Manual

StreptaClick®–HRP Multiplex IHC kit

KROMNIGON
Multiplex IHC made easy

Size:
Large 250 stainings, 100 µL/staining
Small 90 stainings, 100 µL/staining

Kit content and Storage:

StreptaClick®–HRP (antibody labeling reagent):	+4°C
Biotin block buffer:	+4°C
HRP block buffer:	+4°C
3% H_2O_2:	+4°C
Block activator:	+4°C

Not provided in the kit
Tyramide fluorochromes
Tyramide amplification buffer

Introduction

This document contains protocols for (A) StreptaClick labeling of biotinylated antibodies with horseradish peroxidase (HRP), and (B) the use of the HRP-labeled antibodies for tyramide signal amplification (TSA) multiplex immunostaining.

The StreptaClick®–HRP Multiplex IHC kit provides a powerful method for multiplex immunohistochemistry using tyramide signal amplification (TSA), including frozen tissue sections. The method is based on horseradish peroxidase (HRP) that with a click reaction is attached to biotinylated antibodies using a processed form of streptavidin conjugated with HRP (StreptaClick®–HRP). The HRP–labeled biotinylated antibodies can then be used for multiple cycles of immunostainings where each fluorochrome is sequentially developed by TSA (TSA reagents are not provided). The kit contains an HRP block buffer optimized for preserving the morphology and antibody epitopes of frozen tissue sections. The HRP block buffer rapidly quenches the HRP enzyme at room temperature after each TSA cycle, and allows sequential multiplex IHC of up to six antibodies in one day.

Applications

Single and multiplex immunofluorescence staining of frozen tissue sections using TSA to develop fluorescent color(s).

Before you begin

If the antibody is biotinylated 'in house' using a biotinylation kit, excess free biotin must be removed before use (e.g. by a spin column).

Do not use dry milk in the immunostaining buffer. It may contain free biotin that quenches StreptaClick®–HRP during the antibody labeling step. If desired, dry milk can be added after the HRP labeling reaction (Protocol A).

Dilute the biotinylated antibody with your immunostaining buffer of choice (e.g. PBS with 0.5% bovine serum albumin) before mixing with StreptaClick®–HRP. Pre-diluting to a working concentration avoids HRP quenching by sodium azide that is often used as a preservative in antibody stock solutions.

The antibody labeling reaction is not affected by BSA or other stabilizing proteins that may be present in antibody preparations.

The molar ratio between the biotinylated antibody and StreptaClick®–HRP is important for optimal HRP labeling. Table 1 shows how much StreptaClick®–HRP to use for each µl antibody at different antibody stock concentrations. To avoid pipetting errors, use intermediate dilution if using less than 2 µl of the antibody stock solution.

Table 1. Volume of StreptaClick®–HRP and Biotin Block buffer for each µL antibody (antibody stock solution)

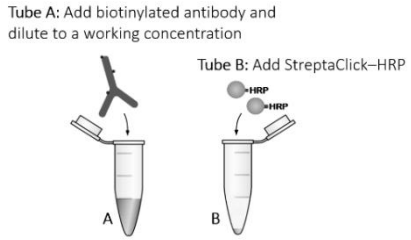
Antibody stock concentration	Antibody (µl)	StreptaClick®-HRP (µl)	Biotin block buffer (µl)
1 mg/mL	1 µL	20 µL	20 µL
0.5 mg/mL	1 µL	10 µL	10 µL
0.1 mg/mL	1 µL	2 µL	2 µL

The antibody labeling is performed at room temperature (RT). Multiple biotinylated antibodies can be labeled with HRP in parallel. You can store the HRP-labeled antibodies at +4°C up to 24 hours. However, use the antibodies within 12 hours when the Biotin block buffer has been added (step 5).

The active HRP block buffer—prepared with H_2O_2 and Block activator—should be stored in the dark (RT or +4°C) and can be used for up to 24 hours.

(A) HRP labeling protocol

The HRP labeling protocol attaches HRP to biotinylated antibodies.



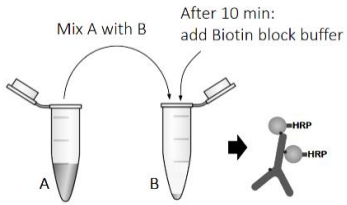
1. Place two tubes of the same size in a rack (e.g. 1.5 ml microfuge tubes).

2. In the first tube (Tube A), prepare your antibody working solution. Note: Only the amount (moles) of the antibody is important. Hence, the volume of the working solution, and thus the concentration, can be altered. Avoid pipetting volumes <1µl. Avoid dry milk in the antibody working solution, since it may contain free biotin.

3. To the second tube (Tube B), add the appropriate amount of StreptaClick®–HRP according to Table 1.

4. Transfer all antibody solution from Tube A to the second tube (Tube B) and mix immediately by pipetting up and

down. Avoid bubbles. This step is important for an even distribution of HRP bound to the antibodies.



5. After 10 minutes or more, add Biotin block buffer according to Table 1 and mix. The Biotin block buffer immediately inactivates any remains of unbound StreptClick–HRP. The biotinylated antibody is now labeled with HRP and ready to be used for TSA immunostaining. Use the HRP-labeled antibodies within 12 hours when the Biotin block buffer has been added.

