D. Standard Protocol

1. Preparation of Buffers and Reagents

The following buffers and reagents are required for slide preparation and TSA application.

Fluorophore Tyramide (FT) Stock Solution

Fluorophore Tyramides (FT) are supplied as solids and must be reconstituted before each use. Add 0.6 ml **(for NEL731,732, and 733)** or 0.3 mL **(for 731A)** of dimethyl sulfoxide (DMSO) or 0.3 mL distilled water **(for NEL734A)** for a FT stock solution. FT Stock Solution, when stored at 4°C, is stable for at least three months. DMSO freezes at 4°C. Thaw the stock solution **before** each use.

Fluorophore Tyramide Working Solution

Before each procedure, make a 1:50 dilution of the FT Stock Solution using **Amplification Diluent**.
Approximately 100-300 µl of FT Working Solution is required per slide.

Wash Buffer

TNT Wash Buffer
0.1M Tris-HCl, pH 7.5 0.15M NaCl 0.05% TWEEN®20

Other wash buffers (such as PBS) may be used as long as 0.5% TWEEN®20 is added.

Blocking Buffer

TNB Blocking Buffer
0.1M Tris-HCl, pH 7.5 0.15M NaCl 0.5% Blocking Reagent (supplied in kit)

Add Blocking Reagent slowly to buffer. Heat gradually (up to 60°C) with continuous stirring to dissolve the Blocking Reagent. Aliquot and store at -20°C for long term use.

The Blocking Reagent supplied in this kit is optimal for use with the TSA kit reagents provided. Other blocking reagents may lead to increased background.

Note:
If the primary antibody requires an incubation buffer other than TNB, use that buffer in place of TNB in step 2 of the Step by Step Protocol.

Procedural Notes

- Do not let slides dry out between steps.
- A humidity chamber is recommended for all incubation steps (i.e., a damp paper towel in a covered box).
- Drain off as much of the incubation solutions as possible, before the addition of the next solution, to prevent dilution and uneven staining.
- Be sure to use enough volume of solutions to cover sections or cells.

2. Step by Step Protocol

Standard Non-Radioactive In Situ Hybridication Technique

The following four steps are a suggested format for slide preparation prior to the introduction of TSA.

Suggested Slide preparation

(1) Prepare slides for detection with TSA-Direct using standard non-radioactive in situ hybridization techniques with biotin, fluorescein or digoxigenin labeled probes up to post hybridization washes.

Blocking Step

(2) Incubate slides with 100 µl of TNB Blocking Buffer in a humid chamber for 30 minutes at room temperature. The use of a coverslip will reduce evaporation.

Suggested Introduction of HRP

(3) Incubate slides, depending on labeled probe, with either:

a) 100 µl of HRP labeled antifluorescein or antidigoxigenin diluted between 1:100 to 1:500 in TNB Buffer

or

b) 100 µl of SA-HRP diluted 1:100 in TNB Buffer. Incubate slides in a humid chamber for 30 minutes at room temperature. The use of a coverslip will reduce evaporation.

TSA Amplification

(4) After HRP incubation, proceed with the following:

a) Wash the slides 3 times for 5 minutes each in TNT Buffer at room temperature with agitation.

b) Pipet 300 µl of the Fluorophore Tyramide (FT) Working Solution onto each slide. Incubate the slides at room temperature for 3 to 10 minutes.

a) Wash the slides 3x for 5 minutes each, in TNT Buffer, at room temperature with agitation.

Visualization of Deposited Fluorophores:

Follow the applicable visualization option fluorescence or chromogenic.

a) Fluorescence Option

Steps

(5) Counterstain if appropriate. Slides are now ready for mounting and for fluorescence microscopy evaluation.

The following is a table of excitation and emission wavelengths for Fluorescein, Tetramethylrhodamine, Coumarin and Cyanine 3 :

Fluorophore	Excitation	Emission
Fluorescein	494 nm	517 nm
Tetramethylrhodamine	550 nm	570 nm
Coumarin	402 nm	443 nm
Cyanine 3	550 nm	570 nm

b) Chromogenic Option for TSA-Direct (GreenFISH) only

Steps

(6) Add approximately 100 µl of diluted Antifluorescein-AP* (1:100 diluted in TNB Buffer) to each slide. The use of a coverslip will reduce evaporation.

(7) Incubate the slides in a humid chamber at room temperature for 30 minutes.

(8) Wash the slides 3x for 5 minutes each in TNT Buffer at room temperature with agitation.

(9) Visualize with standard HRP catalyzed chromogenic substrates such as DAB (diaminobenzidine) and AEC (aminoethyl carbazole) or AP catalyzed substrates such as NBT/BCIP (nitro blue tetrazolium/5-bromo- 4-chloro-indolyl phosphate).

(10) Counterstain if appropriate. Hematoxylin is an effective counterstain for DAB and AEC. Nuclear Fast Red is an effective counterstain for NBT/BCIP. Histomount™ and Clearmount™ may be used for mounting.

* See Complementary Products

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