

PerkinElmer Life and Analytical Sciences, Inc.



**TSA™ PLUS**

**FLUORESCENCE SYSTEMS**

**Tyramide Signal Amplification**  
*For Fluorescence In situ Hybridization  
and Immunohistochemistry*

| Fluorescein                | Tetramethyl-<br>rhodamine  | Cyanine 3                  | Cyanine 5                  |
|----------------------------|----------------------------|----------------------------|----------------------------|
| NEL741<br>50-150 slides*   | NEL742<br>50-150 slides*   | NEL744<br>50-150 slides*   | NEL745<br>50-150 slides*   |
| NEL741B<br>250-750 slides* | NEL742B<br>250-750 slides* | NEL744B<br>250-750 slides* | NEL745B<br>250-750 slides* |

**\* Number of slides determined by volume used per section.**

For Laboratory Use

CAUTION: Research chemicals for research purposes only

## Multiple Dye Combination Kits For Initial Evaluations And Two-Color Fluorescence.

### TSA™ Plus Fluorescence Palette System

Catalog # NEL760

This kit contains all four TSA-Plus Fluorescent System Dyes packaged together in a size convenient to evaluate this technology. There are sufficient reagents to stain 10 to 35 slides with each of the following TSA-Plus dyes: TSA-Plus Fluorescein, TSA-Plus TMR, TSA-Plus Cyanine 3 and TSA Plus Cyanine 5.

The only part of the full sized kit manual which is different for the TSA-Plus Fluorescence Palette System is the volume of DMSO required to reconstitute the solid dye. Please use the following instruction to prepare a stock and working solution for each dye.

#### Fluorophore Tyramide (Amplification Reagent) Stock Solution

**Fluorophore Tyramides (Amplification Reagents)** are supplied as a solid. Each vial must be reconstituted with 60  $\mu$ L DMSO (dimethyl sulfoxide -molecular biology or HPLC-grade) before use. The Fluorophore Tyramide Stock Solution, when stored at 4° C, is stable for at least 3 months. (Note: DMSO freezes at 4° C. Thaw the Stock Solution before each use.)

#### Fluorophore Tyramide(Amplification Reagent) Working Solution

Before each procedure, dilute the Fluorophore Tyramide Stock Solution 1:50 using **1X Plus Amplification Diluent** to make the Fluorophore Tyramide Working Solution. Approximately 100-300  $\mu$ L of Fluorophore Tyramide Working Solution is required per slide. Discard any unused portion of working solution.

### Kits for Two-Color Fluorescence

The following combinations of dyes can be ordered for two color fluorescence staining. Each kit provides sufficient reagents to stain 50 - 150 slides of each dye.

**INSTRUCTIONS FOR PREPARATION OF EACH DYE IN THE COMBINATION KITS ARE IDENTICAL TO THE SINGLE DYE INSTRUCTIONS IN THE MANUAL.**

| Catalog number | Name                                    |
|----------------|---|
| NEL752         | TSA-Plus Cyanine 3 / Cyanine 5 System   |
| NEL753         | TSA-Plus Cyanine 3 / Fluorescein System |
| NEL754         | TSA-Plus Cyanine 5 / Fluorescein System |
| NEL756         | TSA-Plus TMR / Fluorescein System       |

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# I. INTRODUCTION

## A. Background Information

### What is (F) ISH?

In situ hybridization (ISH) is a technique used to detect, visualize and localize DNA and RNA at the cellular level. Radiolabeled or nonrad-labeled probes are hybridized to nucleic acid targets in tissue or cell preps. Probes can be labeled using common labeling techniques such as in vitro transcription, nick translation and 3' end labeling. Detection schemes for in situ hybridization include autoradiography for radioactive probes and dye deposition for enzyme or hapten-labeled probes. In FISH (Fluorescence In Situ Hybridization) fluorophore-labeled probes or reagents are used for detection.

### What is IHC?

Immunohistochemistry (IHC) is a technique to detect, visualize and localize antigens at the cellular level. Common IHC protocols use primary and secondary antibodies to indirectly detect antigens in frozen or paraffin-embedded tissue sections. Detection schemes for IHC include dye deposition for enzyme labeled antibodies, fluorescence for fluorescent labeled antibodies and silver enhancement for systems using gold labeling.

## **What is TSA?**

(Tyramide Signal Amplification) is a powerful, patented technology from NEN Life Science Products that significantly enhances both chromogenic and fluorescent signals. It is easily integrated into standard nonradioactive in situ hybridization (ISH) or IHC protocols, provided that Horseradish Peroxidase (HRP) is in the system.

## **How does TSA™ Plus Fluorescence Systems Signal Amplification work?**

The TSA Plus Fluorescence Systems technology uses HRP to catalyze the deposition of a fluorophore-labeled tyramide amplification reagent onto tissue sections or cell preparation surfaces that have been previously blocked with proteins. The reaction is quick (less than 10 minutes) and results in the deposition of numerous fluorophore labels immediately adjacent to the immobilized HRP enzyme. These fluorophores can then be detected by fluorescence visualization techniques, with significant enhancement of the signal. Because the added labels are deposited proximal to the initial immobilized HRP enzyme site, there is minimal loss in resolution. This signal amplification technique may be applied to both ISH and IHC.

