Protocol

TD-P Revision 2.0

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Mix-n-Stain™ Antibody Labeling Kit

Procedure to Label an Antibody with a CF™ dye

Introduction

Mix-n-Stain™ antibody labeling kits contain everything you need to rapidly label an antibody with GoldBio's CF™ dyes. The labeling procedure comprises simple mixing of your antibody with the reaction buffer and optimally formulated dye provided, followed by a brief incubation. The Mix-n-Stain™ dye is no longer reactive at the end of the labeling, so the conjugate is ready for staining without further purification. After labeling, the dye is covalently linked to the antibody with a degree of labeling of approximately 4-6 dye molecules per antibody molecule. Mix-n-Stain™ labeling is covalent, so Mix-n-Stain-labeled antibodies can be used for multicolor fluorescence staining without transfer of dyes between antibodies.

Mix-n-Stain™ labeling can tolerate sodium azide, Tris, glycine and low levels of glycerol. A microcentrifuge ultrafiltration vial is provided in the kit to rapidly remove incompatible small molecule antibody stabilizers before labeling. The standard Mix-n-Stain™ labeling protocol can be performed in the presence of up to four-fold excess of BSA or gelatin to IgG (by µg amount). Simply choose the kit size that corresponds to the amount of IgG you wish to label. **A modified protocol is provided for labeling IgG in the presence of excess stabilizer protein or ascites fluid.** In this case, choose the kit size that corresponds to the total amount of protein (IgG + stabilizer, or total protein amount in ascites fluid) in the antibody sample you wish to label. The modified protocol also can be used to label amounts of IgG that fall below the lower range of the kit by adding stabilizer protein to the IgG to bring the total protein amount within the kit range. The modified protocol is not recommended for labeling antibodies in crude antiserum or hybridoma cell culture supernatant due to the low concentration of antibody relative to total protein in these formats.

When performing direct immunofluorescence with a fluorescently-labeled antibody, you may need to use a higher concentration of antibody to achieve similar staining intensity compared to indirect immunofluorescence detection using unlabeled primary plus labeled secondary antibody. Typically, indirect immunofluorescence staining results in about 3-fold signal amplification compared to direct immunofluorescence staining. Labeled secondary antibodies will still bind to primary antibodies labeled using Mix-n-Stain™ kits, therefore if multiple primary antibodies from the same species are to be used for multicolor immunofluorescence staining, a secondary antibody cannot be used to distinguish an unlabeled primary antibody from a Mix-n-Stain™ labeled primary antibody from the same species. Mix-n-Stain™ labeled antibodies can be used as a tertiary staining antibody after standard immunofluorescence staining with primary and secondary antibodies.

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Materials

Table 1. Kit Components

Component	5-20 µg labeling	20-50 µg labeling	50-100 µg labeling
Dye	1 vial (Component A)	1 vial (Component A)	1 vial (Component A)
Mix-n-Stain™ reaction buffer, 10X	1 vial (15 µl)	1 vial (15 µl)	1 vial (30 µl)
Mix-n-Stain™ antibody storage buffer	1 vial (60 µl)	1 vial (150 µl)	1 vial (300 µl)
Ultrafiltration vial (MWCO=10K)	1 each	1 each	1 each

Storage/Handling

Store the kit at -20°C. The kit is stable for at least 3 months from date of receipt when stored as recommended.

Preliminary Notes

Mix-n-Stain™ antibody labeling kits are optimized for labeling IgG antibodies. We do not recommend using them to label other proteins, because the degree of labeling may not be optimized. Mix-n-Stain™ labeling conditions may cause IgM antibodies to denature.

Check the compatibility of your antibody with the antibody compatibility guide below. If your primary antibody is a commercial product, please contact the supplier to obtain the antibody concentration and formulation. Mix-n-Stain™ labeling cannot be used to label antibodies in crude serum or hybridoma supernatants. Use a Protein A purification procedure to purify IgG prior to labeling.

An antibody solution free of stabilizers produces the best labeling results, however, low levels of BSA, gelatin, Tris, glycerol in the antibody solution can be tolerated in the standard Mix-n-Stain™ labeling protocol. The labeling is not affected by sodium azide. For the standard Mix-n-Stain™ labeling protocol (Section B), select the kit size that corresponds to the total µg amount of IgG you wish to label.

