FlexiStain™ multi-immunostaining kit

Size: Labeling of 4 x 25 µg biotinylated antibody

Storage: +4 °C

Kit content

- 4 x FlexiStain reagent
- Block buffer (10x)

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

Introduction

FlexiStain™ provides a rapid and flexible method for attaching fluorochromes to biotinylated antibodies. The formed antibody conjugates (FlexiStained antibodies) are stable and can be used similar to directly conjugated antibodies.

FlexiStain can be used for very small conjugation reactions—even less than 0.5 µg antibody—which means that the choice of fluorochromes can be custom made for each experiment.

FlexiStained antibodies can be combined independently of the animal species of the antibodies. Thereby, FlexiStain eliminates the challenges and expenses associated with finding compatible combinations of primary and secondary antibodies when designing new immunopanels.

Storage and Handling

Upon receipt, store the FlexiStain multi-immunostaining kit at 2–6°C. The kit can be stored for 6 months. Do not freeze. Protect fluorescent components from light. **Note:** The FlexiStain conjugation reagents contain 2 mM 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

Biotinylated antibodies are provided by most antibody distributers. Antibodies can also be biotinylated 'in house' using biotinylation kits. The Mix-n-Stain biotin antibody labeling kit from Biotium (biotium.com) works well with FlexiStain. Other biotinylation kits may need titration.

The FlexiStain conjugation step (A) can be performed in advance (for example the day before) to the immunostaining step (B).

The FlexiStain conjugation step (A) can be performed at room temperature or at +4 °C.

The antibody-to-FlexiStain reagent ratio is important. Avoid volumes <2 µl to achieve correct amounts of the components.

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

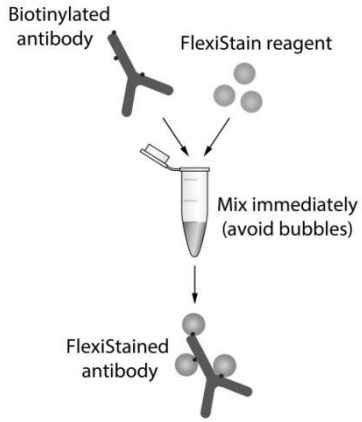
Do not combine FlexiStain with streptavidin protocols.

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

- FlexiStain reagent 488: Ex 495 nm, Em 520 nm
- FlexiStain reagent 546: Ex 542 nm, Em 562 nm
- FlexiStain reagent 594: Ex 590 nm, Em 620 nm
- FlexiStain reagent 647: Ex 650 nm, Em 668 nm

Caution: Make sure what fluorochromes that simultaneously can be separated by your microscope. 594 fluorochromes may overlap with both 546 and 647 fluorochromes. All four fluorochromes can simultaneously be used without overlap using the SpectraSplit™ filter sets from Kromnigon (kromnigon.com).

(A) FlexiStain conjugation protocol



Steps:

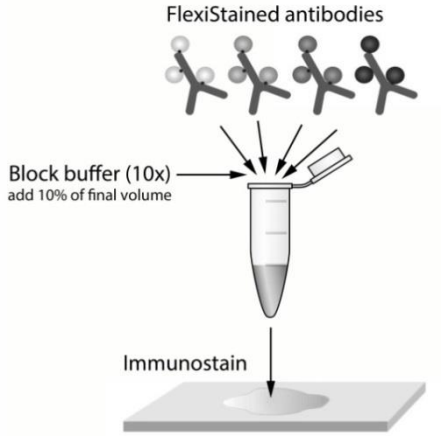
1. **Add the amount of biotinylated antibody to be conjugated into a tube of suitable size.** Make sure that all antibody solution is at the very bottom of the tube and that there are no bubbles. Spin down if needed. Reduce the antibody concentration if the volume is too small (<2 µl). 0.2 ml PCR tubes can be convenient to use.

2. **Add FlexiStain reagent and mix immediately by pipetting up and down**. **Use 10 µl FlexiStain reagent for each µg antibody (see table below).** Make sure that the entire FlexiStain reagent is rapidly mixed with the entire antibody solution. Avoid bubbles. The conjugation step dilutes the antibody concentration (see table below), which must be taken into account in the immunostaining step (next page).

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 at room temperature or at +4 °C for at least 10 minutes**. The FlexiStained antibody is now ready to be used for immunostaining procedures or can be stored for at least one month at +4°C until use, provided that the antibody *per se* tolerates storage at +4°C.

(B) FlexiStain immunostaining protocol



Steps:

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

2. **Calculate the volume of the final immunostaining solution. Add 10% of the final volume of Block buffer (10x) into a new tube.**

3. **Add FlexiStained antibodies (and other antibodies) one by one into the Block buffer (10x).** As a guideline: use the same amount of antibody as in standard immunostaining protocols. The table on page 1 shows how much the original antibody has been diluted during the FlexiStain conjugation step. FlexiStained antibodies can be combined with other primary antibodies. Do not combine with streptavidin protocols. Continue to step 5 within 6 hours.

4. **Fill up with PBS or other buffer to the final volume of the immunostaining solution.** BSA and other additives can be added at this step. If preferred, the PBS can be added to the Block buffer (10x) before FlexiStained antibodies.

5. **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 1-3 hours at RT or 12 h at +4°C.

6. **Wash, mount, and analyze under a fluorescence microscope.** Make sure what fluorochromes that simultaneously can be separated by your fluorescence microscope**.** There is a risk for spillover signals between fluorochromes in the microscope (see Before you begin on page 1).

Trouble shooting guide

Weak signals

The signals should be as bright as with directly conjugated antibodies.

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

- Increase the amount of FlexiStain reagent (up to 2 times more, start with 1.5 times) during the conjugation step (A).

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

- Make sure that the entire FlexiStain reagent during the conjugation step (A) is rapidly mixed with the biotinylated antibody without formation of bubbles.

- Some antibodies can have a biotin conjugated near the antigen-binding site, resulting in sterical hindrance of the active antibody site. This is usually a consequence of over-biotinylation of the antibody. The same antibody from another antibody distributer may work better. Alternatively, biotinylate the antibody in house using biotinylation kit (see Before you begin on page 1).

High background signals

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

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

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

Antibody aggregates are formed

- Avoid bubbles during the mixing step.

- Dilute the FlexiStain reagent 2 times with PBS and dilute the antibody solution to 0.1 mg/ml with PBS prior to the conjugation step (B). Mix the diluted antibody with the double volume of diluted FlexiStain reagent.

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