The **TSA Plus Fluorescence Systems** simplify fluorescence detection because the tyramide amplification reagent is directly labeled with a fluorophore. Once the fluorophore-labeled tyramides have been deposited, results can be immediately visualized via fluorescence microscopy. The TSA Plus Fluorescence Systems include: TSA Plus Fluorescein System (NEL741/NEL741B), TSA Plus Tetramethylrhodamine System (NEL742/NEL742B), TSA Plus

Cyanine 3 System (NEL744/NEL744B) and the TSA Plus Cyanine 5 System (NEL745/NEL745B).

### **What ISH and IHC Mediums are Compatible With The TSA Plus Fluorescence Systems?**

**The TSA Plus Fluorescence Systems** have been successfully applied to the following media: formalin-fixed/paraffin-embedded sections, frozen/fixed sections, chromosome spreads and cultured cells. Cells or tissues must be **fixed** before applying TSA Plus reagents.

#### **B. The TSA Plus Fluorescence Systems**

The **TSA Plus Fluorescence Systems** contain the following components necessary for signal amplification:

**Fluorophore-labeled Tyramide (Amplification Reagent) and 1X Plus Amplification Diluent. Fluorophore labels include Fluorescein (NEL741 / 741B), Tetramethylrhodamine (TMR) (NEL742 / NEL742B), Cyanine 3 (NEL744 / NEL744B), or Cyanine 5 (NEL745 / NEL745B).**

**The TSA Plus Fluorescence Systems** are compatible with a wide variety of standard ISH and IHC protocols. However, HRP must be available for the amplification to occur. All protocols must include the use of an HRP-labeled reagent (SA-HRP, HRP-labeled antibody, ABC reagent, etc.) immediately prior to the addition of the fluorophore-labeled tyramide amplification reagent. HRP reagents must be purchased separately. Amplification is followed by fluorescence visualization techniques.

## **Intended Use**

The intended use of this kit is to amplify signals generated by Horseradish Peroxidase in nonradioactive (F)ISH and fluorescence IHC applications. The reagents in this kit have been optimized for use in slide based assays. These kits are not suitable for use on membranes or microwell plates.

**FOR LABORATORY USE.**

## **Safety Note**

**All reagents are classified as non hazardous. We strongly recommend wearing disposable gloves and safety glasses while working. Thorough washing of hands after handling is also recommended. Do not eat, smoke, or drink in areas in which reagents are handled.**

### C. Components of TSA Plus Fluorescence Systems

|                               |   |
|-------------------------------|---|
| <b>NEL741 (Fluorescein)</b>   | <b>50-150 slides*</b>                               |
| <b>Reagent</b>                | <b>Amount</b>                                       |
| 1X Plus Amplification Diluent | 15 mL   |
| Fluorescein Tyramide          | 2 Vials (25-75 slides each) for 50-150 slides total |

|                               |  |
|-------------------------------|--|
| <b>NEL741B (Fluorescein)</b>  | <b>250-750 slides*</b>                           |
| <b>Reagent</b>                | <b>Amount</b>                                    |
| 1X Plus Amplification Diluent | 75 mL  |
| Fluorescein Tyramide          | 10 Vials 25-75 slides (for 250-750 slides total) |

|                                      |   |
|--------------------------------------|---|
| <b>NEL742 (Tetramethylrhodamine)</b> | <b>50-150 slides*</b>                               |
| <b>Reagent</b>                       | <b>Amount</b>                                       |
| 1X Plus Amplification Diluent        | 15 mL   |
| Tetramethylrhodamine (TMR) Tyramide  | 2 Vials (25-75 slides each) for 50-150 slides total |

|                                       |   |
|---------------------------------------|---|
| <b>NEL742B (Tetramethylrhodamine)</b> | <b>250-750 slides*</b>                                |
| <b>Reagent</b>                        | <b>Amount</b>   |
| 1X Plus Amplification Diluent         | 75 mL   |
| Tetramethylrhodamine (TMR) Tyramide   | 10 Vials (25-75 slides each) for 250-750 slides total |

|                               |   |
|-------------------------------|---|
| <b>NEL744 (Cyanine 3)</b>     | <b>50-150 slides*</b>                               |
| <b>Reagent</b>                | <b>Amount</b>                                       |
| 1X Plus Amplification Diluent | 15 mL   |
| Cyanine 3 Tyramide            | 2 Vials (25-75 slides each) for 50-150 slides total |

|                               |   |
|-------------------------------|---|
| <b>NEL744B (Cyanine 3)</b>    | <b>250-750 slides*</b>                                |
| <b>Reagent</b>                | <b>Amount</b>   |
| 1X Plus Amplification Diluent | 75 mL   |
| Cyanine 3 Tyramide            | 10 Vials (25-75 slides each) for 250-750 slides total |

| <b>NEL745 (Cyanine 5)</b>     | <b>50-150 slides*</b>                               |
|-------------------------------|---|
| <b>Reagent</b>                | <b>Amount</b>                                       |
| 1X Plus Amplification Diluent | 15 mL   |
| Cyanine 5 Tyramide            | 2 Vials (25-75 slides each) for 50-150 slides total |

| <b>NEL745B (Cyanine 5)</b>    | <b>250-750 slides*</b>                                |
|-------------------------------|---|
| <b>Reagent</b>                | <b>Amount</b>   |
| 1X Plus Amplification Diluent | 75 mL   |
| Cyanine 5 Tyramide            | 10 Vials (25-75 slides each) for 250-750 slides total |

