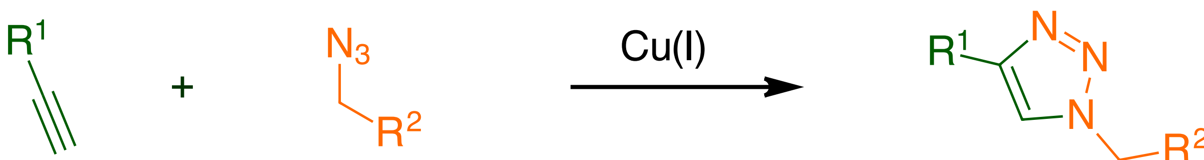
Protocol for conjugation of alkyne- or azide-modified proteins with dye azides or alkynes

Intended for the CuAAC reaction with proteins, protein labeling buffer protects biomolecules from being damaged by reactive oxygen species. This reaction can be used for proteins with alkyne or azide groups, as well as cells or components of cell lysates metabolically labeled with azide groups.

Azide is conjugated to terminal alkyne, resulting in a five-membered heterocycle (1,2,3-triazole). Both groups (azide and alkyne) are extremely rarely found in natural biomolecules, so the reaction is highly specific and effective to handle various tasks.



The reaction proceeds in the presence of copper (I) compounds and almost does not depend on pH. Optimized for operations with proteins, protein labeling buffer contains a salt of copper (II) (a stable precursor of catalytically-active copper (I) compounds), triethylammonium acetate pH 6.8, water-soluble THPTA ligand, and aminoguanidine. It is recommended to use a freshly prepared solution of ascorbic acid to reduce copper (II). THPTA ligand in protein labeling buffer speeds up the reaction by stabilizing catalytically-active compounds of copper (I). The presence of THPTA ligand also allows protein labeling in water medium (without organic solvents) and, owing to stabilization of copper (I) oxidation degree, minimizes production of reactive oxygen species (RAS) and prevents them from damaging proteins by oxidizing histidine, methionine and cysteine. Aminoguanidine in protein labeling buffer prevents chemically reactive aldehydes (dehydroascorbate hydrolysis products) from binding to side chains of arginine, N-terminal cysteine, and lysine.

For this reaction, you will need alkyne- or azide-modified protein in azide sodium-free buffer, dye azide or alkyne, 1.5x protein labeling buffer, and ascorbic acid. It is recommended to perform steps 6 to 9 under an inert gas (nitrogen or argon).

Protocol

We recommend the following protocol for conjugation of modified proteins with dye derivatives:

1. Determine total reaction volume based on the amount of modified protein to be used:

   ! The volume of alkyne- or azide-modified protein solution should be not more than 1/3 of total reaction volume.

Total reaction volume, μL	Amount of protein
100	4 to 20 nmol
200	20 to 40 nmol
400	40 to 80 nmol
600	80 to 600 nmol

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2. Calculate volumes of the reagents for the labeling reaction using the table below:

Reagent	Volume, µL	Concentration of stock solution
Dye azide or alkyne	(amount of protein [nmol]) × 0.3*	10 mM in DMSO or water
Protein labeling buffer	(total reaction volume [µL]) × 0.67	1.5x
Activator (ascorbic acid)	(total reaction volume [µL]) × 0.02	50 mM in water
Water	(total reaction volume [µL] – volume of dye solution [µL] – volume of protein labeling buffer [µL] – volume of activator solution [µL])	—

** the dye excess may vary depending on the number of azide or alkyne groups on the protein molecule. Calculations in the table are shown for 3x excess of the dye. For 1.5–10x excess of the dye, multiply protein amount (nmol) by 0.15–1. But remember that if you use an azide or alkyne that is not soluble in water, in a big excess it may precipitate from the reaction mixture. Use water-soluble dyes, for example, sulfonated cyanine dyes* sulfo-Cyanine.

3. Prepare stock solution of *dye azide or alkyne* (10 mM in DMSO or water, for water-soluble alkynes and azides) and *activator* (ascorbic acid, 50 mM in water).

   Bear in mind that ascorbic acid is readily oxidizable in air. Use only a freshly prepared solution of *activator* (the solution is stable within 1 day). To prepare stock solution, dissolve 10 mg of ascorbic acid in 1.1 mL of water.
4. Add *protein labeling buffer* to modified protein solution and vortex.
5. Add the calculated volume of stock solution of *dye azide or alkyne* and vortex well again.
6. *(recommended)* Degas the mixture to remove oxygen. To do so, connect a disposable pipette tip to a plastic or silicone tubing connected to the pressure regulator of a gas cylinder with inert gas (argon or nitrogen). Turn on a very weak gas flow and put the tip down in the tube so that it can be 3–10 mm higher than the liquid level avoiding touching the liquid and tube walls. The gas flow should make a swirl in the liquid without spattering it. Keep the tip in this position for 10–20 seconds.

   If several labeling reactions are run simultaneously, a centrifugal concentrator can be used for degassing. To do so, place the tubes in the concentrator, turn on rotation, turn on vacuum for 30–40 seconds, then turn off vacuum while feeding inert gas to the input of the system.
7. Add *activator solution (ascorbic acid)*, then purge the tube with inert gas for a few seconds and close it.
8. Vortex the solution.
9. Allow the mixture to stand at room temperature for 8–16 h.
10. Use dialysis or size-exclusion chromatography to isolate the dye-protein conjugate.

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