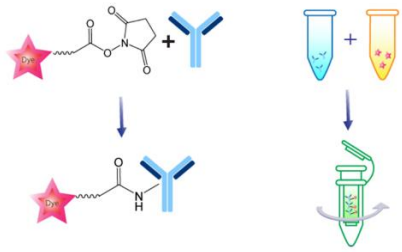
GENERAL PROTOCOL OF DYE NHS ANTIBODY LABELING KIT

Overview

Broadpharm Antibody Conjugation Kits contain most popular sulfonated reactive dyes which are most suitable for the labeling antibodies and other sensitive proteins. The kits provide optimized reagents for labeling antibodies having 2.5-3 labels per antibody, and desalting columns for purifying the labeled molecule. In the reaction, the dye NHS ester is a controlled excess reaction component. It reacts with the amine groups of lysine of the antibody at mild alkaline pH. The purification of antibody is achieved by size exclusion filtration using centrifugal concentrator. The 100 µg kit provides sufficient dye for labeling 50-200 µg of antibody in ~100 µl volume, and the 1 mg kit can label 1-5 mg of antibody in ~500 µl volume.

Note: The kits are not recommended for labeling IgM antibodies, because IgM may denature in alkaline reaction conditions. The kits can be used to label non-immunoglobulin proteins as well. The ratio of dye stock solution to protein amount may require optimization for different proteins. Depending on the protein molecular weight, the dye removal method may need to be modified. To remove free dye by ultrafiltration, the protein should be at least 3X larger than the molecular weight cut-off (MWCO) of the ultrafiltration membrane. For ultrafiltration of proteins with molecular weight of 10-30 kDa, using 3 kDa MWCO ultrafiltration vials are recommended.



Note: Upon receipt, store components NHS at -20°C, and store reaction buffer at 4°C. Allow all the components to reach room temperature, centrifuge the vials briefly before opening, and immediately prepare the required solutions before starting your conjugation. The following SOP is an example for labeling goat anti-mouse IgG antibody.

PREPARATION OF WORKING SOLUTION

1. Antibody working solution: the preferred antibody concentration is 2 mg/ml in supplied reaction buffer component.

   Note:
   - Reaction buffer for BP Fluor 488 and fluorescein is 1M $NaHCO_3$ pH 8.5. For other dyes, 10mM PBS, pH 7.4.
   - Adjust the antibody concentration to 2 mg/ml in reaction buffer if needed. That is, 200 µg antibody in 100 µl reaction buffer for 100 µg kit. For 1 mg kit, scale the content to desire concentration for labeling reaction volume of ~500 µl max.
   - The antibody should be dissolved in supplied buffer component B for labeling reaction.
     If the antibody is dissolved in glycine buffer, it must be dialyzed against 1X PBS or 1M $NaHCO_3$, or use Amicon

Ultra-0.5, 10 kDa molecular weight cutoff (MWCO), (Cat # UFC501008 from Millipore), to remove free amines or ammonium salts (such as ammonium sulfate and ammonium acetate) that are widely used for antibody precipitation.

- Impure antibodies or antibodies stabilized with bovine serum albumin (BSA) or gelatin will not be labeled well.
- Labeling efficiency varies with antibody concentration and dye NHS/ protein mol mixing ratio (MR). For optimal labeling efficiency the final antibody concentration range of 1-10 mg/ml is recommended. In general, the higher the antibody concentration and MR, the higher the labeling efficiency of the dye.
- Note: for antibody lgG (150KDa), MR is the recommended dye to antibody mol ratio.

Table 1: Recommended mixing ratio (MR) of dye NHS: antibody molar ratio.

Dye	Reaction buffer	Mixing Ratio (MR)		
		For low DOL	For optimal DOL	For higher DOL
BP Fluor 488 Fluorescein	1M NaHCO3 pH 8.5	<25	55	>65
Cy3 Cy5 BP Fluor 647 Cyanine 650 800CW	10mM PBS pH 7.4	<=10	20	>=28

For most of the bio-application, optimal DOL of the conjugates is listed here for reference.

