Buccutite™ Rapid Protein Crosslinking Kit *Microscale Optimized for Crosslinking 100 ug Antibody Per Reaction*

Catalog number: 1315
Unit size: 2 Labelings

Component	Storage	Amount
Component A: Buccutite™ MTA	Freeze (< -15 °C), Minimize light exposure	2 Vials (lyophilized)
Component B: Buccutite™ FOL	Freeze (< -15 °C), Minimize light exposure	2 Vials (lyophilized)
Component C: Reaction Buffer	Refrigerated (2-8 °C), Minimize light exposure	1 Vial (50 µL)
Component D: Spin Column	Refrigerated (2-8 °C)	4 Columns

OVERVIEW

Protein-protein conjugations are commonly performed with a bifunctional linker such as SMCC. One end of the SMCC reacts (via NHS ester) with amines (-NH2) found in the amino acid lysine and N-terminus, and the other end reacts (via maleimide) with the thiol groups (-SH) found in the amino acid cysteine. However, SMCC-modified protein is extremely unstable and often self-reactive since proteins often contain both amine and thiol groups that cause significant amount of homo-crosslinking. In addition it is quite difficult and tedious to quantify the number of maleimide groups on a protein. AAT Bioquest has developed a convenient and effective crosslinking method to link two biomolecules with a high conjugation yield. Our method uses one pair of crosslinkers: Buccutite™ MTA and Buccutite™ FOL. MTA is added to one protein, while FOL is added to another protein. Protein-protein cross-linking reaction is initiated by mixing Protein-1-Buccutite ™ MTA and Protein-2-Buccutite ™ FOL. This crosslinking reaction occurs under extremely mild neutral conditions without any catalyst required, and it is robust and efficient.

AT A GLANCE

Protocol Summary

1. Prepare protein solutions
2. Run protein-1 Buccutite™ MTA reaction
3. Run protein-2 Buccutite™ FOL reaction
4. Cross-link protein-1 and protein-2 reactions by mixing and incubating at room temperature

Important Upon receipt, store component A and B at -20 ° C. When stored properly, the kit should be stable for six months. Do not freeze Reaction Buffer (Component C) and Spin Column (Component D). Warm all the components and centrifuge the vials briefly before opening, and immediately prepare the required solutions before starting your conjugation. The following SOP is an example for conjugate 100 ug protein-1 with protein 2. *Note:* Key parameters to achieve best performance:

1. Protein molecular weight: >25,000 Daltons
2. Protein concentration: >=1 mg/mL
3. Protein sample volume: 60~120 µL

PREPARATION OF WORKING SOLUTION

Protein working solution

For labeling 100 µg protein-1 and protein-2 (assuming the concentrations are 1 mg/mL for both proteins), mix 5 µL (5% of the total reaction volume) of Reaction Buffer (Component C) with 100 µL of the each protein solution.

Note The protein should be dissolved in 1X phosphate buffered saline (PBS), pH 7.2-7.4; If it is dissolved in glycine buffer, it must be dialyzed against 1X PBS, pH 7.2-7.4, or use Amicon Ultra-0.5, Ultracel-10 Membrane, 10 kDa (cat# UFC501008 from Millipore) to remove free amines or ammonium salts (such as ammonium sulfate and ammonium acetate) that are widely used for protein precipitation.

Note Stabilizers like bovine serum albumin (BSA) or gelatin will affect the labeling reaction.

SAMPLE EXPERIMENTAL PROTOCOL

Run Protein 1-Buccutite™ MTA reaction

1. Add 105 µL Protein-1 solution directly into the vial of Buccutite ™ MTA (Component A), and mix them well by repeatedly pipetting for a few times or vortex the vial for a few seconds.

2. Keep the Protein-1- Buccutite ™ MTA reaction mixture at room temperature for 30 - 60 minutes. The Antibody-Buccutite™ MTA reaction mixture can be rotated or shaken for longer time if desired.

3. Purify Protein-1- Buccutite ™ MTA through desalting column. Please refer to “Prepare spin column for Antibody purification” part for detailed operations.

4. Calculate the concentration of the Protein-1- Buccutite ™ MTA with 75% yield after desalting. (For example: if starting with 100 µg protein, after desalting column purification, the recovery protein amount is ~75 µg.)

Run Protein 2-Buccutite™ FOL reaction

1. Add the 105 µL Protein-2 solution directly into the vial of Buccutite ™ FOL (Component B), and mix them well by repeatedly pipetting for a few times or vortex the vial for a few seconds.

2. Keep the Protein-2- Buccutite ™ FOL reaction mixture at room temperature for 30 - 60 minutes. The Antibody-Buccutite™ FOL reaction mixture can be rotated or shaken for longer time if desired.

3. Purify Protein-2- Buccutite ™ FOL through desalting column. Please refer to “Prepare spin column for Antibody purification” part for detailed operations.

4. Calculate the concentration of the Protein-2- Buccutite ™ FOL with 75% yield after desalting.

Purify the antibody-Buccutite™ MTA solution

1. Place the column (from Step Prepare spin column for antibody-Buccutite™ MTA purification) in a clean Collecting Tube (1.5 mL, not provided). Carefully load the sample (~105 µL, from Step Antibody-Buccutite™ MTA reaction) directly to the center of the column.

2. After loading the sample, add 5 µL of 1X PBS (pH 7.2-7.4) to make the total volume of 110 µL. Centrifuge the column for 5-6 minutes at 1,000 x g, and collect the solution that contains the desired antibody-Buccutite™ MTA solution.