- **The number of slides is determined by the reagent volume (approximately 100-300  $\mu$  L) which is needed to completely cover the cells or tissue section on the slide. Each vial of tyramide dye is sufficient for 25 to 75 slides.**

#### **Storage and Stability**

Upon receipt, the **TSA Plus Fluorescence Systems** kits should be stored at 4 ° C. The components in this kit are stable for a minimum of 3 months under proper storage conditions. Do not use beyond expiration date listed on kit.

#### **Critical Reagents Required But Not Supplied**

- HRP-labeled reagent (e.g., SA-HRP) is not supplied in the kit. SA-HRP or alternatives including anti-digoxigenin-HRP for use with DIG-labeled probes, HRP-labeled probe or antibody, ABC reagent, etc. must be purchased separately.
- A Blocking reagent may be purchased as a separate component (Cat.No. FP1020).

- DMSO (molecular biology or HPLC grade)
- Buffer components

### Complementary Products

| <b>TSA Kits</b>                 | <b>Catalog Number</b>                                |
|---------------------------------|--|
| TSA Plus DNP (HRP or AP) System | NEL746A, NEL746B,<br>NEL747A, NEL747B                |
| TSA Fluorescence Systems        | NEL701, NEL701A, NEL702,<br>NEL703, NEL704A, NEL705A |
| TSA Biotin System               | NEL700, NEL700A                                      |

| <b>Reagent</b>                      | <b>Catalog Number</b> |
|-------------------------------------|-----------------------|
| BCIP/NBT                            | NEL937                |
| DAB                                 | NEL938                |
| Anti-fluorescein – AP               | NEF709                |
| Anti-fluorescein-HRP                | NEF710                |
| Streptavidin-HRP                    | NEL750                |
| Streptavidin-AP                     | NEL751                |
| Streptavidin-Fluorescein            | NEL720                |
| Streptavidin-Texas Red <sup>®</sup> | NEL721                |
| Streptavidin-Coumarin               | NEL722                |
| Blocking Reagent                    | FP1020                |
| Anti-Human IgG (Goat), HRP-Labeled  | NEF802                |
| Anti-Rabbit IgG (Goat), HRP-Labeled | NEF812                |
| Anti-Mouse IgG (Goat), HRP-Labeled  | NEF822                |

## II. PROTOCOL FOR ISH

### A. Overview Protocol for TSA Plus Fluorescence Systems for In Situ Hybridization

*Standard non-radiometric FISH*

- Quench endogenous peroxidase activity (if needed)
- Tissue permeabilization (if needed)
- Probe hybridization
- Post-hybridization stringency washes



*Blocking Step*

Block slides for 30 min. in TNB buffer @ RT



*Incorporation of HRP*

Incubate slides with appropriate HRP reagent (anti-DIG-HRP, SA-HRP, etc.) for 30 min. @ RT.



Wash slides 3X for 5 min. in TNT buffer @ RT



*TSA Plus Amplification*

Incubate in Fluorophore Tyramide Amplification Reagent working solution for 3 to 10 min. @ RT



Wash slides 3X for 5 min. in TNT buffer @ RT



**Chromogenic- (for NEL741/741B only)**

**Fluorescence**

*Visualization*

Incubate slides in anti-fluorescein-AP or anti-fluorescein-HRP for 30 min. @ RT

Add appropriate counterstain and mount for fluorescence microscopy.



Wash slides 3X for 5 min. in TNT buffer @ RT



Add appropriate chromogen. Counterstain and mount for microscopy

## **B. Suggested ISH Protocol**

The following is a suggested protocol for using TSA Plus Fluorescence Systems for in situ hybridization signal amplification.

TSA technology requires HRP to be an integral part of the detection protocol. This may be accomplished via the use of various hapten-labeled probe/anti-hapten-HRP conjugate combinations such as digoxigenin-labeled probe followed by anti-DIG-HRP, biotin-labeled probe followed by SA-HRP, or with a fluorescein-labeled probe followed by anti-fluorescein-HRP. Once HRP is introduced, the Fluorophore Tyramide (Amplification Reagent) working solution is added. Visualization is then done through the use of standard fluorescence microscopy. For the TSA Fluorescein systems (NEL 741/ NEL741B), the fluorescence signal can be converted to a chromogenic signal. This is possible by following the Fluorescein tyramide step by an anti-fluorescein-enzyme conjugate. Signal is visualized by the addition of an appropriate chromogen. Enzyme conjugates and chromogenic substrates must be purchased separately.