The modified Mix-n-Stain™ labeling protocol (Section C) is based on the total amount of protein in the labeling reaction rather than the amount of IgG in the labeling reaction. The modified protocol should be used to label antibodies in the presence of excess stabilizer protein. Antibodies in ascites fluid can also be labeled using the modified protocol, however you must determine the concentration of total protein in the ascites fluid before labeling (estimation of protein concentration by measuring absorbance at 280 nm is sufficient). Select the kit size that

is appropriate for the total µg amount of protein in the antibody sample that you wish to label. The modified protocol also can be used to label antibody amounts that fall below the lower limit of the kit range by adding additional protein to the IgG to bring the total protein amount within the kit range.

Antibodies labeled in the presence of low levels of BSA and gelatin may show slightly higher background staining compared to antibody labeled without these stabilizers. If the antibody was labeled in the presence of BSA or gelatin, background staining can be greatly reduced by using blocking and wash solutions containing at least 1% BSA or gelatin, respectively.

To remove non-protein components such as Tris, glycine or glycerol, use the ultrafiltration vial provided in the kit to purify your antibody by following the steps in Section A.

The optimal antibody concentration for labeling is 0.5-1.0 mg/ml. The ultrafiltration vial can be used to concentrate antibody solutions by following the steps in Section A (note: stabilizer proteins will also be concentrated by the ultrafiltration vial). If no antibody concentration is required, proceed to the standard antibody labeling protocol (Section B) or the modified antibody labeling protocol (Section C) as appropriate.

Table 2. Mix-n-Stain™ Antibody Compatibility and Labeling Protocol Selection Guide

Component	Compatibility
Sodium Azide	Compatible
Glycerol	≤10%: proceed to standard protocol (Section B) >10%: perform ultrafiltration (Section A)
Tris	≤20mM: proceed to standard protocol (Section B) >20mM: perform ultrafiltration (Section A)
Glycine	Perform ultrafiltration (Section A)
BSA or gelatin	≤4X IgG by µg amount: use standard protocol (Section B) > 4X IgG by µg amount: use modified protocol (Section C)
Ascites fluid	Use modified protocol (Section C)
Serum	Not compatible; purify IgG
Hybridoma cell culture supernatant	Not compatible; purify IgG

Method

A. Ultrafiltration Protocol

Note: Before you begin, use Table 2 (Mix-n-Stain™ Antibody Compatibility and Labeling Protocol Selection Guide) to determine whether your antibody requires ultrafiltration before labeling. If necessary, contact the manufacturer of your antibody to find out the concentration of IgG and antibody stabilizers. If your antibody does not require ultrafiltration, proceed to the appropriate labeling protocol indicated in Table 2.

Note: The ultrafiltration column membrane has a molecular weight cut-off of 10,000 daltons. Therefore, molecules smaller than 10 kDa will flow through the membrane, and molecules larger than 10 kDa, including IgG antibodies, will be retained on the upper surface of the membrane (Figure 1). Take care not to touch the membrane with pipette tips, which could tear or puncture the membrane, resulting in loss of antibody.

Ultrafiltration Vial Capacities	Volume
Maximum Sample Volume	500 µl
Final Concentrate Volume	15 µl
Filtrate Receiver Volume	500 µl
Hold-up Volume (Membrane/Support)	<5 µl

1. Add an appropriate amount of antibody to the membrane of the ultrafiltration vial, being careful not to touch the membrane. Spin the solution at 14,000 x g in a microcentrifuge for one minute. Check to see how much liquid has filtered into the filtrate collection tube (lower chamber). Repeat the centrifugation until all of the liquid has filtered into the collection tube. Discard the liquid in the collection tube.

2. For antibody concentration, proceed to Step 3. For clean-up, add an equal volume of 1X PBS to the membrane. Spin the vial at 14,000 x g until the liquid has filtered into the filtrate receiving tube.

3. Add an appropriate concentration of PBS to the membrane to obtain a final antibody concentration of 0.5-1.0 mg/ml. Pipet the PBS carefully up and down over the upper surface of the membrane to recover and resuspend the antibody.

4. Transfer the recovered antibody solution to a fresh microcentrifuge tube.

5. If you are using the modified antibody labeling protocol, save the ultrafiltration vial to concentrate your antibody after labeling.

