Avoid freeze / thaw cycles

For in vitro use only
Quality guaranteed for 12 months
Store at -20°C

Keto-Fluorescein and Coumarin-Thioester must be stored in the dark

### Description
Fluorescein/Coumarin Protein-Dual-Labeling Kit provides a highly efficient and easy-to-handle tool for one-pot dual-labeling of proteins via two chemoselective reactions. The C-terminal modification of the protein is based on a fast oxymineketone ligation. For N-terminal labeling, the N-terminal cysteine undergoes a native chemical ligation with a fluorophorethioester.

The kit contains Keto-Fluorescein and Coumarin-Thioester as fluorescent labels.

### Background
Multi-color labeling is a valuable technique for the characterization of proteins with respect to their structure, folding and interactions both as single molecules and in cellular investigations. The key technique for such studies is based on fluorescence resonance energy transfer (FRET). FRET applications require the attachment of donor and acceptor molecules to specific sites of a given protein or proteins. Such labeling is typically achieved through conjugation at cysteine residues or amino groups or by genetic fusion to different fluorescent proteins. Recent advancements in chemical methods have substantially expanded tools that are available for site-specific modification of proteins. However, site-specific incorporation of multiple fluorophores into a single protein remains a considerable challenge. This kit provides an efficient method for C- and N-terminal dual-labeling of proteins based on chemoselective reactions.

### Kit contents

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTWIN Vector (red cap)</td>
<td>5 µg modified pTWIN vector</td>
</tr>
<tr>
<td>Bisoxymine (blue cap)</td>
<td>111 µl 0.9 M in Reaction Buffer</td>
</tr>
<tr>
<td>MPAA (white cap)</td>
<td>35 mg (4-carboxymethyl) thiophenol</td>
</tr>
<tr>
<td>MESNA (yellow cap)</td>
<td>35 mg (Sodium 2-mercaptoethanesulfonate)</td>
</tr>
<tr>
<td>Keto-Fluorescein (purple cap)</td>
<td>7.5 µl 100 mM</td>
</tr>
<tr>
<td>Coumarin-Thioester (purple cap)</td>
<td>12 µl 50 mM</td>
</tr>
<tr>
<td>Aniline</td>
<td>10 mg</td>
</tr>
<tr>
<td>TEV protease (N’6His-modified)</td>
<td>230 µg</td>
</tr>
</tbody>
</table>

### Structure

![Structure Diagram](attachment:image.png)

**Bisoxymine**

**MPAA (4-carboxymethyl) thiophenol**

**Keto-Fluorescein**

**Coumarin-Thioester**
Protein Dual-Labeling Kit
Universal C/N-terminal Protein Labeling with Fluorescein/Coumarin

Protein Labeling

**Spectroscopic data of fluorescein**
Excitation maximum: $\lambda_{ex} = 495$ nm  
Emission maximum: $\lambda_{em} = 520$ nm

**Spectroscopic data of coumarin**
Excitation maximum: $\lambda_{ex} = 440$ nm  
Emission maximum: $\lambda_{em} = 475$ nm

**Procedure**

Preparation of proteins with N-terminal cysteine and C-terminal oxyamine.

1. Clone target gene containing N-terminal tobacco etch virus (TEV) protease recognition site (ENLYFQKC) into modified pTWIN vector using Ndel and SapI sites.
2. Express fusion protein (TEV-Cys-proteinIntein-His) in BL21 (DE3) cells.
3. Collect cells in 25 mL icecold **Breaking Buffer** freshly supplemented with 1 mM PMSF. CRITICAL: PMSF should be added freshly. Don’t add any reducing substances.
4. Lyse cells using a microfluidizer or ultrasonication.
5. Add 1% Triton X-100 to cell lysate and centrifuge at 35,000 rpm, 4°C for 30 min.
6. Filter supernatant through a 0.2 µm filter.
7. Load cell lysate onto a Ni-NTA column equilibrated with Buffer A.
8. Wash column with Buffer A and continue with 2% Buffer B until absorbance reaches baseline.
9. Elute column with a gradient of 2% Buffer A and 100% Buffer B. Collect eluted fractions.
10. Identify and collect fractions of interest by SDS-PAGE.
11. Add MESNA powder to protein solution to a concentration of 0.5 M and incubate overnight at 20°C (intein cleavage).
12. Dilute solution with 5-fold volume of Buffer A.
13. Load onto a Ni-NTA column equilibrated with Buffer A containing 10 mM MESNA. Collect flowthrough
14. Wash column with 2.5% Buffer B containing 10 mM MESNA. Collect and pool flowthrough and concentrate protein.
15. Run a gel filtration on a Superdex column using Elution Buffer. CRITICAL: Prepare fresh solution, filter buffer through a 0.2 µm filter and degas on a vacuum-membrane pump by stirring for 0.5 h at room temperature.
16. Identify and collect fractions of interest by SDS-PAGE. Concentrate protein.
17. Incubate 200 µL Cys-proteinIntein-Thioester (5-25 mg/mL) with 111 µL Bisoxymine (0.9 M stock solution in reaction buffer, final 333 mM) in Reaction Buffer on ice overnight.
   The reaction is monitored by ESI-MS.
18. Dialyze protein twice against 1 L **Dialysis Buffer** at 4°C.
19. Incubate 50 µM resulting TEVCys-protein-ONH₂ with 5 µM TEV protease for 1 h at room temperature.
20. Load solution onto a Ni-NTA column equilibrated with Buffer A containing 2 mM β-mercaptoethanol. Collect flowthrough.
21. Wash column with 2.5% Buffer B containing 2 mM β-mercaptoethanol. Collect and pool flowthrough and concentrate protein.
22. Remove peptide and exchange buffer using a desalting column pre-equilibrated with Elution Buffer.

Protein N- and C-terminal Labeling

23. Incubate 50 µM Cys-protein-ONH₂ with 0.5 mM Coumarin-Thioester and 0.5 mM Keto-Fluorescein in the presence of 200 mM MPAA and 100 mM Aniline in Reaction Buffer on ice for 24 h. The reaction is monitored by ESI-MS.
24. Remove excess dyes using a desalting column pre-equilibrated with Dialysis Buffer.
Protein Dual-Labeling Kit
Universal C/N-terminal Protein Labeling with Fluorescein/Coumarin

Protein Labeling

To be provided

Breaking Buffer
25 mM NaH₂PO₄ pH 7.5, 0.5 M NaCl

PMSF

Triton X-100

Buffer A
50 mM NaH₂PO₄ pH 8.0, 0.3 M NaCl

Buffer B
50 mM NaH₂PO₄ pH 8.0, 0.3 M NaCl, 0.5 M imidazole

Elution Buffer
25 mM NaoPi pH 7.2, 30 mM NaCl, 10 mM MESNA

Reaction Buffer
30 mM NaoPi pH 7.5, 50 mM NaCl

Dialysis Buffer
30 mM Naphosphate pH 7.5, 50 mM NaCl, 2 mM DTE

β-mercaptoethanol

Elution Buffer
30 mM Naphosphate, pH 7.0, 50 mM NaCl
Protein Dual-Labeling Kit

Universal C/N-terminal Protein Labeling with Fluorescein/Coumarin

Protein Labeling

Selected References