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. However, the kit can also be used for FFPE 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 and FFPE 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 ratio between the biotinylated antibody and StreptaClick®–HRP is important for optimal HRP labeling (Table 1). Make sure that you know the approximate antibody stock concentration. 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)

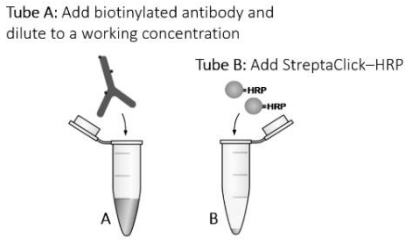
Antibody stock concentration	Antibody (µl)	StreptaClick®-HRP (µl)	Biotin block buffer (µl)
1 mg/mL	1 µL	20 µL	20 µL
0.5 mg/mL	1 µL	10 µL	10 µL
0.1 mg/mL	1 µL	2 µL	2 µL

The antibody labeling is performed at room temperature (RT). Multiple biotinylated antibodies can be labeled with HRP in parallel. Store the HRP-labeled antibodies at +4°C, and use them for TSA immunostaining within 8 hours.

The HRP block buffer—prepared with H2O2 and Block activator—should be stored at room temperature and can be used for up to 24 hours.

(A) HRP labeling protocol

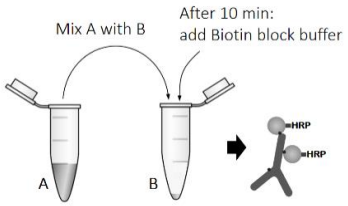
The HRP labeling protocol attaches HRP to biotinylated antibodies that will be used for TSA immunostaining.



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. As a guidance, use a 1:500-1:1000 dilution from a 0.5 mg/ml antibody stock solution. To avoid pipetting errors use intermediate dilution if using less than 2 µl of the antibody stock solution. Avoid dry milk in the immunostaining buffer, since it may contain free biotin.

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



StreptaClick®–HRP Multiplex IHC kit

KROMNIGON
Multiplex IHC made easy

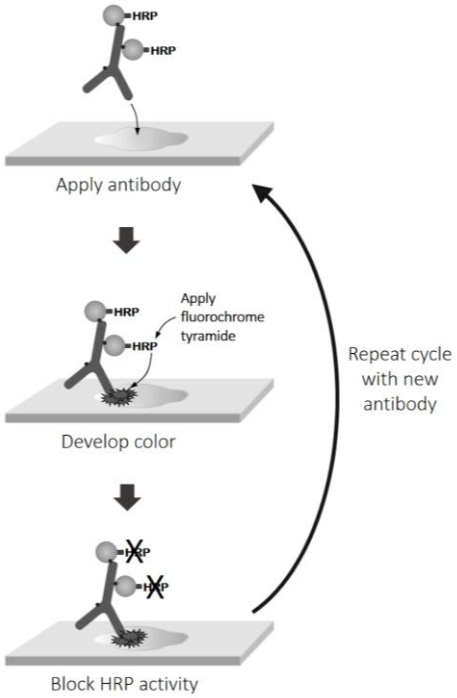
5. Transfer all antibody solution from Tube A to the second tube (Tube B) and mix immediately by pipetting up and down. Avoid bubbles. This step is important for an even distribution of HRP bound to the antibodies.

6. 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.

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.



The protocol does not use heat treatment between cycles, which allows TSA multiplex immunostaining also on frozen tissue sections. Each staining cycle contains three main procedures – Antibody incubation, Color development, and HRP block.

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

2. Block endogenous peroxidases with the provided HRP block buffer. To activate the HRP block buffer, 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 5 and can be stored at room temperature for 24 hours.

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

3. Apply 100 µl HRP-labeled antibody to your tissue sections and incubate 20-60 minutes at RT. For short incubation (20-25 min) placing the slides on a rotation shaker at 50 rpm is recommended. Wash in distilled water twice (2x 30 seconds) and 5 min in PBS.

4. Dilute tyramide fluorochrome 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 10 minutes at room temperature.

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.

5. Apply 100 µl of the activated HRP block buffer to each tissue section and incubate for 12 minutes at RT. This step is only needed when a sequential immunostaining cycle will be performed.

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

6. Repeat steps 3-5 with the next HRP-labeled antibody.

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

Troubleshooting guide

<u>Weak/No signals</u>

- 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).

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

- Increase the amount of tyramide fluorochrome during the color developing step. Twice the amount of tyramide fluorochrome can improve the signal significantly.

- 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. Use the HRP block activator at room temperature.

To strong signals or unspecific signals

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

- Decrease the amount of tyramide fluorochrome during the color developing step.