# BC-$NH_2$

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S9236S 004121115111

# S9236S

**2 mg** **Lot: 0041211**

**Store at: –20°C** **Exp: 11/15**

## Introduction

$BC\text{-}NH_2$ is an amine-terminated building block for the one-step synthesis of CLIP-tag™ substrates from NHS esters or other activated carboxylic esters of labels or surfaces. This building block allows you to make custom CLIP-tag substrates for labeling CLIP-tag fusion proteins. The BC-NH2 is particularly useful for the synthesis of fluorescent CLIP-tag substrates.

The CLIP-tag protein labeling system enables the specific, covalent attachment of virtu-ally any molecule to a protein of interest. CLIP-tag is a protein tag based on human $O^6$-alkylguanine-DNA-alkyltransferase (hAGT). CLIP-tag substrates are derivatives of benzylcytosine (BC). In the labeling reaction, the substituted benzyl group of the substrate is covalently attached to the reactive cysteine of CLIP-tag forming a stable thioether bond. Although CLIP-tag is based on the same protein as SNAP-tag®, the benzylcytosine substrates form a separate class of substrates, different from the benzylguanine substrates recognized by SNAP-tag. CLIP-tag and SNAP-tag can be used for orthogonal and complementary labeling of two proteins simultaneously in the same cells.

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 in this document.

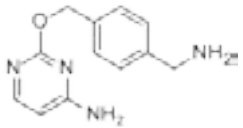

***Figure 1.*** *Structure of $BC\text{-}NH_2$ (MW 230.3 g/mol)*

## Storage

Dry $BC\text{-}NH_2$ should be stored at –20°C

The $BC\text{-}NH_2$ building block is soluble in anhydrous DMF. Choose a solvent volume that will be compatible with the final use. Heating at 37°C, vortexing and/or sonication will aid dissolution. Unused $BC\text{-}NH_2$ substrate dissolved in DMF should be stored at –20°C.

## Quality Control

**Purity and Characterization:** Purity of $BC\text{-}NH_2$ was determined to be 97% by HPLC analysis. Molecular weight $[M+H]^+$ was determined by MS to be 231.1 (231.1 expected)

## Reaction Conditions for Chemical Coupling

We recommend that coupling reactions between $BC\text{-}NH_2$ and NHS esters take place in anhydrous N,N-dimethylformamide (DMF) using triethylamine as a base.

Coupling reactions typically use an excess of the $BC\text{-}NH_2$ (1.5 equivalents) relative to the desired NHS label (1 equivalent) at 5–20 mM final concentration. Add a 1.5-fold molar excess of triethylamine in DMF. The reaction is generally performed at 30°C overnight.

The purification strategy will depend on the label used. Good results have been obtained with both HPLC and silica gel chromatography.

**Example Reaction: Coupling of $BC\text{-}NH_2$ to Alexa Fluor® 350 NHS-ester:**

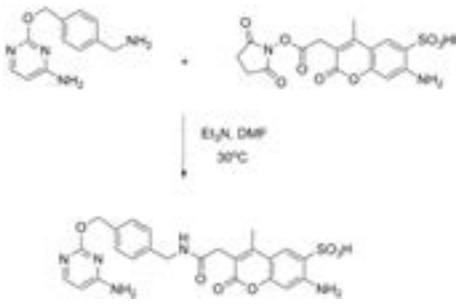

***Figure 2.*** *Coupling of $BC\text{-}NH_2$ to Alexa Fluor® 350 NHS ester. A solution of 2.4 mg (5.8 µmol) Alexa 350 NHS-ester in 500 µl DMF was added to 2.0 mg (8.7 µmol) $BC\text{-}NH_2$ and 1.2 µl triethylamine (8.7 µmol) in 500 µl DMF. The reaction was shaken at 30°C overnight. The solvent was evaporated under vacuum. The reaction was diluted in 1 ml water / acetonitrile (9:1) and the product purified by reverse phase HPLC.*

## Protocol for Labeling CLIP-tag Fusion Proteins with BC-substrates

Once the building block has been converted into a BC-substrate it can be used to label CLIP-tag fusion proteins. These can be labeled in cell lysates or as purified protein.