### **First Time Users**

First time nonradioactive ISH users should assess the need for various tissue pre-treatment conditions which may be necessary to improve penetration of reagents and/or to reduce background. A balance must be achieved between making the target accessible versus causing loss of target and/or destruction of tissue morphology. Reagent penetration may be improved by protein digestion or detergent permeabilization prior to probe hybridization. Common protein digestion methods include the use

of 0.005-0.1% pepsin in 0.01M HCl or Proteinase K (1-10  $\mu$  g/mL) in TRIS-HCl / 0.05M EDTA. Cell preparations are often permeabilized with detergents such as saponin or Triton X-100. Background may be reduced using procedures such as acetylation of tissue and/or inhibition of endogenous enzyme (peroxidase or alkaline phosphatase) activity.

First time TSA Plus Fluorescence Systems users should apply this to a proven ISH system.

## **Controls**

Always run control slides with each experiment. These should include an unamplified control slide (i.e., include specific probe but eliminate TSA reagents) and an amplified negative control slide (i.e., hybridize with either no probe, a nonspecific probe, or a mix of labeled specific probe plus a 100-fold excess unlabeled probe and include TSA reagents in detection procedure). In addition to proving validity of results, control slides are often beneficial in determining the cause of non-specific background.

## **Reagent Titration**

In general, most researchers have found that TSA requires lower probe and conjugate concentrations for optimal results when compared with standard unamplified nonradioactive methods.

### **1. Probe titration:**

Probe concentration must be optimized. It should be assessed using the standard concentration used in unamplified nonradiometric procedures and at reduced concentrations of 2 to 20-fold - less in the hybridization mix. Failure to establish appropriate probe concentration can result in little to no signal development.

## 2. **Titration of HRP enzyme conjugate:**

HRP must be present in the staining system in order to use the TSA Plus Fluorescence Systems. This could be done by using hapten-labeled probes followed by an appropriate anti-hapten-HRP conjugate (see p.14 for suggestions). HRP conjugates must be purchased separately. Appropriate HRP conjugate concentrations to assess include supplier's recommended starting concentration, 2-fold less and 5-fold less. In cases where no signal and no background are seen, it may be necessary to use an increased concentration instead. For example, if the recommended starting titer is 1:100, run HRP titration slides with HRP conjugate at 1:50, 1:100, 1:200 and 1:500.

## **Quenching Endogenous Peroxidase**

Activation and covalent binding of the Fluorophore Tyramides (Amplification Reagents) are catalyzed by HRP. To minimize background, endogenous peroxidase activity, if present, must be quenched. Users should establish the need for doing this and optimal methodology specific to the tissues or cells being stained.

Options include:

1. 0.3% H<sub>2</sub>O<sub>2</sub> to 3% H<sub>2</sub>O<sub>2</sub>
2. Methanol or PBS as diluent for H<sub>2</sub>O<sub>2</sub>.
3. Incubation time of 10 to 60 minutes.

For paraffin-embedded tissues, quenching can be done after dewaxing and alcohol rehydration but before the protease

digestion step. For frozen tissue or cell preps, quenching can be done following fixation and before the protease digestion step. After quenching wash with TN or 1X PBS buffer for 5 minutes.

Failure to establish optimum tissue pre-treatments and reagent concentrations may result in poor signal amplification and/or increased background.

## **Volumes**

The protocol is written for minimal volumes of reagent (e.g., 100-300  $\mu$ L). Reagent volumes used should be sufficient to completely cover cells or tissue sections on slide. If larger volumes are necessary to cover the samples, make the appropriate changes in the protocol.

## **Technical Support**

If there are any further questions regarding TSA in your ISH system, please contact PerkinElmer Life and Analytical Sciences Technical Support Department before proceeding. Refer to the Troubleshooting Guide section for local listing.

Technical assistance is also available through our web site at [www.PerkinElmer.com](http://www.PerkinElmer.com).

## C. Standard ISH Protocol

### 1. Preparation of Buffers and Reagents

The following buffers and reagents are required for slide preparation and TSA Plus Fluorescence Systems amplification.

#### **Fluorophore Tyramide (Amplification Reagent) Stock Solution**

Fluorophore Tyramides (Amplification Reagents) are supplied as a solid. Each vial must be reconstituted with 0.15 mL DMSO (dimethyl sulfoxide -molecular biology or HPLC-grade) before use. The Fluorophore Tyramide Stock Solution, when stored at 4° C, is stable for at least 3 months. (Note: DMSO freezes at 4° C. Thaw the Stock Solution before each use.)

#### **Fluorophore Tyramide(Amplification Reagent) Working Solution**

Before each procedure, dilute the Fluorophore Tyramide Stock Solution 1:50 using 1X Plus Amplification Diluent to make the Fluorophore Tyramide Working Solution. Approximately 100-300 µL of Fluorophore Tyramide Working Solution is required per slide. Discard any unused portion of working solution.

## Wash Buffer

TNT Wash Buffer

0.1 M TRIS-HCl, pH 7.5

0.15 M NaCl

0.05% Tween<sup>®</sup> 20

**NOTE: Other wash buffers (such as PBS) may be used. Substitution of 0.3% Triton X-100 for the 0.05% Tween-20 is also possible. Users should validate the use of wash buffer alternatives with their own systems.**

## Blocking Buffer

TNB Blocking Buffer

0.1 M TRIS-HCl, pH 7.5

0.15 M NaCl

0.5% Blocking Reagent (not supplied in kit)

Add Blocking Reagent slowly in small increments to buffer while stirring. Heat gradually to 60 ° C with continuous stirring to completely dissolve the Blocking Reagent. (This may take up to several hours. Preparation of volumes less than 100 mL allows for more even heating.) Aliquot and store at -20 ° C for long term use. Discard any unused blocking buffer which has been stored for greater than 24 hours at room temperature. The Blocking Reagent (Cat# FP1020) must be ordered as a separate component. It has been found to be optimal for use with the TSA kit reagents. The user should validate the use of alternative blocking reagents.