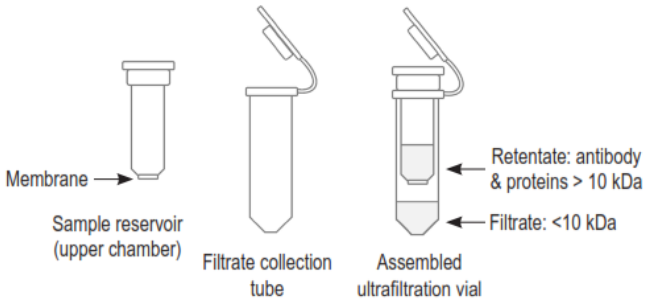

Figure 1: Ultrafiltration vial components

B. Standard Mix-n-Stain™ Labeling Protocol

Note: Before you begin, use Table 2 (Mix-n-Stain™ Antibody Compatibility and Labeling Protocol Selection Guide) to determine whether your antibody requires ultrafiltration before labeling. If necessary, contact the manufacturer of your antibody to find out the concentration of IgG and antibody stabilizers.

1. Use your antibody at a concentration of 0.5-1.0 mg/ml for optimal labeling. If the antibody is in a lyophilized form or is more concentrated, reconstitute or dilute the antibody in PBS. Transfer the antibody to be labeled to a clean tube. Make sure the µg amount of IgG in the labeling reaction falls within the range of the kit.

2. Warm up the Mix-n-Stain™ Reaction Buffer vial and the Mix-n-Stain™ Storage Buffer vial to room temperature before use. Centrifuge the vials briefly to collect the solutions at the bottom of the vials.

3. Mix the 10X Mix-n-Stain™ Reaction Buffer with the antibody solution at a ratio of 1:10 so that the antibody solution contains a final concentration of 1X Reaction Buffer (for example, mix 9 µl of antibody with 1 µl of 10X reaction buffer). Mix the solutions by pipetting up and down a few times.

4. Transfer the entire solution from Step 3 to the vial containing the CF™ dye. There is no need to measure the amount of the dye in the vial. Vortex the vial for a few seconds.

5. Incubate the vial in the dark for 30 minutes at room temperature.

6. Dilute the labeled antibody solution with the provided Storage Buffer. Simply transfer the entire labeled antibody solution into the Storage Buffer. The antibody is now ready to use for staining. The concentration of the CF dye-labeled antibody is approximately the amount of your starting antibody divided by the total volume.

   Note: Antibody Storage Buffer contains 2mM sodium azide.

7. The labeled antibody is stable for at least 6 months when stored 4°C, protected from light. Alternatively, the antibody can be stored in single use aliquots at -20°C for longer term storage.

   Note: If you prefer not to use the antibody dilution buffer, you can store the solution in single use aliquots at -20°C. Without repeated freeze-thaws, the labeled antibody solution should be stable for at least 6 months.

C. Modified Mix-n-Stain™ Labeling Protocol

Note: Before you begin, use Table 2 (Mix-n-Stain™ Antibody Compatibility and Labeling Protocol Selection Guide) to determine whether your antibody requires ultrafiltration before labeling. If necessary, contact the manufacturer of your antibody to find out the concentration of IgG and antibody stabilizers.

1. Use your antibody solution at a concentration of 0.5-1.0 mg/ml total protein (IgG plus stabilizer protein) for optimal labeling, using PBS to dilute the solution if necessary. Make sure the µg amount of total protein in the labeling reaction falls within the range of the kit. If you wish to label an amount of IgG that falls below the lower limit of the kit, add BSA to bring to the total protein concentration (IgG + BSA) within the range of the kit and proceed with labeling based on total protein concentration.

2. Warm up the Mix-n-Stain™ Reaction Buffer vial and the Mix-n-Stain™ Storage Buffer vial to room temperature before use. Centrifuge the vials briefly to collect the solutions at the bottom of the vials.

3. Mix the 10X Mix-n-Stain™ Reaction Buffer with the antibody solution at a ratio of 1:10 so that the antibody solution contains a final concentration of 1X Reaction Buffer (for example, mix 9 µl of antibody with 1 µl of 10X reaction buffer). Mix the solutions by pipetting up and down a few times.

