CLIP-Cell™ Fluorescein

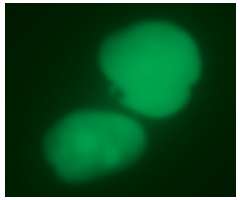
1-800-632-7799
info@neb.com
www.neb.com

S9218S 002120315031

S9218S

50 nmol **Lot: 0021203**

Store at: –20°C **Exp: 3/15**



Live CHO-K1 cells transiently transfected with pCLIP-H2B. Cells were labeled with CLIP-Cell Fluorescein (green) for 60 minutes.

Introduction

CLIP-Cell™ Fluorescein is a green fluorescent substrate that can be used to label CLIP-tag™ fusion proteins inside living cells or *in vitro*. This cell-permeable substrate (BC-PF) is based on dipivaloyl-fluorescein and is suitable for standard fluorescein filter sets. Dipivaloylfluorescein is essentially non-fluorescent, but it becomes fluorescent inside the cell when it is hydrolyzed by non-specific esterases, yielding fluorescein. It has an excitation maximum at 500 nm and emission maximum at 524 nm. This substrate has limited photostability. If this presents a problem, we recommend using CLIP-Cell 505, which has similar spectral characteristics, but much greater photostability. This package includes 50 nmol of CLIP-Cell Fluorescein substrate, sufficient to make 10 ml of a 5 µM CLIP-tag fusion protein labeling solution.

The CLIP-tag is a novel tool for protein research, allowing the specific, covalent attachment of virtually any molecule to a protein of interest. CLIP-tag is a small polypeptide based on mammalian O^6-alkylguanine-DNA-alkyltransferase (AGT). CLIP-tag substrates are derivatives of benzylcytosine (BC). In the labeling reaction, the substituted benzyl group of the substrate becomes covalently attached to the reactive cysteine of CLIP-tag forming a stable thio-ether link. Although CLIP-tag is based on the same protein as SNAP-tag®, the benzylcytosine substrates form a separate class of substrates, different

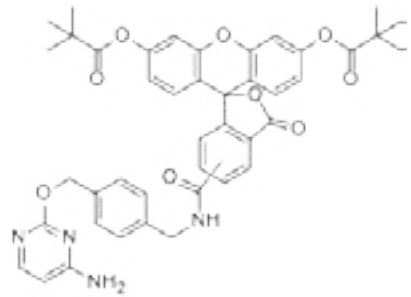


Figure 1. *Structure of CLIP-Cell Fluorescein (MW 756.8 g/mol).*

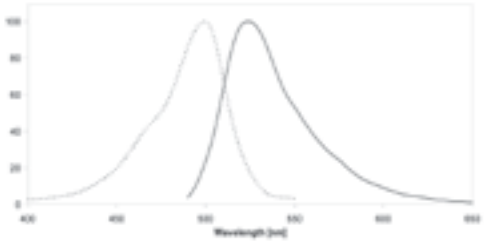


Figure 2. *Excitation (dotted line) and emission spectra of CLIP-Cell Fluorescein (fluorescent product of CLIP-Cell Fluorescein in cells) coupled to SNAP-tag / CLIP-tag in buffer at pH 7.5*

from the benzylguanine substrates recognized by SNAP-tag. CLIP-tag and SNAP-tag can be used for orthogonal simultaneous labeling.

There are two steps to using this system: subcloning and expression of the protein of interest as a CLIP-tag fusion, and labeling of the fusion with the CLIP-tag substrate of choice. Expression of CLIP-tag fusion proteins is described in the documentation supplied with CLIP-tag plasmids. The labeling of the fusion proteins with the CLIP-tag substrate is described below.

Materials Required but not Supplied:

Cells expressing CLIP-tag fusion proteins

Tissue culture materials and media

Transfection reagents

Fluorescence microscope with suitable filter set

DMSO

Storage

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

Quality Controls

Purity and Characterization: Purity of CLIP-Cell Fluorescein was determined by HPLC analysis to be 63%. Molecular weight $[M+H]^+$ was determined by MS to be 757.3 (757.3 expected).

***In vitro* protein labeling:** Reaction of CLIP-Cell Fluorescein (10 µM) with purified CLIP-tag protein (5 µM) *in vitro*, followed by mass spec analysis, indicated an efficiency of labeling of 90%.

Cellular Protein Labeling: Cells transfected with CLIP-tag vectors expressing Histone H2B (intracellular) were labeled with 5 µM CLIP-Cell Fluorescein for 60 minutes and visualized by confocal microscopy. The intracellular target was efficiently labeled.

Instructions for Cellular Labeling

CLIP-tag fusion proteins can be expressed by transient or by stable transfection. For expression of fusion proteins with CLIP-tag, refer to instructions supplied with the CLIP-tag plasmids. For cell culture and transfection methods, refer to established protocols.

Dissolve one vial of CLIP-tag substrate (50 nmol) in 50 µl of 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.

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 in DMSO up to at least 10 mM.

Protocol for Labeling Reaction:

1. Dilute the labeling stock solution 1:200 in medium to yield a labeling medium of 5 µM dye substrate. 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 will be consumed within one hour.

2. Replace the medium on the cells expressing a CLIP-tag fusion protein with the CLIP-tag 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

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).

3. Wash the cells three times with tissue culture medium with serum and incubate them in fresh medium for 30 minutes. Replace the medium one more time to remove unreacted CLIP-tag substrate that has leaked out of the cells.

4. Image the cells using an appropriate filter set. CLIP-tag fusion proteins labeled with CLIP-Cell Fluorescein should have an excitation maximum at 500 nm and an emission maximum at 524 nm, and can be imaged with standard fluorescein filter sets.

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

Notes

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-tag fusion protein to generate a control. This can be achieved using a nonfluorescent 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 is included with CLIP-Cell Block (NEB #S9220).

(see other side)

Optimizing Labeling

Optimal substrate concentrations and reaction times range from 1–10 µM and 30–60 minutes, respectively, depending on experimental conditions and expression levels of the CLIP-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 CLIP-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.

Fixation of Cells

After labeling the CLIP-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 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 medium prior to the first wash step (Step 3) as a DNA counterstain and leave this on the cells for 2 minutes prior to completing the wash steps. 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.

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 CLIP-tag fusion protein via Western blot using Anti-SNAP-tag Antibody (NEB #P9310). This antibody shows high crossreactivity with the CLIP-tag and can be used for Western blot detection. Alternatively, CLIP-Vista Green (NEB #S9235) can be used to confirm presence of CLIP-tag fusion in cell extracts following SDS-PAGE, without the need for Western blotting.

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. 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.

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

CLIP-Cell Fluorescein has only limited photostability. Plan your experimental protocol accordingly. Minimize the cells' exposure to light during and after labeling and to the excitation light. If problems with photobleaching are experienced when working with labeled fixed cells, addition of a commercially available anti-fade reagent may be helpful.

If the fluorescence signal decreases rapidly, it could also 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.

Instructions for Labeling of Proteins *in vitro*:

1. Dissolve the vial of CLIP-Cell Fluorescein substrate (50 nmol) in 50 µl of DMSO to yield a 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 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 CLIP-tag 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 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 if handling at temperatures above 4°C is minimized.

CLIP-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 CLIP-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 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 poor labeling continues, we recommend checking the activity of the CLIP-tag using CLIP-Vista Green.

If the CLIP-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 CLIP-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, you can try reducing the labeling time or decreasing the labeling temperature. We recommend overnight incubation when labeling at 4°C.