Tip: You can prepare all antibodies for the multistaining experiment at the same time and store them at +4°C until use.

(B) TSA immunostaining protocol

The HRP-labeled antibodies can be used for multiplex TSA immunostaining. As a guidance for the TSA immunostaining, use a 1:250-1:500 dilution from a 0.5 mg/ml antibody stock solution (1-2 µg/ml working solution).

The protocol does not use heat treatment between cycles, which makes the protocol suited for frozen tissue sections.

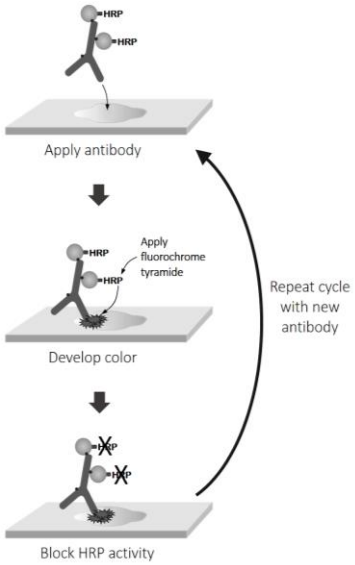
Each staining cycle contains three main procedures – Antibody incubation, Color development, and HRP block.

6. Prepare your tissue sections for immunostaining according to standard protocols. There is no need for avidin/biotin blocking.

7. Block endogenous peroxidases. Add 10 µl 3% H_2O_2 and 50 µl Block activator to each ml of HRP block buffer. Apply 100 µl of the activated HRP block buffer to each tissue section and incubate for 12 minutes at RT. The activated HRP block buffer will also be used in step 10 and can be stored in the dark for 24 hours.

Wash briefly in distilled water or PBS twice (2x 30 seconds) and 30 seconds in PBS.

8. Antibody incubation. Apply 100 µl HRP-labeled antibody to your tissue sections and incubate 20-60 minutes at RT. For short incubation (20-30 min) placing the slides on a rotation shaker at 50 rpm is recommended. Wash briefly in distilled water or PBS twice (2x 30 seconds) and 5 min in PBS.



9. Color development. Dilute the tyramide dye in tyramide amplification buffer and apply to your samples. Tyramide reagents are not provided in the kit. Apply 100 µl to your tissue sections and incubate 12 minutes at room temperature. The development time can be adjusted between 10-15 minutes. In addition, the working concentration of the tyramide dye can be adjusted to obtain the desired signal intensity.

Wash in distilled water three times (3x 30 seconds). It is important to only wash in water (and not PBS) before applying the HRP block buffer.

10. HRP block. Apply 100 µl of activated HRP block buffer (with added H_2O_2 and Block activator) to each tissue section and incubate for 12 minutes at RT. This step is only needed when a sequential immunostaining cycle will be performed. The step can be prolonged to 15 min if the HRP activity is not fully quenched.

Wash briefly in distilled water or PBS twice (2x 30 seconds) and 30 seconds in PBS.

11. Repeat steps 8-10 with the next HRP-labeled antibody.

12. Wash, mount, and analyze under a fluorescence microscope.

Troubleshooting guide

<u>Weak/No signals</u>

- Increase the amount of antibody used during the immunostaining step.

- Increase the color development time (step 9) to 15 minutes.

- Increase the amount of tyramide dye during the color developing step (step 9).

- Check that your biotinylated antibody works using a two-step immunostaining with the StreptaClick®–HRP; 1) Incubate tissue section with the biotinylated antibody. 2) Wash and incubate tissue sections with StreptaClick®–HRP alone diluted 1:20 in PBS. Wash and develop color with TSA.

- The ratio between the biotinylated antibody and StreptaClick®–HRP is important. Check the concentration of the antibody. If you do not know the exact concentration, test different amounts of labeling reagent during antibody

labeling. Loading too much StreptaClick®–HRP will interfere with the antibody function.

- It is important to first prepare an antibody working solution before mixing with StreptaClick®–HRP. Pre-diluting to a working concentration avoids HRP quenching by sodium azide that is often used as a preservative in antibody stock solutions.

- Fast mix between the antibody working solution and the StreptaClick®-HRP is important. Make a full transfer of the antibody working solution (Tube A) into the tube of StreptaClick®-HRP reagent (Tube B).

- The HRP block buffer may not have been washed away properly before adding the next HRP–labeled antibody to the sample.

- Some antibodies have biotin conjugated near the antigen-binding site, resulting in sterical hindrance when attaching the HRP to the antibody. Using less StreptaClick®–HRP during the labeling step may help.

- Do not use dry milk in the immunostaining buffer, since it may contain free biotin that quenches the StreptaClick®–HRP. If desired, dry milk can be added after the antibody labeling reaction.

Cross-over signal from other antibodies

- The biotin block step may be incomplete. Check that the Biotin block buffer is properly added to the antibodies before applying HRP-labeled antibodies to the tissue.

- The HRP block step may be incomplete. Check that H_2O_2 and Block activator is properly added to the HRP block buffer. Increase the HRP block step (step 10) to 15 minutes. Use the HRP block activator at room temperature.

To strong signals or unspecific signals

- Decrease the tyramide dye concentration during the color developing step.

- Lower the concentration of the antibody working solution during the immunostaining step.