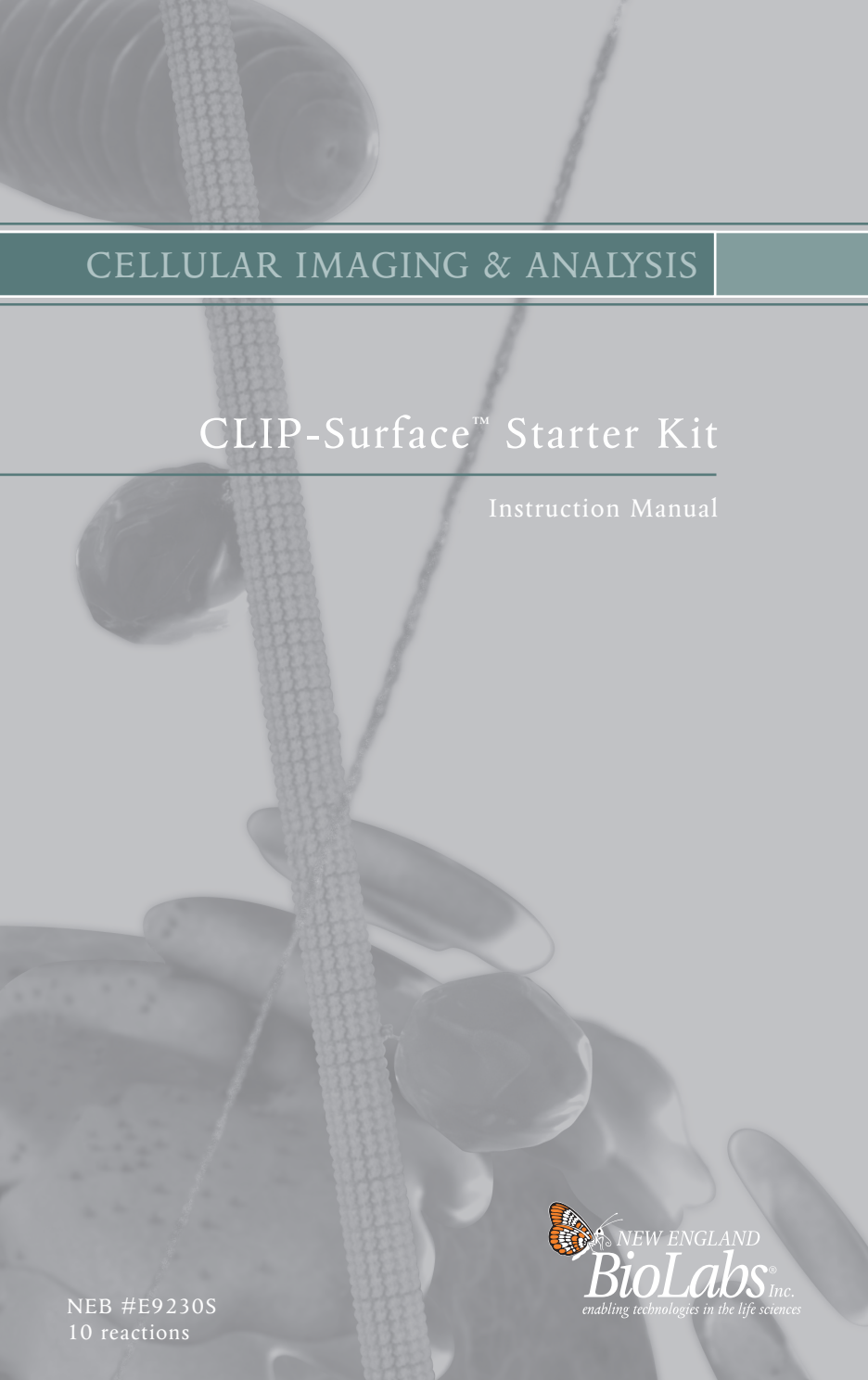


CELLULAR IMAGING & ANALYSIS

CLIP-Surface™ Starter Kit

Instruction Manual

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

NEB #E9230S
10 reactions

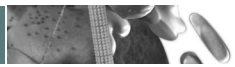


Table of Contents:

Kit Components:

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

pCLIP$_f$ Vector ..... 20 µg
Supplied in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), 0.5 µg/µl

pCLIP$_f$-NK1R Control Plasmid ..... 20 µg
Supplied in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), 0.5 µg/µl

CLIP-Surface™ 488 ..... 10 nmol
Supplied dry. Prepare a stock solution in DMSO as described on page 10.