## 2. **Procedural Notes**

- Do not let slides dry out between steps.
- A humidified 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 reagent dilution and uneven staining. Blot area around, but not on, tissue section using a labwipe.
- Be sure to use enough volume of each reagent to completely cover sections or cells.
- Optional: Use of coverslips may reduce reagent evaporation, especially during steps, which require long incubation at elevated temperatures (such as probe hybridization). However care must be taken upon removal to prevent damage to tissues or cells.

## 3. **Step by Step Protocol**

The following is a suggested protocol for the use of TSA Plus Fluorescence Systems in a non-radioactive ISH protocol.

### **Step 1: Slide Preparation**

Prepare tissues or cells using standard fixation and embedding techniques. Dewax and rehydrate slides according to standard procedures.

## **Step 2: Standard Non-radioactive In Situ Hybridization Technique**

Follow standard non-radioactive in situ hybridization techniques. Include tissue permeabilization (if needed) and quenching of endogenous peroxidase activity (if needed). Probe hybridization (with digoxigenin, biotin, or fluorescein-labeled probes) should be done using concentration determined in optimization studies (see p. 9) followed by post-hybridization stringency washes.

**NOTE: Always run an unamplified control slide and an amplified negative control slide with each experiment.**

## **Step 3: Blocking Step**

Incubate slides with 100-300  $\mu$ L of TNB Buffer in a humidified chamber for 30 minutes at room temperature.

## **Step 4: Introduction of HRP**

Incubate slides for 30 minutes at room temperature in a humidified chamber with appropriate HRP-labeled reagent using **either**:

- a. DIG-labeled probes: 100-300  $\mu$ L of anti-digoxigenin-HRP (Boehringer-Mannheim anti-DIG-POD Cat. # 1-207-733) diluted in TNB Buffer. A suggested range is 1:100 to 1:1000.

*or*

- b. Biotin-labeled probes: 100-300  $\mu$ L of **SA-HRP** (Cat. # NEL750) diluted in TNB Buffer. A suggested range is 1:500 to 1:2500.

*or*

- c. Fluorescein-labeled probes: 100-300  $\mu$ L of anti-fluorescein-HRP (Cat. # NEF710) diluted in TNB Buffer. A suggested range is 1:125 to 1:500.

**NOTE: HRP-labeled reagents are available from a variety of vendors. Concentrations listed above are suggested starting titers. Appropriate concentration for use MUST be established as per optimization studies suggested on p.9.**

### **Step 5: Wash**

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

### **Step 6: TSA Plus Fluorescence Systems Amplification**

Pipet 100-300  $\mu$ L of the **Fluorophore Tyramide (Amplification Reagent) Working Solution** (p. 11) onto each slide. Incubate the slides at room temperature for 3 to 10 minutes.

### **Step 7: Wash**

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

### **Step 8: Visualization of Deposited Fluorophores**

Follow desired fluorescence or chromogenic visualization option.

#### **a) Fluorescence Option**

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, Cyanine 3 and Cyanine 5.

| <b>Fluorophore</b>   | <b>Excitation</b> | <b>Emission</b> |
|----------------------|-------------------|-----------------|
| Fluorescein          | 494 nm            | 517 nm          |
| Tetramethylrhodamine | 550 nm            | 570 nm          |
| Cyanine 3            | 550 nm            | 570 nm          |
| Cyanine 5            | 648 nm            | 667 nm          |