Cross-linking Protein-1 and Protein-2 Reaction

1. Cross-linking reaction is initiated by mixing Protein-1- Buccutite ™ MTA and Protein-2- Buccutite ™ FOL at the desired molar ratio. Usually, Buccutite ™ FOL-modified Protein-2 is mixed with Buccutite ™ MTA-modified Protein-1 in 1.5-2.0 molar ratio to drive the crosslinking reaction to completion. The mixing ratio can be reversed depending on your downstream applications, and the cost of your proteins.

2. Rotate the mixture for 1~2 hour at room temperature or stay at 4 °C overnight. The reaction mixture is now ready to use, or to be stored at 4 °C. Desalting is optional.

Optional: Conjugate Analysis and Purification

1. A small amount of reaction mixture (for example: 2~4 mg) could be analyzed using 4-12% Bis-Tis Protein Gel in a SDS running buffer system to check the conjugation result.

2. The conjugation reaction mixture contains the desired conjugate along with small amount of unlinked Protein-2 (used in excess). If required, the reaction mixture can be purified by size exclusion chromatography (SEC), and the desired conjugate fractions are pooled and combined.

Prepare spin column for purification

1. Invert the provided spin column (Component D) several times to re-suspend the settled gel and remove any bubbles.

2. Snap off the tip and place the column in a washing tube (2 mL, not provided). Remove the cap to allow the excess packing buffer to drain by gravity to the top of the gel bed. If column does not begin to flow, push cap back into column and remove it again to start the flow. Discard the drained buffer, and then place the column back into the Washing Tube. However, centrifuge immediately if the column is placed into a 12 x 75 mm test tube (not provided).

3. Centrifuge for 2 minutes in a swinging bucket centrifuge at 1,000 x g (see Centrifugation Notes section) to remove the packing buffer. Discard the buffer.

4. Apply 1-2 mL 1X PBS (pH 7.2-7.4) to the column. After each application of PBS, let the buffer drain out by gravity, or centrifuge the column for 2 minutes to remove the buffer. Discard the buffer from the collection tube. Repeat this process for 3-4 times.

5. Centrifuge for 2 minutes in a swinging bucket centrifuge at 1,000 x g (see Centrifugation Notes section) to remove the packing buffer. Discard the buffer.

6. Place the column in a clean Collecting Tube (1.5 mL, not provided). Carefully load the sample (~105 µL) directly to the center of the column.

7. After loading the sample, add 10 µL of 1X PBS (pH 7.2-7.4), centrifuge the column for 5-6 minutes at 1,000 x g, and collect the solution that contains the desired protein-1-Buccutite™ MTA solution or Protein-2- Buccutite ™ FOL solution.

Centrifugation Notes

Spin column (Component D) can fit into 2 mL microcentrifuge tubes or 12 x 75 mm test tubes for sample collection during centrifugation. Use the 2 mL microtube with the columns for the initial column equilibration step.

Swinging bucket centrifuges capable of generating a minimum force of 1,000 x g are suitable for Bio-Spin column use. The gravitational force created at a particular revolution speed is a function of the radius of the microcentrifuge rotor. Consult the swinging bucket centrifuge instruction manual for the information about conversion from revolutions per minute (RPM) to centrifugal or g-force. Alternatively, use the following equation to calculate the speed in RPM required to reach the gravitational force of 1,000 x g:

$$\mathbf{RCF\ (x\ g) = (1.12 \times 10^{-5})\times(RPM)\times 2\times r}$$

RCF is the relative centrifugal force

r is the radius in centimeters measured from the center of the rotor to the middle of the Bio-Spin column

RPM is the speed of the rotor

EXAMPLE DATA ANALYSIS AND FIGURES

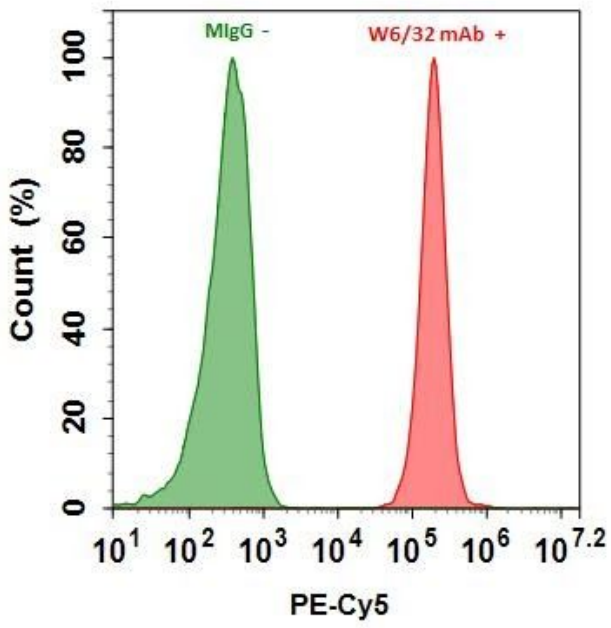

Figure 1.
Flow cytometry analysis of HL-60 cells stained with 1ug/ml Mouse IgG control (Green) or with 1ug/ml mouse Anti-Human HLA-ABC (W6/32 mAb) (Red) and then followed by Goat Anti-Mouse IgG-PE-Cy5 conjugate prepared with Buccutite™ Rapid PE-Cy5 Tandem Antibody Labeling Kit (Cat#1315).

DISCLAIMER

AAT Bioquest provides high-quality reagents and materials for research use only. For proper handling of potentially hazardous chemicals, please consult the Safety Data Sheet (SDS) provided for the product. Chemical analysis and/or reverse engineering of any kit or its components is strictly prohibited without written permission from AAT Bioquest. Please call 408-733-1055 or email info@aatbio.com if you have any questions.