are located upstream of the ACP-tag.
2. To subclone a gene of interest into pACP-tag(m)-2 as a C-terminal fusion to the ACP-tag use the available restriction sites downstream of the ACP-tag: SbfI, BamHI, PmeI (blunt), XhoI, PacI and NotI.

Note: When fusing the gene of interest to the C-terminus of the ACP-tag, note that there is a stop codon between the PacI and NotI sites, so SbfI, BamHI, PmeI, 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. When cloning a secretion peptide upstream of the ACP-tag, ensure that a start codon is included. The addition of a Kozak sequence immediately

upstream from the start codon (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 (including the secretion peptide) 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 pACP-tag(m)-2 as a fusion with the ACPwt gene, the resulting plasmid can be used for transient or stable expression of the ACP-tag fusion protein in a suitable cell line.

Direct Cloning

1. Direct cloning can also be used to make fusions with the ACP-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 the ACP-tag, note that there is a stop codon between the PacI and NotI sites, so SbfI, BamHI, PmeI, 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 ACP-tag Fusions

Transient Expression

Expression of the fusion protein cloned in pACP-tag(m)-2 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. ACP-tag fusion proteins can be observed 24 hours post-transfection. We recommend using pACP-ADRβ2 as an expression control plasmid. Both ACP-tag fusion proteins and the pACP-ADRβ2 localization control plasmid 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.

We recommend using TransPass D2 (NEB #M2554) in combination with TransPass V (NEB #M2561) or Roche's FuGENE® 6 Transfection Reagent for both transient and stable transfections.

Stable Expression

pACP-tag(m)-2 and pACP-ADRβ2 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 ACP-tag expression. In addition monoclonal cell lines can be isolated and characterized, if desired.

Use of the ACP-tag Control Plasmid pACP-ADRβ2:

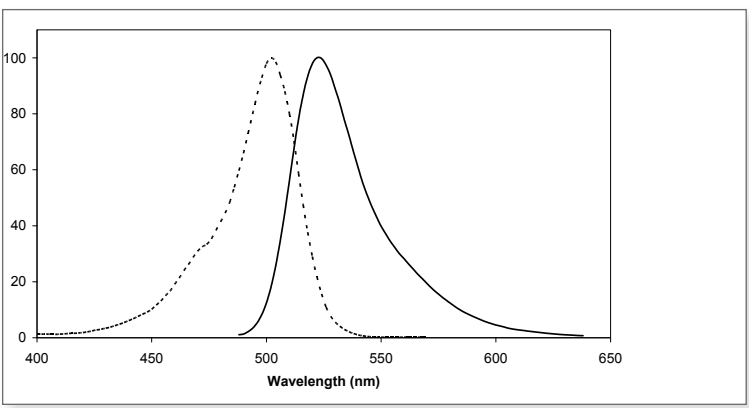
This control plasmid contains a Beta-2 Adrenergic Receptor sequence cloned C-terminal to the ACP-tag coding sequence in pACP-tag(m)-2. This sequence directs the fusion protein to the plasma membrane with the ACP tag exposed on the outside surface, giving a surface localized control signal when labeled with CoA substrates (Figure 2). The Beta-2 Adrenergic Receptor is a member of the G protein coupled receptors and mediates the catecholamine-induced activation of adenylate cyclase through the action of G proteins. The full sequence and map for pACP-ADRβ2 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 ACP-tag Fusion Proteins

The kit includes two substrates for the included SFP Synthase, CoA 488 and CoA 547. Both can be used to label ACP-tag or MCP-tag fusion proteins on cell surfaces or in solution.