4. Transfer the entire solution from Step 3 to the vial containing the CF™ dye. There is no need to measure the amount of the dye in the vial. Vortex the vial for a few seconds.

5. Incubate the vial in the dark for 30 minutes at room temperature.

6. **Optional**: you can transfer the entire labeling reaction to the tube of antibody storage buffer provided. However, this may result in a highly dilute IgG solution, which may not be practical for subsequent use. To transfer the antibody to storage buffer without additional dilution, follow the steps below.

Note: Antibody Storage Buffer contains 2mM sodium azide.

7. Transfer the labeling reaction to the membrane of the ultrafiltration vial provided (or saved after antibody clean-up, above. Centrifuge the vial at 14,000 x g until all of the liquid has filtered into the receiving vial as described in section A.

8. Resuspend the labeled antibody in antibody storage buffer at the desired final concentration of IgG. Carefully pipette the storage buffer up and down over the upper surface of the membrane to recover and resuspend the antibody.

Note: Antibody Storage Buffer contains 2mM sodium azide.

9. Transfer the recovered antibody solution to a fresh microcentrifuge tube. The antibody is now ready to use for staining.

10. The labeled antibody is stable for at least 6 months when stored 4°C, protected from light. Alternatively, the antibody can be stored in single use aliquots at -20°C for longer term storage.

Table 4. Reference Guide to Ordering GoldBio CF™ dyes.

Label/Dye	Ex (nm)	Em (nm)	GoldBio Catalog #
CF™350	347	448	CF-350
CF™405S	404	431	CF-405L
CF™405M	408	452	CF-405M
CF™405L	395	545	CF-405S
CF™440	440	515	CF-440
CF™488A	490	515	CF-488A
CF™514	516	548	CF-514
CF™532	527	558	CF-532
CF™543	541	560	CF-543
CF™555	555	565	CF-555
CF™568	562	583	CF-568
CF™594	593	614	CF-594
CF™633	630	650	CF-633
CF™640R	642	662	CF-640R
CF™647	650	665	CF-647
CF™660C	667	685	CF-660C
CF™660R	663	682	CF-660R
CF™680	681	698	CF-680
CF™680R	680	701	CF-680R

CF™750	755	777	CF-750
CF™770	770	797	CF-770
CF™790	784	806	CF-790
FITC	494	518	F-825

Frequently Asked Questions

How do I remove any unconjugated free dye from the labeled antibody since there is no purification step?

This question relates to a key element of our invention. The unique formulations of our dyes and buffers and the labeling strategy have completely removed this concern, which normally has to be dealt with when using conventional antibody labeling methodology. The exact mechanism on how this problem is solved is proprietary information.

Can I use Mix-n-Stain™ labeled antibodies for multi-color immunofluorescence staining, or will the dye transfer between antibodies?

Mix-n-Stain™ labeling results in covalent linkage of dye and antibody, so there will be no dye diffusion or transfer. Please refer to the Introduction section on page 1 for more details.

Can I use a Mix-n-Stain™ kit for labeling proteins other than antibodies?

Mix-n-Stain™ kits are optimized for labeling IgG antibodies. We do not recommend them for labeling other proteins. Mix-n-Stain™ labeling conditions may cause denaturation of IgM antibodies.

Is staining with Mix-n-Stain™ labeled antibodies as sensitive as staining with unlabeled primary and fluorescent secondary antibodies?

Direct immunofluorescence detection can be less sensitive than indirect detection. Please refer to the Introduction section on page 1 for more details.

What are the advantages of using directly labeled conjugates compared to indirect staining with labeled secondary antibodies?

Direct immunofluorescence staining eliminates the need for secondary antibody incubation and wash steps, and allows the use of multiple primary antibodies from the same species for multicolor detection, or staining of animal tissues with antibodies raised in the same species without secondary antibody cross-reactivity (e.g. mouse-on-mouse staining).

What are the advantages of Mix-n-Stain™ kits over Invitrogen's Zenon® antibody labeling kits?

