FlexiStain™ antibody labeling kit

Size: 500 µl (labels 50 µg biotinylated antibody)

Concentration: 0.1 mg/ml FlexiStain streptavidin

Storage: +4 ºC, protect from light

Kit content

- FlexiStain reagent, 500 µl (contains 0.09% sodium azide)
- Biotin block buffer, 500 µl (biotin in PBS)

This document contains protocols for (A) antibody labeling with FlexiStain, and (B) the use of FlexiStained antibodies for immunostaining of samples.

Introduction

FlexiStain™ provides a rapid, robust, and flexible method for attaching fluorochromes to biotinylated antibodies. The antibody-FlexiStain complex (FlexiStained antibody) is stable and can be used in the same way as antibodies conjugated directly with fluorochromes.

FlexiStain is a unique immunolabeling tool as it enables multiplexing with multiple biotinylated antibodies. There is no spillover of FlexiStain reagent between antibodies and antibodies of the same animal host species can be used.

The FlexiStain labeling reaction is efficient and can be run in such small volumes that it becomes practical to label individual antibodies with fluorochromes depending on the experiment.

Storage and Handling

Upon receipt, store the FlexiStain antibody labeling kit at +4 ºC. The kit can be stored for 6 months after receipt. Do not freeze. Protect fluorescent components from light. Note: The FlexiStain reagent contains 0.09% sodium azide.

Applications

FlexiStained antibodies can be used for immunostainings in histology and flow cytometry. Other immunostaining applications have not been tested. FlexiStained antibodies can be combined with standard immunolabeling protocols, such as primary + secondary antibodies or directly conjugated antibodies, but not with streptavidin.

Before you begin

If the antibody is biotinylated 'in house' using a biotinylation kit, excess free biotin must be removed (eg by a spin column). Occasionally, antibodies have lysine residue that are used for biotinylation near the antigen-binding site. In these cases FlexiStain can sterically affect the binding to the antigen. Kits that specifically biotinylate the Fc-portion of the antibody will circumvent this potential problem.

The FlexiStain labeling step (A) can be performed several days in advance of immunostaining step (B). Note: When Biotin block buffer is added, the FlexiStained antibody must be used within 24 hours.

The FlexiStain labeling step (A) can be performed at room temperature (RT) or at +4 ºC.

The antibody-to-FlexiStain reagent ratio is important. Check the concentration of the antibody. Avoid volumes <2 µl to achieve correct amounts of the components. Pre-dilute the antibody and/or the FlexiStain reagent with PBS when volumes become small. Dilution does not influence labeling efficiency.

The FlexiStain reaction does not require removal of bovine serum albumin (BSA) or other stabilizing proteins that may be present in antibody preparations.

Do not combine FlexiStain with protocols where standard streptavidin is used.

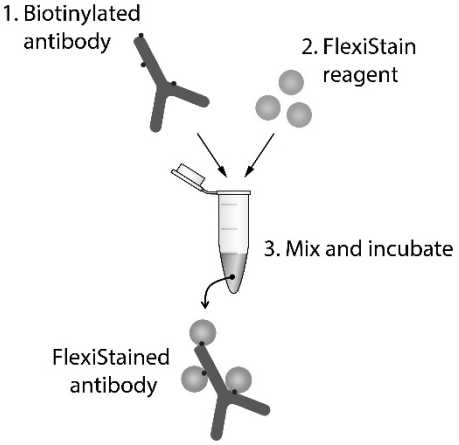
The FlexiStain fluorochromes have the following excitation (Ex) and emission (Em) peaks:

- FlexiStain, AlexaFluor488: Ex 495 nm, Em 520 nm
- FlexiStain, Atto542*: Ex 542 nm, Em 562 nm
- FlexiStain, AlexaFluor 594: Ex 590 nm, Em 620 nm
- FlexiStain, AlexaFluor 647: Ex 650 nm, Em 668 nm

** Atto542 is a Cy3 equivalent*

Caution: Make sure what fluorochromes that can be simultaneously separated by your microscope. 594 fluorochromes may overlap with both 542 and 647 fluorochromes. All four fluorochromes can simultaneously be used without spectral overlap using the SpectraSplit™ filter sets from Kromnigon AB (kromnigon.com).