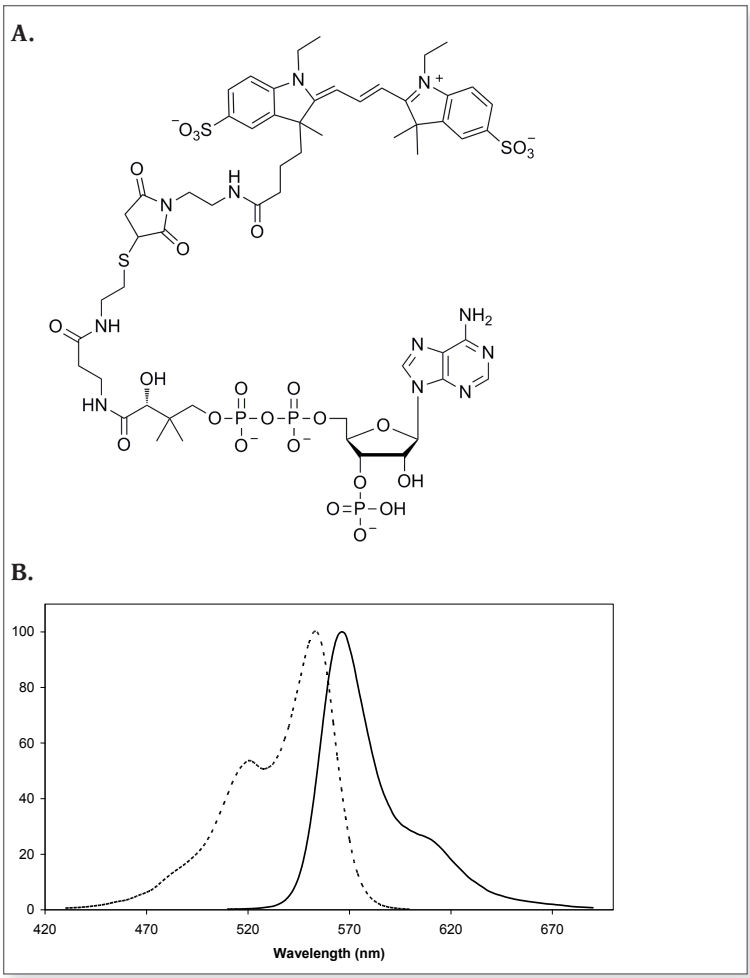
CoA 488 is a non-cell-permeable photostable green fluorescent substrate that is based on the ATTO-TEC dye ATTO 488 and is suitable for standard fluorescein filter sets. It has an excitation maximum at 506 nm and an emission maximum at 526 nm (Figure 4). This kit contains 10 nmol of CoA 488 substrate, sufficient to make 2 ml of a 5 µM ACP-tag or MCP-tag fusion protein labeling solution.

Figure 4.



Excitation (dotted line) and emission spectra of CoA 488 after coupling to ACP-tag in buffer at pH 7.5.

Figure 5.



(A) Structure of CoA 547 (MW 1528.4 g/mol), (B) Excitation (dotted line) and emission spectra of CoA 547 coupled to ACP-tag in buffer at pH 7.5.

CoA 547 is a non-cell-permeable red fluorescent substrate that is based on the Dyomics dye DY-547 and is suitable for use with standard TAMRA and Cy3 filter sets. It has an excitation maximum at 554 nm and an emission maximum at 568 nm (Figure 5). This kit contains 10 nmol of CoA 547 substrate, sufficient to make 2 ml of a 5 µM ACP-tag or MCP-tag fusion protein labeling solution.

Instructions for Cellular Labeling

Preparation of Labeling Stock Solution

Dissolve one vial of CoA substrate (10 nmol) in 10 µl of fresh DMSO to yield a labeling stock solution of 1 mM. 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 your requirements. The substrates are soluble up to at least 10 mM.

Protocol for Cell Surface Labeling Reaction

1. Dilute the labeling stock solution 1:200 in medium to yield a labeling medium of 5 µM CoA substrate. Mix dye with medium thoroughly by pipetting up and down 10 times (necessary for reducing backgrounds). 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 (1:100 dilution of supplied 1 M stock solution). Finally, add SFP Synthase (supplied) or ACP Synthase (NEB #P9301) 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.

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

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 an ACP-tag fusion protein with the labeling medium and incubate at 37°C, 5% CO_2 for 30 minutes.
3. Wash the cells three times with tissue culture medium containing serum.
4. Image the cells using an appropriate filter set. ACP-tag and MCP-tag fusion proteins labeled with CoA 488 should have an excitation maximum at 506 nm and an emission maximum at 526 nm, and can be imaged with standard fluorescein filter sets. ACP-tag and MCP-tag fusion proteins labeled with CoA 547 should have an excitation maximum at 554 nm and an emission maximum at 568 nm, and can be imaged with standard TAMRA or Cy3 filter sets.