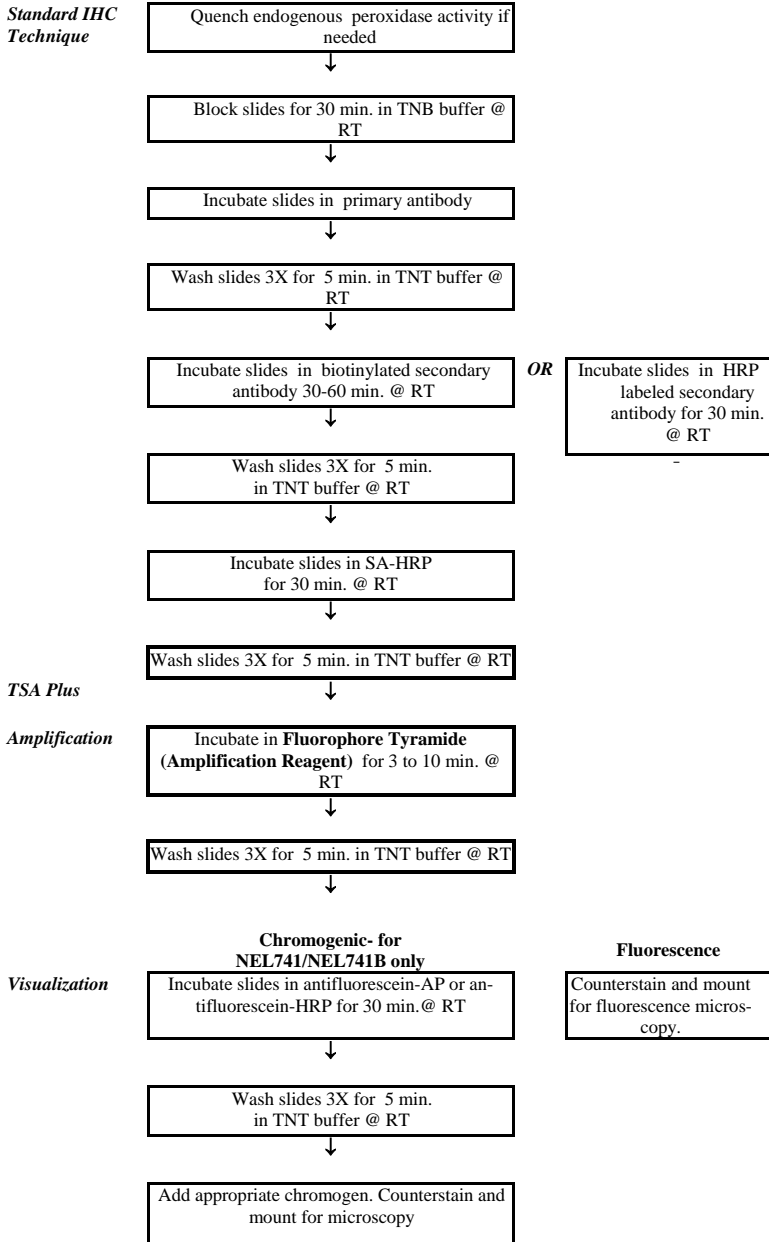
**b) Chromogenic Option for TSA Plus Fluorescein (NEL741/NEL741B) only**

**Steps**

- Add approximately 100  $\mu$ l of Anti-fluorescein- HRP\* (1:25) or Anti-fluorescein-AP\* (1:100) diluted in TNB Buffer to each slide. The use of a coverslip will reduce evaporation. (\* See Complementary products, p. 6.)
- Incubate the slides in a humid chamber at room temperature for 30 minutes.
- Wash the slides 3x for 5 minutes each in TNT Buffer at room temperature with agitation.
- 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).
- 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.

### III. PROTOCOL FOR IHC

#### A. Overview Protocol for TSA Plus Fluorescence Systems Immunohistochemistry



## **B. Suggested IHC Protocol**

The following is a suggested protocol for using **TSA Plus Fluorescence Systems** for immunohistochemistry signal amplification.

TSA technology requires HRP to be an integral part of the detection protocol. This may be accomplished by using either an HRP labeled secondary antibody or a biotin labeled secondary antibody followed by SA-HRP. Once HRP is introduced, the **Fluorophore Tyramide (Amplification Reagent) Working Solution** is added. Visualization is then done through the use of standard fluorescence microscopy. For the TSA Fluorescein System (NEL741 / NEL741B), the fluorescence signal can be converted to a chromogenic signal. This is possible by following the Fluorescein tyramide step by an antiluorescein-enzyme conjugate. Signal is visualized by the addition of an appropriate chromogen. Enzyme conjugates and chromogenic substrates must be purchased separately.

### **First Time Users**

First time users should apply the TSA Plus Fluorescence Systems to a proven IHC system.

### **Controls**

**Always run control slides with each experiment.** Include at least one negative control slide (eliminating primary antibody but including the **TSA Fluorescence Systems** reagents) and one unamplified control slide (include all reagents except **TSA reagents**). In addition to proving validity of results, control slides may be beneficial in determining the cause of non-specific background.

## **Reagent Titration**

Failure to establish optimum reagent concentrations may result in poor amplification and/or increased background.

Primary and/or secondary antibody dilutions should be optimized when applying TSA for the first time. The following test slides are recommended:

### **Primary Antibody Titration**

#### **Test slide 1:**

Primary or Secondary Ab at manufacturer's recommended dilution.

#### **Test slide 2:**

5 fold dilution of slide #1 Ab concentration.

#### **Test slide 3:**

5 fold dilution of slide #2 Ab concentration.

#### **Test slide 4:**

5 fold dilution of slide #3 Ab concentration.

More than the above dilutions may be necessary. In cases where low signal is obtained, increasing the dilution of the primary antibody often leads to better signal amplification.

### **Secondary Antibody and/or HRP Conjugate Titrations.**

Test 2-fold serial dilutions starting with the manufacturer's recommended dilution. In cases where no signal and no background are seen, it may be necessary to use an increased concentration of reagent. For example, if the recommended starting dilution is 1:1000, run test slides using concentrations of 1:500, 1:1000 and 1:2000.

## Quenching Endogenous Peroxidase

Activation and covalent binding of the **Fluorophore Tyramides (Amplification Reagents)** are catalyzed by HRP. To minimize background, endogenous peroxidase activity, if present, must be quenched before the immunostaining protocol. **Users should establish the need for doing this and optimal methodology specific to the tissues or cells being stained.**

Options include:

1. 0.3% H<sub>2</sub>O<sub>2</sub> to 3% H<sub>2</sub>O<sub>2</sub>
2. Methanol or PBS as diluent for H<sub>2</sub>O<sub>2</sub>.
3. Incubation time of 10 to 60 minutes.