CLIP-Surface™ 547 ..... 10 nmol
Supplied dry. Prepare a stock solution in DMSO as described on page 10.

CLIP-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 two years dry or 3 months dissolved in DMSO.

Required Materials Not Included

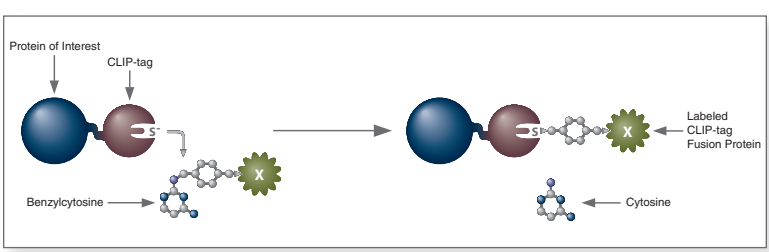
Mammalian Cell Lines
DNA Transfection Reagents
Standard Tissue Culture Media and Plasticware
DMSO
Hoechst 33342 for Nuclear Staining (optional)

Introduction:

The SNAP-tag® and CLIP-tag™ are novel tools 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 CLIP-tag is a 20 kDa mutant of the human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (hAGT) that reacts specifically and rapidly with benzylcytosine (BC) derivatives, leading to covalent labeling of the CLIP-tag with a synthetic probe (Figure 1). The CLIP-tag has a number of features that make it ideal for a variety of protein labeling applications. The rate of the reaction of the CLIP-tag with these derivatives is largely independent of the nature of the synthetic probe attached to BC, permitting the labeling of CLIP fusion proteins with a wide variety of functional groups. Many of these CLIP-tag substrates are non-cell-permeable, allowing live-cell imaging of protein expression and localization on the cell surface (Figure 2). The ability to turn on the signal at will, together with the availability of a nonfluorescent blocking agent (CLIP-Cell™ Block), allows time-resolved pulse-chase analysis of protein trafficking to the cell surface, as well as subsequent internalization. Finally, the availability of orthogonal protein labeling systems from NEB permits simultaneous labeling of multiple proteins in a single cell (SNAP-tag, another hAGT variant that reacts exclusively with O^6-benzylguanine substrates, and the ACP/MCP tags, small protein tags which can be enzymatically labeled on the cell surface with Coenzyme A derivatives).

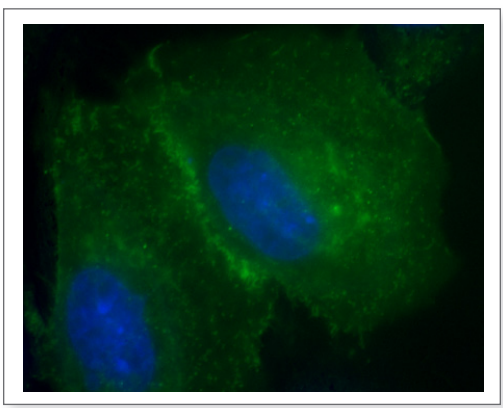
Figure 1.



CLIP-tag Reaction.

The CLIP-Surface Starter Kit contains a mammalian expression plasmid ($pCLIP_f$) encoding the CLIP-tag flanked by restriction sites for cloning a gene of interest, and two non-cell-permeable fluorescent CLIP-tag substrates. A positive control plasmid ($pCLIP_f$-NK1R), encoding a CLIP-tagged protein (neurokinin-1 receptor) with a well-characterized cell surface localization, is also included. Lastly, a negative control "blocking agent" (CLIP-Cell™ Block) is included that interacts with the CLIP-tag, but is not fluorescent. There are two steps to using this system: subcloning and expression of the protein of interest as a $CLIP_f$ fusion, and labeling of the fusion with the CLIP-tag substrate of choice.

