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

Tyramide dye 3-color and 5-color kits (Cat. no. 60031-1 and 60051-1) are available from Kromnigon and have been specifically optimized for use with the StreptaClick® HRP kit.

Applications

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

Overview

This document contains protocols for (A) StreptaClick labeling of biotinylated antibodies with horseradish peroxidase (HRP), and (B) the subsequent application of HRP-labeled antibodies in tyramide signal amplification (TSA) immunostaining. The TSA protocol is optimized to preserve both tissue morphology and antigen epitopes. It does not involve heat treatment and is therefore particularly well suited for frozen sections and for applications where maintaining histological integrity after multiple TSA staining cycles is essential.

StreptaClick® is a processed form of streptavidin that prevents aggregation when mixed with biotinylated antibodies in solution. This enables rapid and straightforward labeling of biotinylated antibodies with horseradish peroxidase (HRP). Simply mix each antibody with the StreptaClick® HRP labeling reagent, and the antibodies become directly conjugated to HRP. This is a one-step, 10-minute procedure without washing steps. The labeling reaction is not affected by bovine serum albumin (BSA) or other stabilizing proteins that may be present in antibody preparations. The resulting HRP-labeled antibodies are then used for multiplex IHC based on TSA (figure 1).

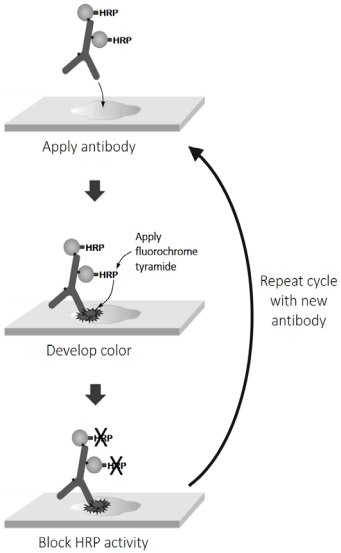


Figure 1: *One staining cycle using StreptaClick®-labeled antibodies consists of three steps: (1) antibody incubation, (2) color development, and (3) blocking of HRP enzyme activity.*

Before you begin

If the antibody is biotinylated 'in house' using a biotinylation kit, excess free biotin must be removed before use. We recommend using Kromnigon's BiotinPure kit (Cat. no. 9000051-1) or oYo-Link® Single-Biotin from AlphaThera.

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

(A) StreptaClick® HRP labeling protocol

Antibody labeling with StreptaClick® HRP is performed at room temperature (RT).

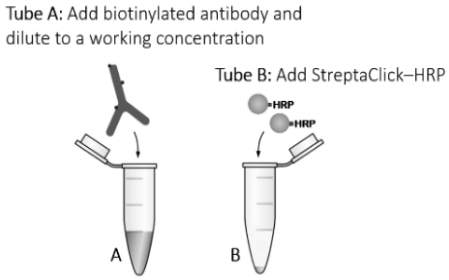
Antibodies should be diluted to their final working concentration prior to mixing with the StreptaClick® HRP labeling reagent (step 2 below). The term *working concentration* refers to the antibody concentration that will be used when added to the tissue section (immunostaining step). This predilution step prior to mixing with the StreptaClick® HRP labeling reagent prevents HRP quenching caused by sodium azide, which is commonly used as a preservative in antibody stock solutions.

Procedure:

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), dilute the biotinylated antibody with your immunostaining buffer of choice (e.g., PBS with 0.5% BSA) to the desired volume and final working concentration.

Tip: *For TSA immunostaining, a 1:250–1:1000 dilution from a 0.5 mg/ml antibody stock solution is recommended, corresponding to a 0.5–2 µg/ml working concentration.*



The molar ratio between the biotinylated antibody and the StreptaClick® HRP labeling reagent is critical for optimal

antibody labeling. Therefore, avoid pipetting volumes smaller than 2 µl of the antibody stock solution.

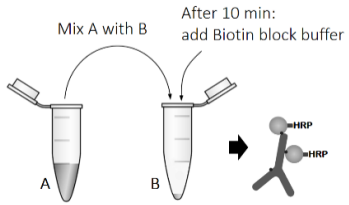
Tip: *If, for example, only 0.8 µl of the antibody stock is required for the experiment, prepare an intermediate dilution of the stock antibody. For instance, prepare a 5-fold diluted antibody stock aliquot and then pipette 4 µl (0.8 µl × 5) from this dilution.*

3. In the second tube (Tube B), add the appropriate volume of StreptaClick® HRP labeling reagent according to Table 1 below. For example, if 3 µl of biotinylated antibody at a stock concentration of 0.5 mg/ml is added to Tube A, then add 30 µl (3 × 10 µl) of StreptaClick® HRP labeling reagent to Tube B.

Table 1. *The volume of StreptaClick® HRP labeling reagent to be used for each µL of biotinylated antibody at different antibody stock concentrations.*

Antibody stock conc.	Antibody (µl)	StreptaClick® HRP labeling reagent (µl)
1 mg/mL	1 µL	20 µL
0.5 mg/mL	1 µL	10 µL
0.1 mg/mL	1 µL	2 µL

4. Transfer the entire antibody solution from Tube A into Tube B (containing the StreptaClick® HRP labeling reagent) and mix immediately by pipetting up and down. Avoid creating bubbles.