For paraffin-embedded tissues quenching can be done after dewaxing and alcohol rehydration but before the blocking step. For frozen tissue or cell preps, quenching can be done following fixation and before the blocking step.

After quenching wash with TNT buffer for 5 minute.

## Volumes

The protocol is written for minimal volumes of reagent (e.g., 100-300  $\mu$ L). Reagent volumes used should be sufficient to completely cover cells or tissue sections on slide. If larger volumes are necessary to cover the samples, make the appropriate changes in the protocol.

## Technical Support

If there are any further questions regarding TSA in your IHC system, please contact NEN Life Science Products Technical Support Department before proceeding. Refer to the Troubleshooting Guide section for local listing. Technical assistance is also available through our web site at [www.PerkinElmer.com](http://www.PerkinElmer.com).

### C. Standard IHC Protocol

#### 1. Preparation of Buffers and Reagents

The following buffers and reagents are required for slide preparation and **TSA Plus Fluorescence Systems** amplification.

##### **Fluorophore Tyramide (Amplification Reagent) Stock Solution**

**Fluorophore Tyramides (Amplification Reagents)** are supplied as a solid. Each vial must be reconstituted before use with 0.15 mL of DMSO (dimethyl sulfoxide - molecular biology or HPLC-grade). The Fluorophore Tyramide Stock Solution, when stored at 4 ° C, is stable for at least 3 months. (Note: DMSO freezes at 4 ° C. Thaw the Stock Solution before each use.)

##### **Fluorophore Tyramide (Amplification Reagent) Working Solution**

Before each procedure, dilute the Fluorophore Tyramide Stock Solution 1:50 using **1X Plus Amplification Diluent** to make the Fluorophore Tyramide Working Solution. Approximately 100-300 µL of Fluorophore Tyramide Working Solution is required per slide. Discard any unused portion of working solution.

## **Wash Buffer**

TNT Wash Buffer

0.1 M TRIS-HCl, pH 7.5

0.15 M NaCl

0.05% Tween<sup>®</sup> 20

Other wash buffers such as PBS may be used. Substitution of 0.3% Triton X-100 for the 0.05% Tween-20 is also possible. Users should validate the use of wash buffer alternatives with their own systems.

**NOTE: When staining cell surface/membrane targets, do NOT include detergent in wash buffer or diluents. Detergents may cause stripping or alteration of cell surface antigens**

## **Blocking Buffer**

TNB Blocking Buffer

0.1 M TRIS-HCl, pH 7.5

0.15 M NaCl

0.5% **Blocking Reagent** (not supplied in kit)

Add **Blocking Reagent** slowly in small increments to buffer while stirring. Heat gradually to 60 °C with continuous stirring to completely dissolve the **Blocking Reagent**. (This may take up to several hours. Preparation of volumes less than 100 mL allows for more even heating.) Aliquot and store at -20 °C for long term use. Discard any unused blocking buffer which has been stored for greater than 24 hours at RT.

The **Blocking Reagent** (Cat.# FP1020) must be ordered as a separate component. It has been found to be optimal for use with the **TSA** kit reagents. The user should validate the use of alternative blocking reagents.

## 2. **Procedural Notes**

- Do not let slides dry out between steps.
- A humidified 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. Blot area around, but not on, tissue section using a labwipe.
- Be sure to use enough volume of solutions to cover sections or cells.
- Optional: Use of coverslips may reduce reagent evaporation. However care must be taken upon removal to prevent damage to tissues or cells.

## 3. **Step by Step Protocol**

The following is a **suggested** protocol for the use of **TSA Plus Fluorescence Systems** in IHC applications.

### **Step 1: Slide Preparation**

Prepare tissues or cells for using standard fixation and embedding techniques. Dewax and rehydrate using standard protocols. Quench endogenous peroxidase activity if necessary.

**NOTE: Always run an unamplified control slide and an amplified negative control slide with each experiment.**

### **Step 2: Blocking Step**

Incubate slides with 100-300  $\mu\text{L}$  of TNB Buffer in a humidified chamber for 30 minutes at room temperature. (Note: PBS may be substituted for the TRIS-NaCl buffer.)

### **Step 3: Primary Antibody Incubation**

Drain off the TNB Buffer and apply 100-300  $\mu\text{L}$  of the primary antibody, diluted in TNB Buffer. Incubate the primary antibody preparation per the manufacturer's instructions regarding incubation time and temperature requirements. (Use concentration determined in optimization studies -see p.19.).

### **Step 4: Wash**

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

### **Step 5: Introduction of HRP**

Incubate slides with HRP by doing one of the following:

- a) 100-300  $\mu$ L of HRP labeled secondary antibody diluted in TNB Buffer (Use concentration determined in optimization studies- see p.19)

*or*

- b) 100-300  $\mu$ L of biotinylated secondary antibody diluted in TNB Buffer. (Use concentration determined in optimization studies- see p. 19) Incubate 30-60 minutes in a humidified chamber. Wash the slides for 3 X 5 minutes TNT buffer at room temperature with agitation. Follow by 100-300  $\mu$  L of SA-HRP diluted in TNB Buffer. (Use SA-HRP concentration determined in optimization studies- see p.19) Incubate slides in a humidified chamber for 30 minutes at room temperature.