For labeling, we recommend using a 1.5 fold excess of substrate to CLIP-tag fusion protein and incubation for 1 hour at room temperature. Typical concentrations are 30 µM substrate and 20 µM CLIP-tag fusion protein. The labeling incubation can be followed by a separation step such as dialysis or spin column separation to remove unreacted substrate, if desired.

## Notes

Unlabeled protein samples should be stored at –20°C, or at –80°C for long-term storage. Many proteins benefit from the addition of glycerol for frozen storage, typically 20% v/v. Handling at temperatures above 0°C should be minimized by thawing the unlabeled protein samples shortly before use, and keeping them on ice until just before the labeling reaction.

We recommend the routine addition of 1 mM DTT to all buffers used for handling, labeling and storage of CLIP-tag fusion proteins. The stability of the CLIP-tag is improved in the presence of reducing agents; however it can also be labeled in their absence.

If a particular fusion protein requires buffers without reducing agents, minimize all handling steps of the protein above 4°C prior to the labeling reaction.

The CLIP-tag labeling reaction works well between pH 5.0 and pH 10.0 The salt concentration may need to be optimized for your particular fusion protein, although the CLIP-tag labeling reaction has been shown to work at a broad range of ionic strengths (NaCl concentrations between 15 mM and 1 M). If the buffer conditions used for labeling lead to insolubility of your protein, we recommend testing a range of pH and ionic strengths. For a range of fusion proteins we have found ionic strengths for monovalent salts (e.g. sodium chloride) from 50 mM to 250 mM helpful.

Where stickiness of the fusion protein is a problem we recommend adding Tween 20 at a final concentration of 0.05% to 0.1%. At this concentration Tween 20 does not affect the performance of the CLIP-tag. Ionic detergents (e.g. SDS) should be avoided.

The maximum degree of labeling possible with the CLIP-tag is one molecule of label per molecule of fusion protein.

## Storage and Handling of Labeled CLIP-tag Fusion Proteins

Labeled CLIP-tag fusion proteins should be stored at concentrations of 1 mg/ml or less at 4°C. Sodium azide may be added to 2 mM final concentration to prevent bacterial growth. Under these conditions the labeled protein should be stable at 2–6°C for several months. If freezing is required, it may be best to shock freeze by rapidly cooling the sample to a temperature significantly below 0°C in the presence of 20% (v/v) glycerol.

Labeled protein is linked to the CLIP-tag label by a covalent bond. Therefore the conjugates are highly stable. DTT or other reducing agents are not required in the storage buffers. Since the fusion protein is covalently labeled, labeled protein may be detected after harsh or even denaturing treatments, such as chromatographic or electrophoretic separation including SDS-PAGE.

Avoid repeated freezing and thawing. Protect from light if the protein is labeled with a fluorophore.

## Troubleshooting

### Labeling Reaction

If exhaustive labeling of a protein sample is not achieved using the recommended conditions, try the following protocol modifications: Double the incubation time to two hours total at 25°C or to 24 hours at 4°C; or double the ratio of label to protein in the labeling reaction. Both approaches may be combined. If you still have poor labeling results, we recommend checking the activity of the CLIP-tag.

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.

### Inefficient Labeling

DMSO at greater than 1% of the volume of protein solution may slow the reaction rate. Lower substrate concentrations also slow the reaction rate. Increasing the reaction time may be helpful in these cases.

*(see other side)*

Activity of the CLIP-tag can sometimes be partially or completely lost. This can be caused by extended storage of non-reacted CLIP-tag fusion proteins at 4°C or greater. The sensitivity of the CLIP-tag to inactivation is also significantly increased if no reducing agent is added.

If you believe that the activity of the CLIP-tag is affected, we recommend analyzing a small fraction of it on an SDS-PAGE gel using the CLIP-Vista Green (NEB #S9235S) to confirm that the CLIP-tag is active.

If you encounter problems with the activity we recommend thawing another sample of your protein or reexpressing and repurifying the CLIP-tag fusion protein following the advice given in the CLIP-tag plasmid instructions.

**Reference**

1. Gautier A. et al. (2008) *Chemistry & Biology,* 15, 128-136.