Figure 2.



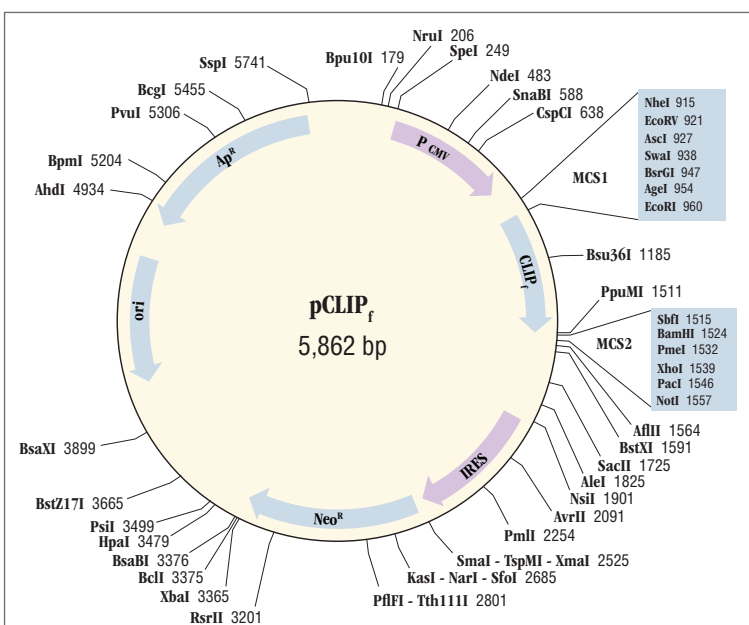
Live CHO-K1 cells transiently transfected with $pCLIP_f$-NK1R. Cells were labeled with CLIP-Surface™ 488 (green) for 30 minutes and counterstained with Hoechst 33342 (blue).

Construction and Expression of CLIP-tag Fusion Proteins:

The mammalian expression plasmid pCLIP$_f$ is intended for the cloning and stable or transient expression of CLIP-tag protein fusions in mammalian cells. This plasmid encodes the CLIP$_f$ 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 CLIP$_f$ for the efficient selection of stable transfectants. Codon usage of the gene is optimized for expression in mammalian cells. pCLIP$_f$ contains two multiple cloning sites to allow cloning of the fusion partner as a fusion to the N- or C-terminus of the CLIP-tag.

pCLIP$_f$ contains an improved version of CLIP-tag, termed CLIP$_f$. CLIP$_f$ 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.



pCLIP$_f$ plasmid map.

Detailed Description of pCLIP$_f$

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 CLIP$_f$, which is a mutant form of the human gene for O^6-alkylguanine-DNA-alkyltransferase (hAGT). The codon usage of the gene is optimized for expression in mammalian cells. In the plasmid sequence, the CLIP$_f$ gene is encoded from bp 969 to 1514.

This plasmid is intended for the cloning and stable or transient expression of CLIP-tag protein fusions in mammalian cells. It is particularly suitable for the efficient production of stable cell lines expressing CLIP-tag gene fusions. The plasmid contains the CMV promoter followed by the genes for CLIP$_f$ 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 CLIP$_f$ 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 CLIP$_f$ coding sequence, as a fusion to the N- or C-terminus of the CLIP$_f$. pCLIP$_f$ can also be used as an expression control plasmid, expressing the CLIP-tag alone, in which case the CLIP-tag protein is distributed throughout the cell. The CLIP$_f$ 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 CLIP-tag Fusions in pCLIP$_f$ Vector