### **Step 6: Wash**

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

### **Step 7: TSA Plus Fluorescence Systems Amplification**

Pipet 100-300  $\mu$ L of the **Fluorophore Tyramide (Amplification Reagent) Working Solution** (p. 21) onto each slide. Incubate the slides at room temperature for 3 to 10 minutes.

### **Step 8: Wash**

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

### **Step 9: Visualization of Deposited Fluorophores**

Follow desired fluorescence or chromogenic visualization option:

#### **a) Fluorescence Option**

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, Cyanine 3 and Cyanine 5:

| <b>Fluorophore</b>   | <b>Excitation</b> | <b>Emission</b> |
|----------------------|-------------------|-----------------|
| Fluorescein          | 494 nm            | 517 nm          |
| Tetramethylrhodamine | 550 nm            | 570 nm          |
| Cyanine 3            | 550 nm            | 570 nm          |
| Cyanine 5            | 648 nm            | 667 nm          |

#### **b) Chromogenic Option for TSA Fluorescein (NEL 741/NEL741B) only**

##### **Steps**

- Add approximately 100  $\mu$ l of Antifluorescein- HRP\* (1:25) or Antifluorescein-AP\* (1:100) diluted in TNB Buffer to each slide. The use of a coverslip will reduce evaporation. (\*See Complementary products p. 6.)

- Incubate the slides in a humid chamber at room temperature for 30 minutes.
- Wash the slides 3x for 5 minutes each in TNT Buffer at room temperature with agitation.
- 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).
- 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.

#### IV. TROUBLESHOOTING GUIDE

##### A. In Situ Hybridization (ISH)

| PROBLEM                | REMEDY  |
|------------------------|---|
| <b>Low Signal</b>      | <ul style="list-style-type: none"><li>• Titer HRP conjugate used for visualization to determine optimum concentration for signal amplification.</li><li>• Increase concentration of <b>Fluorophore Tyramide (Amplification Reagent)</b> solution and/or lengthen incubation time.</li><li>• Add tissue permeabilization step to facilitate penetration of reagents.</li></ul>   |
| <b>Excess Signal</b>   | <ul style="list-style-type: none"><li>• Decrease concentration of HRP conjugate.</li><li>• Decrease probe concentration.</li><li>• Decrease <b>Fluorophore Tyramide (Amplification Reagent)</b> incubation time.</li><li>• Decrease concentration of enzyme conjugate used for chromogenic visualization.</li></ul>   |
| <b>High Background</b> | <ul style="list-style-type: none"><li>• Decrease concentration of HRP conjugate.</li><li>• Decrease probe concentration.</li><li>• Shorten chromogen developing time.</li><li>• Lengthen endogenous peroxide quenching step.</li><li>• Samples may contain endogenous biotin. Switch to fluorescein or digoxigenin labeled probes.</li><li>• Filter buffers.</li><li>• Increase number and/or length of washes.</li><li>• Nonqualified or contaminated blocking reagent used.</li></ul> |

## B. Immunohistochemistry (IHC)

| PROBLEM                | REMEDY  |
|------------------------|---|
| <b>Low Signal</b>      | <ul style="list-style-type: none"> <li>• Titer primary and/or secondary antibodies to determine optimum concentration for signal amplification</li> <li>• Increase concentration of <b>Fluorophore Tyramide (Amplification Reagent)</b> solution and/or increase incubation time.</li> <li>• In some cases it may be necessary to utilize antigen retrieval techniques to unmask the target.</li> </ul> |
| <b>Excess Signal</b>   | <ul style="list-style-type: none"> <li>• Decrease concentration of primary and/or secondary antibody or HRP conjugates.</li> <li>• Decrease <b>Fluorophore Tyramide (Amplification Reagent)</b> incubation time.</li> <li>• Decrease concentration of enzyme conjugates used for chromogenic visualization.</li> </ul>  |
| <b>High Background</b> | <ul style="list-style-type: none"> <li>• Filter buffers</li> <li>• Decrease concentration of primary and/or secondary antibody or HRP conjugates.</li> <li>• Lengthen endogenous peroxide quenching step.</li> <li>• Increase number and/or length of washes.</li> <li>• Shorten chromogen development time.</li> <li>• Nonqualified or contaminated blocking reagent used.</li> </ul>                  |

### **C. Customer Technical Support Services**

For further technical information, or to place an order contact:

**In the U.S.:** 1 (800) 762-4000.

**Outside the U.S.:** <http://www.perkinelmer.com/lasoffices>

**Web site is:** <http://www.perkinelmer.com>

### **V. REFERENCES**

A complete updated reference list is available upon request from Customer Technical Support as well as through the NEN web site at [www.perkinelmer.com](http://www.perkinelmer.com).

## **Patents**

TSA and its use are protected under U.S. Patents 5, 196, 306, 5, 583, 001, 5, 731, 158 and 5, 863, 748 and patents pending and foreign equivalents thereof.

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