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

Notes for Cellular Labeling

Dual labeling of ACP-tag and MCP-tag fusion proteins with different substrates

The included SFP Synthase will efficiently label both ACP-tag and MCP-tag fusion proteins displayed on the cell surface, whereas the ACP Synthase (NEB #P9301S) will modify ACP-tag fusions only. This permits sequential dual labeling of two different surface-localized proteins in the same cell, one protein expressed as an ACP-tag fusion and the other as an MCP-tag fusion.

Simply carry out the labeling procedure described above using ACP Synthase and one CoA substrate (to label the ACP-tag fusion), then wash 3 times with label-free medium to remove unreacted substrate and the synthase. Then repeat the labeling procedure with a second CoA substrate and the SFP Synthase to label the MCP-tag fusion.

Optimizing Labeling

Optimal substrate concentrations and reaction times range from 1–20 µM and 30–60 minutes, 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 1–5 µM substrate and 30 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 ACP-tag or MCP-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. As an alternative to visualize proteins with fast turnover rates, ACP-tag and MCP-tag 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 ACP-tag or MCP-tag 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 ACP-tag or MCP-tag with any fixation method.

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 labeling medium after the 30 minute incubation (step 2 above) as a DNA counterstain for nuclear visualization

of live cells. DAPI should be used for counterstaining of cells after fixation and permeabilization.

Immunocytochemistry

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

Experimental conditions that do not allow fetal calf serum

If fetal calf serum has to be omitted due to the experimental setup, the labeling can be carried out in medium without serum. Higher background levels might be observed because 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.

Instructions for Labeling of Proteins *in vitro*:

1. Dissolve the vial of CoA 488 substrate or CoA 547 substrate (10 nmol) in 10 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 Purified Protein	5 µl	5 µM
40 µM ACP or 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 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 ACP-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 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 (e.g. for redox-sensitive proteins) 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 your ACP- or MCP-tag 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 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 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.

Troubleshooting

Cloning of the Gene of Interest

If subcloning of the gene of interest into pACP-tag(m)-2 does not work, reconfirm all the cloning steps (primer design, choice of restriction site, etc.). If all steps are confirmed as being correct, try cloning using different restriction sites. Be sure to include a positive and negative control for the ligation reaction. Alternatively try to subclone the ACP-tag gene into an expression vector already containing your gene of interest.

Expression

In general we have not experienced problems expressing ACP-tag protein fusions. However if your fusion protein does not appear to be expressed, try expressing the ACP-ADRβ2 protein fusion as a positive control using cells transiently transfected with the included pACP-ADRβ2 localization control plasmid. Labeling these cells with a fluorescent CoA substrate should show strong surface-localized fluorescence. Note that the intensity of this fluorescence may vary depending on cell line and substrate used. Expression of ACP-ADRβ2 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 ACP-tag or MCP-tag fusion protein (e.g. by Western blot). If no antibody against the fusion partner is available, label cells with CoA Biotin (NEB #S9351) following the procedure above, then run a Western blot and probe with commercially available labeled streptavidin or anti-biotin antibody.

An alternate explanation may be that the terminus of the protein fused to the tag is not localized on the extracellular surface of the cell membrane and therefore the ACP-tag or MCP-tag is not available for labeling by ACP or SFP Synthase; placing the tag at the other terminus of the protein (keeping the secretion peptide at the N-terminus) may alleviate this problem.

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 within the range of 1–20 µM and 30–60 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 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 CoA substrate to surfaces. Addition of DNAse I (10 µM/ml final concentration) may also help reduce the background that may be caused by non-transfected plasmid DNA aggregating at the surfaces 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 generally not a problem as both CoA 488 and CoA 547 are very photostable. However, if you experience problems with photobleaching, addition of a commercially available anti-fade reagent may be helpful.

