Tyramide Signal Amplification (TSA)

This tyramide signal amplification (TSA) protocol can be used to detect signals in peroxidase-labeled samples by either immunostaining or *in situ* hybridization. All incubations are carried out on a shaker.

Reagents:

- 2-4% formaldehyde, pH 7.4
- phosphate buffer (PBS), pH 7.4
- PBT: 0.1% Tween-20; PBS, pH 7.4
- 1mM sodium azide in PBT
- 30% H_2O_2
- 1 mg/ml fluorescently labeled tyramide in labeling grade DMSO

Optionally:

- dextran sulfate (DS)
- 4-iodophenol
- acidic glycine buffer: 0.1 M glycine; pH 2.0; 0.1% Tween-20

Protocol:

1. Fix the sample in the required manner. Usually, fixation is carried out with cold 2-4% formaldehyde (pH 7.4). Wash the sample from the fixative with phosphate buffer (PBS, pH 7.4) and **PBT** (0.1% Tween-20; PBS, pH 7.4).
2. Inhibit endogenous peroxidase activity by incubating the sample in an **inhibitory solution** (1 mM sodium azide in PBT) for 30-60 min at room temperature.

   Optionally. Instead of 1 mM sodium azide, 3% H_2O_2 in PBT or 0.02 N HCl can be used for inhibition. For subsequent immunochemistry, this stage can be combined with blocking nonspecific binding of antibodies. To do this, blocking serum or 1% BSA must be added to the azide or H_2O_2 solutions.

   Note! In the case of low background and for *in situ* hybridization, this step can be skipped; go directly to step 4.
3. Thoroughly wash the sample from the inhibitory solution with three 10-minute incubations in PBT at room temperature.
4. Perform all immunochemistry or *in situ* hybridization steps to label samples with peroxidase-conjugated antibodies.

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5. Wash the sample thoroughly to remove antibodies with three 10-minute incubations in PBT at room temperature.
6. Prepare **reaction buffer** immediately before use. To do this, dilute PBT twice with 30% H_2O_2, 1:100 each time, to the final concentration of 0.003% (1:10000).

   Optionally. To increase the sensitivity of the method, the following components can be added to the reaction mixture (separately or in combination):

   - 2% dextran sulfate (DS) is used to increase the viscosity of the reaction mixture.
   - 500 µg/ml 4-iodophenol is used to enhance the peroxidase reaction. It is more convenient to use as a 1:200 dilution of the stock solution (100 mg/ml in ethanol).
7. Add 1 to 10 µg/ml labeled tyramide to the reaction buffer (the optimal concentration must be determined experimentally). Mix by shaking.
8. Incubate the sample with the reaction buffer in the dark at room temperature for 5–30 min (the exact time is determined experimentally; 15 min can be used as a starting point).
9. Stop the reaction by incubating the sample in inhibitor solution (1 mM sodium azide in PBT) for 10 min at room temperature in the dark.
10. Wash the sample with PBS three times for 10 min.
11. To repeat the antibody-TSA labeling cycle, treat the sample with **acidic glycine buffer** (0.1% Tween-20; 0.1 M glycine, pH 2.0) for 10 min at room temperature. This procedure detaches antigen-bound antibodies without affecting the tyramide covalently bound to proteins.
12. Mount the sample under a coverslip using a mounting medium.

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Troubleshooting

PROBLEM	RECOMMENDED ACTION
Low signal	• Optimize probe / antibody concentration • Titer HRP conjugate to determine optimum concentration for signal amplification • Add tissue permeabilization step to facilitate penetration of reagents • Lengthen the incubation time with the tyramide working solution • Use antigen retrieval techniques to unmask the target epitopes
Excess signal	• Optimize probe / antibody concentration • Decrease concentration of HRP conjugate • Decrease the tyramide concentration in working solution • Shorten the incubation time with the tyramide working solution
Low resolution or blurry signal	• Shorten the incubation time with the tyramide working solution • Check the dilution of the stop reagent
High background	• Decrease probe / primary antibody concentration • Use a lower concentration of secondary antibody • Decrease HRP conjugate concentration • Check for endogenous biotin (if using streptavidin conjugates) • Check for antibody specificity • Shorten the incubation time with the tyramide working solution • Lengthen endogenous peroxidase quenching step • Increase number and/or length of washes • Nonqualified or contaminated blocking reagent used • Filter buffers
Bright dots on background	• Centrifuge secondary antibody tube before use

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