Dye	optimal DOL
Fluorescein	4-9
BP Fluor488	4-9
Cy3	2-4
Cy5	3-5
BP Fluor 647	3-7
Cyanine 650	3-7
800CW	2-4

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2. Calculate appropriate volume of reactive dye stock solution.

Equation 1

$$\frac{[(Mass/MW) * 1000] * MR}{Conc.} = \mu l \text{ reactive dye to add to sample}$$

Where

Mass: antibody mass (µg)
MW: antibody molecular weight
MR: the reactive dye: antibody ratio from the table
Conc: the concentration (nmol/µl) of the reactive dye stock solution prepared below
Note: 2 nmol/µl for 100 µg kit; 10 nmol/µl for 1mg kit

SAMPLE EXPERIMENTAL PROTOCOL

1. For 100 µg kit, prepare antibody stock at a concentration of 2 mg/ml in supplied buffer component B. Aliquot 100 µl into a vial for use. (Post labeling dye removal by spin column has a capacity to process a max of 130 µl).
   For 1 mg kit, scale the antibody content to desire concentration in supplied buffer component B. For labeling reaction volume ~500 µl max.
2. Allow a vial of dye NHS to warm up to room temperature, and then add 50 µl anhydrous DMSO to it. Vortex to dissolve the dye. The concentration of this reactive dye stock solution is 2 nmol/µl for 100ug kit; 10 nmol/µl for 1mg kit.
3. Add the appropriate volume of reactive dye solution, based on equation 1, to antibody buffer vial and mix well. Protect the antibody/dye solution from light by wrapping the vial in aluminum foil and incubate the reaction for 1 hour at room temperature with gentle rocking.

Buffer Exchange and Excess dye Removal

100 µg kit excess dye removal by spin column:

1. Remove bottom closure of Zeba Spin desalting column, and then loosen but do not remove the cap.
2. Place column in a 1.5-2.0 ml collection tube (microcentrifuge tube). Centrifuge at 1,500 × g for 1 minute to remove storage solution.
3. Place a mark on the side of the column where the compacted resin is slanted upward. Place column in the microcentrifuge with the mark facing outward in all subsequent centrifugation steps.
4. Transfer the column to a new collection tube, uncap to apply labeling reaction sample to the top of the compact resin bed, slowly (volume not to exceed a 130 µl).
5. Centrifuge at 1,500 × g for 2 minutes to collect the sample. Discard the desalting column after use.
6. Store dye-antibody at 4°C for < 1 month. For longer periods, store at -20°C or -80°C, optionally with stabilizers (e.g., 0.1% bovine serum albumin) and 0.02-0.05% sodium azide.

1 mg kit excess dye removal by protein concentrator:

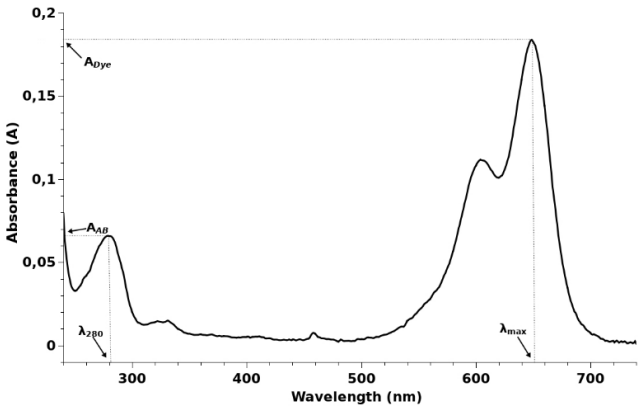
1. Hydrate concentrator membrane 'filter device' with 400 to 500 µl of reaction buffer or DI water, and microcentrifuge 14,000 x g, for 3 minutes. Discard, liquid from filter device and collection tube.
2. Spin down by adding the dye-antibody to the concentrator/filter device up to 500 µl. Microcentrifuge at 14,000 x g, 8 minutes, or to minimum volume ~ 50 µl left in the filter device. Discard waste from the collection tube.
3. Desalt by adding reaction buffer to the filter device up to 500 µl total volume; usually ~450 µl of buffer. Microcentrifuge at 14,000 x g, 8 minutes, or to minimum volume ~ 50 µl left in the filter device. Discard waste from the collection tube.
4. Repeat step 3, twice.
5. Collect dye-antibody from the filter device into a microcentrifuge tube.
6. Optional for maximum recovery, add a small volume of buffer component B, determined by the user, to the filter device to rinse out residual antibody, microcentrifuge pulse spin, collect antibody/reaction buffer from filter device, add to the microcentrifuge tube from step 5, mix.