Cloning by PCR

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

Note: When fusing the gene of interest to the C-terminus of CLIP$_f$, 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. Proteins of interest can be expressed with the CLIP-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 the included CLIP-Surface substrates. Note that expression of your protein of interest fused to the C-terminus of $CLIP_f$ may require addition of a surface localization signaling peptide (e.g., from the 5HT3A serotonin receptor, found on the included $pCLIP_f$-NK1R control plasmid) to the free N-terminus of $CLIP_f$.
3. 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.
4. For fusions upstream of $CLIP_f$, 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.
5. 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.
6. Care should be taken to design the cloning so that the fusion partners in the resulting construct are in frame.
7. Perform the PCR reaction and subsequent cloning steps according to established molecular biology protocols.
8. The ligated vector should be transformed into bacteria and the resulting plasmid isolated via a standard miniprep procedure.
9. After subcloning the gene of interest into $pCLIP_f$ as a fusion with the $CLIP_f$ gene, the resulting plasmid can be used for stable or transient expression of the $CLIP_f$ fusion proteins in a suitable cell line.

Direct Cloning

1. Direct cloning can also be used to make fusions with the CLIP-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 $CLIP_f$, note that there is a 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.

Expression of CLIP-tag Fusions

Transient Expression

Expression of the fusion protein cloned in pCLIP$_f$ 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 pCLIP$_f$ or pCLIP$_f$-NK1R as expression control plasmids. Figure 2 shows that the CLIP$_f$-Neurokinin-1 receptor fusion protein (from pCLIP$_f$-NK1R) gives a surface localized signal when labeled with CLIP-Surface substrates. If the empty pCLIP$_f$ plasmid is used as a control vector for transfection, a uniform distribution of the CLIP-tag between nucleus and cytoplasm should be seen (note that cell-permeable CLIP-Cell substrates must be used to visualize unfused CLIP$_f$). Both pCLIP$_f$ 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. 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

pCLIP$_f$ and pCLIP$_f$-NK1R 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 CLIP-tag expression. In addition, monoclonal cell lines can be isolated and characterized if desired.

Use of the CLIP$_f$ Control Plasmid pCLIP$_f$-NK1R:

This control plasmid contains the gene encoding the seven-transmembrane protein Neurokinin-1 receptor (NK1R), a member of the G-protein coupled receptor family. The NK1R was cloned downstream of the CLIP$_f$ coding sequence in pCLIP$_f$, as a fusion to the C-terminus of CLIP$_f$. A signal sequence for import into the endoplasmic reticulum (ER) was cloned upstream of the CLIP-tag. This import signal sequence is based on the serotonin receptor 5HT3A. As these transmembrane fusion proteins are oriented such that the CLIP-tag faces the outside of the cells, labeling using the included non-cell permeable CLIP-Surface substrates will mainly result in a plasma membrane staining pattern (Figure 2). Note that the CLIP$_f$-NK1R fusion protein gives ER, Golgi and plasma membrane labeling when labeled with cell permeable CLIP-Cell substrates. The full sequence and map for pCLIP$_f$-NK1R can be downloaded at www.neb.com.

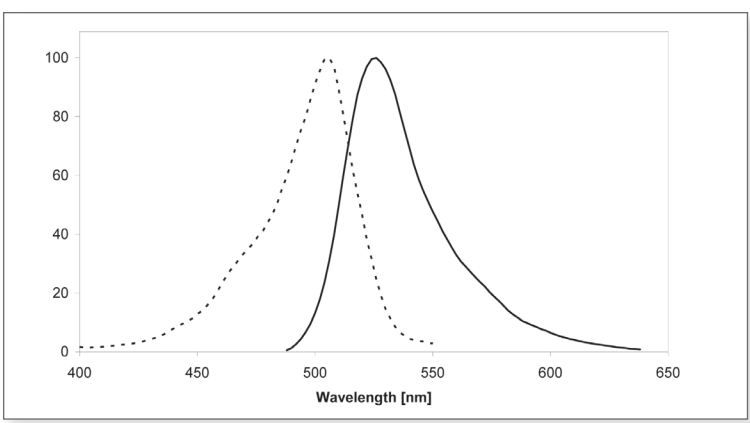
We strongly recommend carrying out parallel expression and labeling experiments with this plasmid as a positive control for your experiment, using the procedure described above.