The major advantages are: 1) the CF dye is covalently attached to the antibody to eliminate dye transfer or diffusion between antibodies during multi-color staining; 2) Mix-n-Stain™ conjugates are stable for several months in storage buffer whereas Zenon labeling reagents are required to be used within 30 minutes; 3) Mix-n-Stain™ conjugates are less bulky because the dyes are directly linked to the antibody, unlike Zenon conjugates which use antibody fragments; 4) No dye/protein optimization is needed, just mix and then stain; 5) No post-staining fixation is required with Mix-n-Stain™; 6) Unlike Zenon, Mix-n-Stain™ labeling is not species-specific.

What are the advantages of Mix-n-Stain™ kits over Innova Bioscience's LightningLink™ Rapid antibody labeling kits?

Mix-n-Stain™ antibody labeling kits use novel CF dyes which are brighter and more photostable than the dyes provided in Lightning Link kits. Mix-n-Stain™ kits are sized for labeling smaller amounts of antibody and are sold as a single labeling, providing more flexibility compared to Lightning Link kits.

What are CF dyes?

CF dyes are highly water soluble, small organic dyes designed for labeling proteins and nucleic acids. With a series of more than 20 colors, many of our CF dyes are brighter and more photostable than competing dyes.

How do I select a Mix-n-Stain™ kit?

For each CF dye, there are three labeling kits for labeling of antibody quantities in three different ranges: 1) 5-20 µg, 2) 20-50 µg, and 3) 50-100 µg. For antibody labeling in the absence of stabilizer protein, select a kit that matches with the amount of your antibody. For antibody labeling in the presence of stabilizer protein or ascites fluid, see Table 2 of the product protocol.

If my antibody amount falls between two kits, which one should I use? For example, if I want to label 50 µg of antibody, should I purchase the 50-100 µg kit or the 20-50 µg kit?

Although either kit will produce good results, it is better to use the smaller kit size if your antibody amount falls between two kit sizes.

What dye/protein ratio should I use to ensure optimal labeling?

There is no need to measure the dye amount or vary the reaction time as long as the amount of your antibody to be labeled falls within the range specified for each kit. With Mix-n-Stain™ labeling kits optimal labeling is ensured because of the proprietary dyes and reaction buffer.

Can I split the kit contents and use it more than one time?

No. The Mix-n-Stain™ kits are optimized for 1 labeling. We do not recommend that you try to split the kit to label more than one antibody or for more than one use.

How important is the antibody concentration?

The kits are optimized for labeling antibodies with a concentration between 0.5-1.0 mg/ml. If your antibody solution is too dilute, you can concentrate it by centrifugation using the ultrafiltration vial provided in the kit. If your antibody solution is too concentrated, you can dilute it with 1x PBS. Antibody concentrations outside the recommended range may result in either under-labeling or over-labeling.

I performed immunofluorescence staining with my labeled antibody, but I don't see any signal. What should I do?

Check with the antibody manufacturer to confirm that the antibody formulation and concentration are compatible with the kit labeling protocol you selected.

You should confirm that your primary antibody is sensitive and specific for your application using indirect labeling before attempting direct labeling. You may need to use a higher concentration of primary antibody to achieve similar signal intensity with direct labeling as with indirect labeling. Please refer to the Introduction section on page 1 for more information.

Covalent labeling may affect the reactivity of certain antibodies. You can test if this is the case by performing indirect immunofluorescence labeling with your Mix-n-Stain™ labeled primary with secondary detection using a fluorescently-labeled secondary antibody to confirm that the primary antibody is still reactive.

If you have access to a fluorescence gel reader or scanner that is compatible with the excitation/emission wavelengths of the dye you are using, you can confirm labeling of your antibody by performing denaturing SDS-PAGE on a small amount (0.1-0.5 µg) of labeled antibody, then imaging the gel fluorescence. You should be able to detect fluorescent bands representing IgG heavy and light chains at ~55 kDa and ~25 kDa.

Table 5. Other Mix-n-Stain™ Antibody Labeling Kits.

GoldBio Catalog #	Product Name
A-825	Mix-n-Stain™ AP Antibody Labeling Kit
B-825	Mix-n-Stain™ Biotin Antibody Labeling Kit
F-825	Mix-n-Stain™ FITC Antibody Labeling Kit
G-825	Mix-n-Stain™ Glucose Oxidase (GOX) Antibody Labeling Kit
H-825	Mix-n-Stain™ HRP Antibody Labeling Kit