Tip*: Rapid mixing of the antibody solution with the StreptaClick® HRP labeling reagent is essential to ensure uniform distribution of HRP on the biotinylated antibodies. Transferring the larger volume from Tube A into the smaller volume in Tube B facilitates efficient mixing.*

5. After 10 minutes or more, add the same volume of Biotin block buffer as StreptaClick® HRP labeling reagent used in step 3 and mix. The Biotin block buffer immediately inactivates any remains of unbound StreptClick® HRP labeling reagent.

The biotinylated antibody is now labeled with HRP and ready to be used for multiplex TSA immunostaining. Use the HRP-labeled antibodies within 12 hours when the Biotin block buffer has been added.

Tip: *All antibodies to be use the same day in the experiment can be labeled with HRP simultaneously* and stored at +4 °C.

Tip: *For an example calculation, see Calculation for a 3-plex IHC staining at the bottom of the next page.*

(B) TSA immunostaining protocol

Each staining cycle consists of three main procedures: antibody incubation, color development, and HRP blocking (Figure 1). The HRP blocking step is performed at RT, which makes the protocol easy to use and does not compromise tissue morphology.

1. Prepare tissue sections for immunostaining according to standard protocols. Avidin/biotin blocking is not required.

2. Block endogenous peroxidases with activated HRP block buffer. To prepare, add 10 µl of 3% H_2O_2 and 50 µl of Block Activator to each ml of HRP block buffer. Apply 100 µl of the activated buffer to each tissue section and incubate for 14 minutes at RT. The activated HRP block buffer will also be used after each color development step (see Step 10).

Tip: *Prepare enough activated HRP block buffer for the entire experiment and store in the dark for up to 24 hours.*

Wash briefly in distilled water or PBS twice (2 × 30 seconds), followed by a 30-second wash in PBS

3. Antibody incubation. Apply 100 µl of HRP-labeled antibody to each tissue section and incubate for 20–60 minutes at RT. For shorter incubations (20–30 minutes), placing the slides on a rotation shaker at 50 rpm is recommended. After incubation, wash briefly in distilled water or PBS twice (2 × 30 seconds), followed by a 10-minute wash in PBS.

4. Color development. Dilute the tyramide dye in tyramide amplification buffer and apply to the tissue sections. Apply 100 µl to each section and incubate for 14 minutes at room temperature. Note: Tyramide reagents may not be provided in the kit.

Tip: *The color development time can be adjusted between 10–20 minutes.*

Tip: *The working concentration of the tyramide dye may be adjusted to achieve the desired signal intensity. For tyramide dyes from Kromnigon the typical dilution range is 1:50-1:200.*

Wash the sections in distilled water three times (3 × 30 seconds).

5. HRP block. Ensure that the sections have been washed in distilled water (not PBS) before applying the HRP block buffer. Apply 100 µl of activated HRP block buffer (prepared with H_2O_2 and Block Activator) to each tissue section and incubate for 14 minutes at RT. This step is required only when a sequential immunostaining cycle will be performed.

Tip: *If HRP activity is not fully quenched, the incubation time may be extended up to 20 minutes*.

Note: *Activated HRP block buffer (prepared with H_2O_2 and Block Activator) should be stored in the dark, either at RT or +4 °C, and can be used for up to 24 hours.*

Wash briefly in distilled water or PBS twice (2 × 30 seconds), followed by 30 seconds in PBS.

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

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

Example: Calculations for a 3-plex IHC staining with different antibody stock concentrations and dilutions

Number of sections: 9 tissue sections

Staining volume: 100 µl / tissue

Calculations below are based on preparing 10 sections × 100 µl = 1000 µl of antibody working solution to account for pipetting loss. The corresponding volumes of StreptaClick® HRP labeling reagent and Biotin Block Buffer are calculated according to Table 1.

Antibodies:

Anti-CD3-biotin (stock 0.1 mg/ml), used at 1:100
Anti-CD4-biotin (stock 0.2 mg/ml), used at 1:400
Anti-CD8-biotin (stock 1.0 mg/ml), used at 1:2000

Calculation table:

Antibody stock solution	Stock conc mg/ml	Dilution factor for IHC	Volume staining buffer	Volume ab stock solution	Strepta-Click HRP	Biotin Block buffer
CD3	0,1	1:100	1000 ul	10 ul	20 ul	20 ul
CD4	0,2	1:400	1000 ul	2,5 ul	10 ul	10 ul
CD8	1,0	1:2000	1000 ul	0,5 ul*	-	-
CD8 (1:10)	0,1	1:200	1000 ul	5 ul	10 ul	10 ul

**** Important:** Avoid pipetting volumes smaller than 2 µl of antibody stock solution. Instead, prepare a 1:10 diluted stock and use this diluted stock to generate the antibody working solution.*

Troubleshooting guide

Weak/No signals

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

- Increase the color development time (step 4) up to 20 minutes.

- Increase the amount of tyramide dye during the color developing step (step 4).

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

- The ratio between the biotinylated antibody and StreptaClick® HRP labeling reagent 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 labeling reagent will interfere with the antibody function.

- It is important to first prepare an antibody working solution before mixing with StreptaClick® HRP labeling reagent. 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 labeling reagent is important. Make a full transfer of the antibody working solution (Tube A) into the tube of StreptaClick® HRP labeling reagent (Tube B).

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

- Do not use dry milk in the immunostaining buffer, since it may contain free biotin that quenches the StreptaClick® HRP labeling reagent. 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. Prolong the HRP block step (step 5) up to 20 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.