Labeling CLIP-tag Fusion Proteins:

The kit includes two non-cell-permeable fluorescent CLIP-tag substrates, CLIP-Surface™ 488 and CLIP-Surface™ 547. Both can be used to label $CLIP_f$ fusion proteins on cell surfaces or in solution.

CLIP-Surface 488 (BC-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 CLIP-Surface 488 substrate, sufficient to make 2 ml of a 5 µM $CLIP_f$ fusion protein labeling solution.

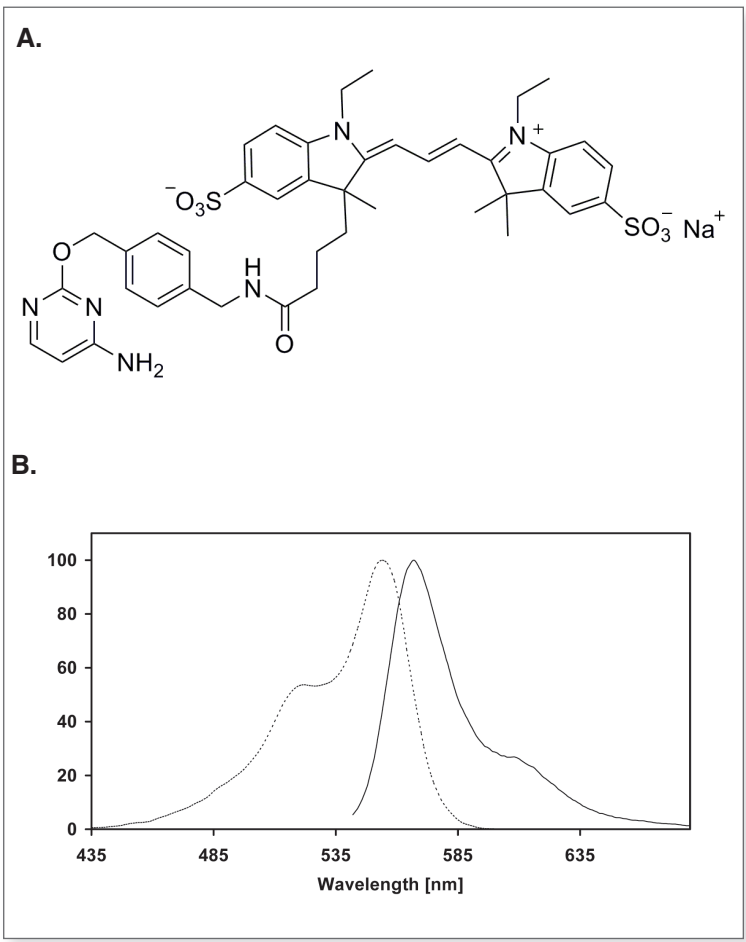
Figure 4.



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

CLIP-Surface 547 (BC-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 or 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 CLIP-Surface 547 substrate, sufficient to make 2 ml of a 5 µM $CLIP_f$ fusion protein labeling solution.

Figure 5.



(A) Structure of CLIP-Surface 547 (MW 850.98 g/mol) (B) Excitation (dotted line) and emission spectra of CLIP-Surface 547 after coupling to CLIP-tag in buffer at pH 7.5.

Instructions for Cellular Labeling

Preparation of Labeling Stock Solution

Dissolve one vial of CLIP-tag substrate in 10 µl of fresh DMSO to yield a labeling stock solution of 1 mM. Mix by vortexing for 10 minutes until all the CLIP-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 Cell Surface Labeling Reaction

1. Dilute the labeling stock solution 1:200 in medium to yield a labeling medium of 5 µM CLIP-Surface 488 or CLIP-Surface 547. Mix dye with medium thoroughly by pipetting up and down 10 times (necessary for reducing backgrounds). For best performance, add the CLIP-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 CLIP-tag substrate 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 a $CLIP_f$ fusion protein with the CLIP-tag 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. $CLIP_f$ fusion proteins labeled with CLIP-Surface 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. $CLIP_f$ fusion proteins labeled with CLIP-Surface 547 should have an excitation maximum at 554 nm and an emission maximum at 568 nm, and can be imaged with standard TAMRA and 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

