TYRAMIDE SIGNAL AMPLIFICATION (TSA+) IMMUNOHISTOCHEMICAL DETECTION

When target expression is low or antibodies are low-affinity, a highly sensitive tyramide signal amplification (TSA) technique can be used, given that horseradish peroxidase (HRP)-conjugated secondary antibody can be introduced. It is very important to block any endogenous HRP activity. TSA amplification reagent is a substrate for HRP, which converts the TSA reagent to highly reactive free radicals that bind covalently to electron-rich amino acids like tyrosine and tryptophan in the immediate proximity to the target. The reaction time is very short, allowing for very good resolution and clarity. The TSA+ technique has increased sensitivity, which allows for detection of low expressed targets and use of low-affinity antibodies. Since primary antibodies can be diluted much more, non-specific background can be reduced, therefore, a proper titration of antibody is crucial for a good result. Finally, TSA offers more options for co-localisation studies since two different antibodies from the same host species can be used (see Multiplexed Immunofluorescence Protocol). TSA+ kit (Perkin Elmer) exists for FITC, TMR, Cy3, Cy3.5, Cy5 and Cy5.5 detection.

IHC-IF TSA+ PROTOCOL.

MATERIALS NEEDED

- Cryo- or paraffin-embedded sections mounted on SuperFrost slides.
- Target retrieval solutions pH6 or pH9 (if needed)
- Incubation chamber with wet Wettex stripes
- DAKO Cytomation pen
- 1xPBS
- Primary and secondary antibodies diluted in 1x PBS, containing 0.3% Triton, 0.01% NaAzide, 0.02% Bacitracin
- Bovine serum albumin (BSA)
- 30% H_2O_2 solution
- TNT buffer
- Blocking reagent (for TNB Buffer) (Perkin Elmer)
- TSA+ kit (FITC, TMR, Cy3 or Cy5 as appropriate) (Perkin Elmer)
- Mounting media (e.g. ProLong Gold with or without DAPI)
- Coverslips
- Eppendorf tubes for dilution of antibodies
- Pipettes and pipette-tips

PROCEDURE

PRIMARY ANTIBODIES PREPARATION

Pre-incubate primary antibody with BSA (0.5%, 1 h at RT) prior to application to the tissue. Dilute primary antibody in antibody diluent to a working concentration.

SECTION PRE-TREATMENT PRIOR TO IHC

CRYO-SECTIONS

- **Perfusion-fixed,** 10-30% sucrose cryoprotected sections cut in a microtome at 14 µm, thaw-mounted on Super Frost slides. Dry sections on slides for additional 2 h at room temperature (RT). Rehydrate in 1xPBS 2x15 min, or proceed with antigen retrieval.
- **Snap-frozen** sections cut in a microtome at 14 µm, thaw-mounted on Super-Frost slides and dried for 2 h at RT. Pre-fix sections in ice-cold paraformaldehyde (4% PFA) for 20 min (if using stored sections, put frozen sections directly into 4% PFA, do not allow thawing). Rinse in 1x PBS 15 min.

Antigen retrieval for cryosections (optional)

If target requires antigen retrieval, standard target retrieval solutions can be used, but the temperature and time should be reduced.

Heat-induced epitope retrieval (HIER)

1. HIER buffer pH 6.1, a modified citrate buffer, 60-70 C, 10 min.
2. HIER buffer pH 9, a Tris/EDTA buffer, 60-70 C, 10 min.

PARAFFIN SECTIONS

Paraffin embedded sections cut at 4 µm thick, mounted on Super Frost slides, dried at +50C overnight.

Antigen retrieval for paraffin sections

Heat-induced epitope retrieval (HIER)

1. HIER buffer pH 6.1, a modified citrate buffer, 97 C, 20 min.
2. HIER buffer pH 9, a Tris/EDTA buffer, 97 C, 20 min.

If antigen retrieval is not required:

1. Deparaffinise sections in xylene (2x5 min)
2. Rehydrate sections: 100% EtOH 2x3 min, 95% EtOH 2x3 min, 70% EtOH 2x3 min, rinse in ddH20
3. Proceed with IHC procedure.

IHC TSA+ PROCEDURE

DAY I

1. Draw a circle around each section using DAKO Cytomation pen.
2. Rinse slides in 1x PBS 15 min at RT
3. Block endogenous peroxidase:
   a. Cryo sections: Put slides in 0.03 -0.1% H2O2 in 1xPBS for 5 min at RT to quench endogenous peroxidase activity
   b. Paraffin sections: Put slides in 0.3% H2O2 in 1xPBS for 15 min at RT to quench endogenous peroxidase activity
4. Rinse in PBS 15 min at RT
5. Add primary antibody diluted in 1x PBS, containing 0.3% Triton, 0.01% NaAzide, 0.02% Bacitracin), approx. 180µl/ slide
6. Incubate in a humidified chamber overnight (18-48 h) at 4ºC.

The proper antibody titration is crucial for the results and should be defined by the user. The first standard titration curve can be run in a following way.

Start with concentration suggested by a supplier, and then run 3 more 5x dilutions.

E.g.: 1:400, 1:2,000, 1:10,000, 1:50,000

Generally, paraffin-embedded tissues will require higher titre of primary AB then cryo tissue.

DAY II

7 Rinse slides in 1xTNT wash buffer, 15-20 min at RT
8 Block slides in 1xTNB buffer 30 min at RT (on slides, 180 µl/ slide)
9 Incubate with HRP-labelled secondary AB (1:200) diluted in TNB 30 min at RT on slides (E.g. DAKO SwARb-HRP if the primary antibody is raised in rabbit)
10 Rinse in 1xTNT buffer 15-20 min at RT
11 Incubate with biotinyl tyramide conjugated fluorochrome (Ex. FITC 1:100 or Cy3 1:200 – 1:500 in amplification diluent (TSA Kit) 10 min at RT, under a black cover
12 Rinse in 1x TNT buffer 15-20 min at RT, under a black cover
13 Mount in anti-fading solution (e.g ProLong Gold)
14 Leave slides for 24h at 4C before microscopy/scanning
15 Store at 4C

Protocol optimized for Triple A Polyclonals™ and PrecisA Monoclonals™ from Atlas Antibodies.

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