Appendix: Sequence of ACP-tag Region of pACP-tag(m)-2 Vector

Unique restriction sites in the regions flanking the ACPwt gene are displayed above the coding strand. The complete sequence of pACP-tag(m)-2 and pACP-ADRβ2 can be downloaded at www.neb.com.

```
 721     aaaatcaacgggactttccaaaatgtcgtaacaactccgccccattgacgcaaatgggcg

 781     gtaggcgtgtacggtgggaggtctatataagcagagctctctggctaactagagaaccca

 841     ctgcttactggcttatcgaaattaatacgactcactatagggagacccaagcttggtacc
                                                MCS1
                        ------------------------------------------------>
                         NheI   EcoRV   AscI        SwaI   BsrGI   AgeI
 901     gagctcggatcgtcGCTAGCGATATCGGCGCGCCAGCATTTAAATCTGTACAGACCGGTG
                        A  S  D  I  G  A  P  A  F  K  S  V  Q  T  G
         ------>
          EcoRI
 961     AATTCaagcttaccatgagcactatcgaagaacgcgttaagaaaattatcggcgaacagc
         E  F  K  L  T  M  S  T  I  E  E  R  V  K  K  I  I  G  E  Q

1021     tgggcgttaagcaggaagaagttaccaacaatgcttctttcgttgaagacctgggcgcgg
         L  G  V  K  Q  E  E  V  T  N  N  A  S  F  V  E  D  L  G  A

1081     attctcttgacaccgttgagctggtaatggctctggaagaagagtttgatactgagattc
         D  S  L  D  T  V  E  L  V  M  A  L  E  E  E  F  D  T  E  I

1141     cggacgaagaagctgagaaaatcaccaccgttcaggctgccattgattacatcaacggcc
         P  D  E  E  A  E  K  I  T  T  V  Q  A  A  I  D  Y  I  N  G
                                             MCS2
                 ------------------------------------------------>
                  SbfI    BamHI   PmeI        XhoI    PacI     NotI
1201     accaggcgCCTGCAGGCGGATCCGCGTTTAAACTCGAGGTTAATTAATGAGCGGCCGCata
         H  Q  A  P  A  G  G  S  A  F  K  L  E  V  N  *

1261     gataactgatccagtgtgctggaattaattcgctgtctgcgagggccagctgttggggtga

1321     gtactccctctcaaaagcgggcatgacttctgcgctaagattgtcagtttcc
```

5´ MCS

```
      NheI  EcoRV   AscI         SwaI    BsrGI   AgeI   EcoRI
         |     |       |            |        |       |      |
GTCGCTAGCGATATCGGCGCGCCAGCATTTAAATCTGTACAGACCGGTGAATTCACC
CAGCGATCGCTATAGCCGCGCGGTCGTAAATTTAGACATGTCTGGCCACTTAAGTGG
R  R     R  Y  R  R  A  S  I     I  C  T  D  R     I  H
 V  A  S  D  I  G  A  P  A  F  K  S  V  Q  T  G  E  F  T
  S  L  A  I  S  A  R  Q  H  L  N  L  Y  R  P  V  N  S  P
```

3´ MCS

```
      SbfI  BamHI       PmeI XhoI       PacI     NotI
         |     |           |    |          |        |
CCTGCAGGCGGATCCGCGTTTAAACTCGAGGTTAATTAATGAGCGGCCGC
GGACGTCCGCCTAGGCGCAAATTTGAGCTCCAATTAATTACTCGCCGGCG
 P  A  G  G  S  A  F  K  L  E  V  N        A  A  A
  L  Q  A  D  P  R  L  N  S  R  L  I  N  E  R  P
P  C  R  R  I  R  V     T  R  G     L  M  S  G  R
```

Note

NEB 10-beta Competent *E. coli* (High Efficiency) (NEB #C3019) is recommended for propagating and subcloning of the vector and control plasmid. If using the ClaI site for subcloning, *dam-/dcm-* Competent *E. coli* (NEB #C2925) is recommended. The restriction endonuclease ClaI (NEB #R0197) is methylation sensitive.

Ordering Information

PRODUCT	NEB #	SIZE
ACP-Surface Starter Kit	E9300S	10 reactions
COMPANION PRODUCTS		
pACP-tag(m)-2 Vector	N9322S	20 µg
pACP-ADRβ2 Control Plasmid	N9321S	20 µg
CoA 488	S9348S	50 nmol
CoA 547	S9349S	50 nmol
CoA 647	S9350S	50 nmol
CoA Biotin	S9351S	50 nmol
ACP Synthase	P9301S	25 nmol
SFP Synthase	P9302S	25 nmol

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