Blocking Unreacted CLIP-tag with CLIP-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 CLIP-tag activity in a cell sample expressing the $CLIP_f$ fusion protein to generate a control. This can be achieved using the included nonfluorescent CLIP-tag substrate, CLIP-Cell Block (bromothenylcytosine, BTC). CLIP-Cell Block may also be used in pulse-chase experiments to block the CLIP-tag reactivity during the chase between two pulse-labeling steps. A protocol for blocking with CLIP-Cell Block can be found 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 $CLIP_f$ fusion protein. Best results are usually obtained at concentrations between 1 and 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 $CLIP_f$ 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, $CLIP_f$ 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 $CLIP_f$ 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 CLIP-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 CLIP-tag substrate for simultaneous microscopic detection. We routinely add 5 µM Hoechst 33342 to the labeling medium after the 30 minutes incubation (Step 2 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 CLIP-tag labeling and fixation of the cells according to standard protocols without loss of the CLIP-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 one vial of CLIP-Surface 488 or CLIP-Surface 547 (10 nmol) in 10 µl of fresh DMSO to yield a labeling stock solution of 1 mM CLIP-tag substrate. Mix by vortexing for 10 minutes until all the CLIP-tag 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	32 µl	
5X CLIP-tag Reaction Buffer	10 µl	1X
50 mM DTT	1 µl	1 mM
50 µM CLIP-tag Purified Protein	5 µl	5 µM
250 µM CLIP-tag 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 you may wish to separate the nonreacted substrate from the labeled $CLIP_f$ fusion protein. You can use gel filtration or dialysis. Please refer to the vendor's instructions for the separation tools you are using.

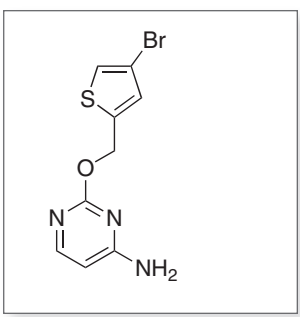
Note for Labeling in vitro

We recommend the routine addition of 1 mM DTT to all buffers used for handling, labeling and storage of the CLIP-tag. The stability of the CLIP-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. $CLIP_f$ fusion proteins can be purified before labeling, but the labeling reaction also works in non-purified protein solutions (including cell lysates).

Use of CLIP-Cell Block to Block Reactivity of CLIP$_f$ Fusion Proteins:

CLIP-Cell Block (bromothenylcytosine, BTC) is a non-fluorescent compound that blocks the reactivity of the CLIP-tag on the surface of live cells (Figure 6). It can be used to generate inactive controls in live and fixed cell labeling experiments performed with CLIP$_f$ fusion proteins. CLIP-Cell Block reacts with the CLIP-tag irreversibly, inactivating it for subsequent labeling steps. Note that CLIP-Cell Block is highly membrane permeable and once in the cell will also inactivate any intracellularly localized CLIP-tag fusions.

Figure 6.



Structure of CLIP-Cell Block (MW 286.15 g/mol)

Instructions for Use with CLIP-Surface 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 CLIP-tag activity in a cell sample expressing the CLIP$_f$ fusion protein to generate a control. This is done by a pre-incubation of the cells with CLIP-Cell Block, followed by the incubation with the labeling solution. CLIP-Cell Block may also be used in pulse-chase experiments to block the CLIP-tag reactivity during the chase between two pulse-labeling steps.

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

Preparation of Stock Solution

Dissolve one tube of CLIP-Cell Block (20 nmol) in 10 µl of fresh DMSO to give a stock solution of 2 mM. Mix by vortexing for 10 minutes, until all the CLIP-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 µM, which is a 1:200 dilution of this stock solution.