(A) FlexiStain antibody labeling protocol



Steps:

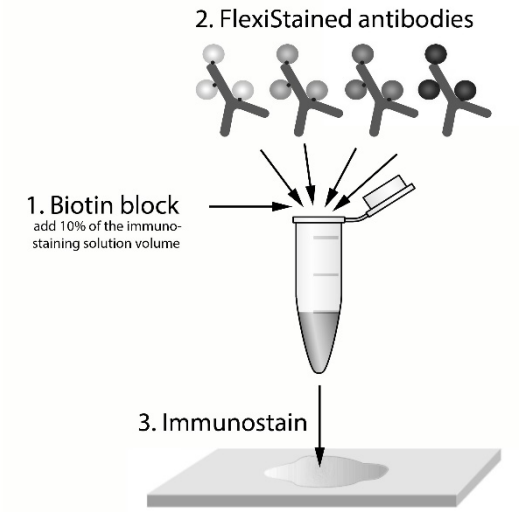
1. Add the desired amount of biotinylated antibody to be labeled to a suitably sized tube. Pre-dilute the antibody if the volume is less than 2 ul. Make sure that all of the antibody solution is at the bottom of the tube. Antibody concentration as low as 0.01 mg/ml can be used. 0.2 ml PCR tubes are convenient to use for small reactions.

2. Add the appropriate amount of FlexiStain reagent (see table) and mix immediately by pipetting up and down. Avoid bubbles. Adding FlexiStain reagent above the surface of the antibody solution is the most efficient way to obtain a rapid even mixture. Pre-dilute the FlexiStain reagent up to 10 times if the volume is small. The volume of FlexiStain should preferably be larger than the volume of the antibody.

Antibody concentration	µL antibody	µL FlexiStain reagent	Dilution
1 mg/mL	1	10	11 times
0.5 mg/mL	1	5	6 times
0.2 mg/mL	1	2	3 times
0.1 mg/mL	1	1	2 times

3. Incubate for 5 minutes at RT or at +4ºC. The FlexiStained antibody is now ready to be used for immunostaining procedures or can be stored for at least one week at +4ºC until use, provided that the antibody *per se* tolerates the storage.

(B) FlexiStain immunostaining protocol



The FlexiStained antibodies can be used for multi-immunostaining similar to directly conjugated antibodies. When multiplexing with several FlexiStained antibodies, the immunostaining solution shall contain Biotin block to inactivate any free FlexiStain reagent. The Biotin block buffer is not necessary if only one FlexiStained antibody is used.

Steps:

Prepare your tissue samples for immunostaining according to standard protocols. There are no restrictions for standard pre-blocking reagents (e.g. serum, IgGs, BSA, Fc-block, biotin/streptavidin block). FlexiStained antibodies do not need an avidin/biotin blocking step.

1. Add 10% Biotin block buffer to the immunostaining solution for multistainings. The Biotin block buffer quenches any excess reactive FlexiStain. The amount of Biotin block can vary between 5-10%. If convenient, Biotin block buffer can be added to each FlexiStained antibody after step 3 in protocol A (approximately 0.5 µl biotin block / µg antibody, must be used within 24 hour, see 2.)
2. Add FlexiStained antibodies (and other antibodies) one by one into the immunostaining solution. As a guideline: Use the same amount of antibody or up to twice as much as in standard immunostaining protocols. The table on page 1 shows how much the antibody has been diluted during the FlexiStain labeling step. FlexiStained antibodies can be combined with other primary antibodies. Continue to the next step within 24 hours when Biotin block has been added.
3. Apply the immunostaining solution to your samples and incubate. Continue the immunostaining procedure according to standard protocols. For immunostaining of tissue sections, the incubation times normally are 0.5-3 hours at RT or overnight at +4°C.
4. Wash, mount, and analyze under a fluorescence microscope. Make sure what fluorochromes that can be separated by your fluorescence microscope without spectral overlap (see 'Before you begin' on page 1).

Trouble shooting guide

The brightness of antibodies labeled with FlexiStain is similar to directly conjugated antibodies.

Weak/No signals

- Check that your biotinylated antibody works using a 2-step immunostaining with FlexiStain. Incubate your sample with the biotinylated antibody, wash, and then add FlexiStain reagent (diluted 1:40 in PBS) in a separate second step.

- Some antibodies have biotin conjugated near the antigen-binding site, resulting in sterical hindrance when attaching FlexiStain to the antibody. Reducing the amount of added FlexiStain reagent by 50% in the labeling step (A) will then improve the signal.

- Increase the amount of FlexiStained antibody used during the immunostaining step (B).

- Take care that the entire FlexiStain reagent during the labeling step is rapidly mixed with the biotinylated antibody without formation of bubbles. Adding FlexiStain on top of the antibody solution facilitates a rapid mixture. It is easier to mix rapidly if the FlexiStain volume is >2 times the antibody volume.

- The antibody-to-FlexiStain reagent ratio is important. Check that the concentration of the biotinyated antibody is correct.

- Consider changing fluorochrome. FS647 has the highest signal to noise ratio.

High background signals

- Use less FlexiStain reagent during the labeling step (A).

- Lower the amount of antibody during the immunostaining step (B).

- Spin the biotinylated antibody at full speed on a table top centrifuge (~21000 x g) for 10 minutes before labeling with FlexiStain, to remove potential antibody aggregates.

- Wash with 0.01% Triton-X100 after the antibody incubation step of the sample.