Dye/Protein ration calculation

To calculate dye to antibody ratio, measure absorption spectrum of the conjugate, or absorption at 280 nm (A_{Ab}), and at dye absorption maximum (A_{dye}). A typical absorption spectrum of a dye labeled antibody is shown below. Depending

on the dye, wavelength of the maximum may vary.

Dye	MW	Absorption Max	Emission Max	Extinction coefficient	CF_{280}
Fluorescein NHS	473.4	487	517	83,000	0.35
CY3 NHS	735.8	548	569	162,000	0.06
BP Fluor 488 NHS	630.5	496	519	71,000	0.11
Cy5 NHS	778	646	665	271,000	0.04
Cyanine dye 650 NHS	890	650	665	270,000	0.04
BP Fluor 647 NHS	1022	647	664	270,000	0.04
800CW NHS	1166	778	794	300,000	0.03



$$\frac{Dye}{Ab} = \frac{A_{dye} \times \varepsilon_{Ab}}{(A_{Ab} - CF \times A_{dye)} \times \varepsilon_{dye}}$$

A_{dye}: Absorbance of dye
ε_{Ab}: Molar extinction coefficient at 280 nm of biomolecule
A_{Ab}: Absorbance at 280 nm of the dye-protein conjugates
CF: Correction factor at 280nm accounting for the absorption of the dye at 280 nm
ε_{dye}: Molar extinction coefficient of the dye

Storage of Antibody-Dye

The antibody conjugate should be stored in the presence of a carrier protein

Troubleshooting

Problem	Possible cause	Solution
Low or no conjugation	Buffer containing primary amine	Buffer exchange the antibody into a non-amine-containing buffer such as the PBS by desalting columns or dialysis
	Dye NHS was hydrolyzed	Use reagent immediately upon reconstitution
	Carrier protein was present in the antibody solution	Remove carrier protein before conjugation by using Protein A, G or A/G resin or an antibody clean-up kit, this will reduce competition for labeling
The downstream application was unsuccessful	Protein not labeled	Determine if the protein was labeled by calculating the dye-to-protein ratio
Low yield	Improper centrifugation	Make sure to use the indicated centrifugation speed
	Unstable protein	Equilibrate the column with PBS or other suitable buffer before adding the labeled protein
Problems with image capture	Photostability	Wrapped with aluminum foil during the labeling process

FAQ

Q: What is the optimized D/P ratio?

A: It will depend on the dye and the application. For example, for 800 CW, a D/P ratio of 1:1 - 2:1 for an IgG antibody is suitable for both Western and In-Cell Western applications. Higher D/P ratios (3:1 - 4:1) for an IgG antibody may still be usable for Western blot detection, but may exhibit increased background and therefore not perform optimally for other applications, but for BP Fluor 488 and fluorescein, most application need D/P 4-9.

For in vivo imaging applications, the dye/protein ratio of the conjugate may affect biological or biochemical activity of the protein, signal-to-noise ratio, blood clearance, and biodistribution.

Q: Can I label other proteins by these dye NHS labeling kits?

A: Yes. Check the reference table for BP Fluor 647 below; for other dyes, use this table accordingly.

Table 1. Recommended BP Fluor 647 dye NHS : protein molar ratios (MR), 10mM PBS pH 7.4, and typical yields for labeling 12–150 kDa proteins

Protein (MW in kDa)	**Dye NHS /protein (MR)**			**% Yield**
	For Lower DOL	**For Optimal DOL**	**For Higher DOL**	
parvalbumin	<=6	7	>=9	60
soybean trypsin inhibitor	<6	10	>12	60-80
thrombin	<6	12	>18	70
streptavidin	<=6	12	>15	84-92
transferrin	<=8	12	>17	68-80
F(ab)2	<9	15	>16	78-84
IgG	<10	20	>28	79-97