Blocking CLIP-tag Activity with CLIP-Cell Block

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

1. Prepare two cell samples suitable for labeling, each expressing the $CLIP_f$ fusion protein of interest.
2. Mix an appropriate amount of medium with CLIP-Cell Block stock solution in a ratio of 1:200 to give a blocking medium of 10 µM CLIP-Cell Block. For best performance, add the dissolved CLIP-Cell Block to complete medium, including serum. Do not prepare more medium with CLIP-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% CO_2 for 30 minutes.
5. Remove CLIP-Cell Block or DMSO-containing medium by washing both samples of cells twice with complete medium.
6. Label both cell samples with the CLIP-Surface 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 $CLIP_f$ fusion protein is present in the cell.

Note that there is a constant turnover and resynthesis of proteins in the cell. Protein transport to the membrane and internalization followed by degradation or recycling, are constantly ongoing processes. After having blocked all existing $CLIP_f$ fusion proteins on the cell membrane, newly synthesized protein may be transported to the cell surface and may get labeled during incubation with a fluorescent CLIP-Surface 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 CLIP-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 pCLIP$_f$ 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 CLIP$_f$ gene into an expression vector already containing your gene of interest.

Expression

In general we have not experienced problems expressing CLIP$_f$ protein fusions. However if your fusion protein does not appear to be expressed, try expressing the CLIP$_f$-NK1R protein fusion as a positive control using cells transiently transfected with the included pCLIP$_f$-NK1R. Labeling of such cells with a fluorescent CLIP-Surface substrate should show strong surface-localized fluorescence. The empty pCLIP$_f$ plasmid can also be used as a control (uniform cytosolic and nuclear fluorescence when using cell-permeable CLIP-Cell substrates). Note that the intensity of this fluorescence may vary depending on cell line and substrate used. Expression of 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 CLIP$_f$ fusion protein. If no antibody against the fusion partner is available, Anti-SNAP-tag Antibody (NEB #P9310) can be used. Alternatively, CLIP-Vista Green (NEB #S9235) can be used to confirm presence of CLIP$_f$ fusion in cell extracts following SDS-PAGE, without the need for Western blotting. An alternate explanation is that the CLIP$_f$ is fused to the end of the protein that is not localized on the extracellular surface of the cell membrane; reversing the orientation of the fusion protein may solve this problem.

Weak Labeling

Weak labeling may be caused by insufficient exposure of the fusion protein to the substrate. Try increasing the concentration of CLIP-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 CLIP-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 CLIP-tag from the N- to the C-terminus or vice versa. Photobleaching is generally not a problem as both CLIP-Surface 488 and CLIP-Surface 547 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 $CLIP_f$ 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 CLIP-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 CLIP-tag using CLIP-Vista Green (NEB #S9235).

If the $CLIP_f$ 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 $CLIP_f$ fusion protein, and store the fusion protein at –20˚C.

Using less than the recommended amount of substrate stock solution (1%) 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 CLIP-tag Region of pCLIP$_f$ Vector

Unique restriction sites in the regions flanking the CLIP$_f$ gene are displayed above the coding strand. The complete sequence of pCLIP$_f$ and pCLIP$_f$-NK1R can be downloaded at www.neb.com.

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

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

Ordering Information

PRODUCT	NEB #	SIZE
CLIP-Surface™ Starter Kit	E9230S	10 reactions
COMPANION PRODUCTS		
CLIP-Vista Green	S9235S	50 nmol
CLIP-Biotin	S9221S	50 nmol
pCLIP$_f$-H2B Control Plasmid	N9218S	20 µg
pCLIP$_f$-Cox8A Control Plasmid	N9217S	20 µg
CLIP-Surface™ 488	S9232S	50 nmol
CLIP-Surface™ 547	S9233S	50 nmol
CLIP-Surface™ 647	S9234S	50 nmol
CLIP-Cell™ Block	S9220S	100 nmol
CLIP-tag™ Purified Protein	P9311S	100 µg
Anti-SNAP-tag® Antibody (Polyclonal)	P9310S	100 µl

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