PerkinElmer, Inc.

TSA™PLUS Fluorescence Kits

Tyramide **S**ignal **A**mplification

For in Situ *Hybridization and Immunohistochemistry*

TSA Plus Fluorescence Kits		
TSA Plus Fluorescein Kit	25-75 slides	NEL741E001KT
	50–150 slides	NEL741001KT
	250–750 slides	NEL741B001KT
TSA Plus TMR Kit	50–150 slides	NEL742001KT
	250–750 slides	NEL742B001KT
TSA Plus Cyanine 3 Kit	25-75 slides	NEL744E001KT
	50–150 slides	NEL744001KT
	250–750 slides	NEL744B001KT
TSA Plus Cyanine 5 Kit	25-75 slides	NEL745E001KT
	50–150 slides	NEL745001KT
	250–750 slides	NEL745B001KT
TSA Plus Multi-Fluor Combination Kits		
TSA Plus Cyanine 3/Cyanine 5 Kit	50–150 slides	NEL752001KT
TSA Plus Cyanine 3/Fluorescein Kit	50–150 slides	NEL753001KT
TSA Plus Cyanine 5/Fluorescein Kit	50–150 slides	NEL754001KT
TSA Plus Fluorescein/TMR Kit	50–150 slides	NEL756001KT
TSA Plus Fluorescence Palette Kit (Fluorescein, TMR, Cyanine 3 and Cyanine 5)	10–35 slides	NEL760001KT

* number of slides determined by volume used per section

For Laboratory Use
CAUTION: A research chemical for research purposes only.

TABLE OF CONTENTS

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. Labeled probes are hybridized to nucleic acid targets in tissue or cell preps. Probes can be labeled using common 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. Immunocytochemistry (ICC) refers specifically to detection of antigens in cells rather than tissue. Immunofluorescence is a commonly used term for the use of fluorescence to visualize sub-cellular localization of antigens.

What is TSA?

TSA (Tyramide Signal Amplification) is a powerful, patented technology from PerkinElmer that significantly enhances both chromogenic and fluorescent signals. It is easily integrated into standard ISH, IHC, ICC or immunofluorescence protocols, provided that horseradish peroxidase (HRP) may be introduced. .

How does TSA Plus Fluorescence 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 locked 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.

TSA Plus Fluorescence simplifies fluorescence detection because the amplification reagent is directly labeled with a fluorophore. Once the fluorophore-labeled reagents have been deposited, results can be immediately visualized via fluorescence microscopy. The TSA Plus Fluorescence Kits include: TSA Plus Fluorescein (NEL741***), TSA Plus Tetramethylrhodamine (NEL742***), TSA Plus Cyanine 3 (NEL744***) and the TSA Plus Cyanine 5 (NEL745***).

What ISH and IHC mediums are compatible with TSA Plus Fluorescence?

TSA Plus Fluorescence has 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. TSA Plus Fluorescence Kits

TSA Plus Fluorescence Kits contains the following components necessary for signal amplification:

- TSA Plus Fluorophore Amplification Reagent,
- 1X Plus Amplification Diluent

Intended Use

The intended use of this kit is to amplify signals generated by horseradish peroxidase in (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 microtiter plates. Labeled streptavidin, blocking reagent and other components should be purchased separately.

FOR LABORATORY USE

Safety Note

All reagents are classified as nonhazardous. 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 Kits

TSA Plus Fluorescein Kit	25-75 slides	NEL741E001KT	1X Plus Amplification Diluent 7.5 mL Fluorescein Plus Amplification Reagent 1 Vial
	50–150 slides	NEL741001KT	1X Plus Amplification Diluent 15 mL Fluorescein Plus Amplification Reagent 2 Vials
	250–750 slides	NEL741B001KT	1X Plus Amplification Diluent 75 mL Fluorescein Plus Amplification Reagent 10 Vials
TSA Plus TMR Kit	50–150 slides	NEL742001KT	1X Plus Amplification Diluent 15 mL Tetramethylrhodamine Plus Amplification Reagent 2 Vials
	250–750 slides	NEL742B001KT	1X Plus Amplification Diluent 75 mL Tetramethylrhodamine Plus Amplification Reagent 10 Vials
TSA Plus Cyanine 3 Kit	25-75 slides	NEL744E001KT	1X Plus Amplification Diluent 7.5 mL Cyanine 3 Plus Amplification Reagent 1 Vial
	50–150 slides	NEL744001KT	1X Plus Amplification Diluent 15 mL Cyanine 3 Plus Amplification Reagent 2 Vials
	250–750 slides	NEL744B001KT	1X Plus Amplification Diluent 75 mL Cyanine 3 Plus Amplification Reagent 10 Vials
TSA Plus Cyanine 5 Kit	25-75 slides	NEL745E001KT	1X Plus Amplification Diluent 7.5 mL Cyanine 5 Plus Amplification Reagent 1 Vial
	50–150 slides	NEL745001KT	1X Plus Amplification Diluent 15 mL Cyanine 5 Plus Amplification Reagent 2 Vials
	250–750 slides	NEL745B001KT	1X Plus Amplification Diluent 75 mL Cyanine 5 Plus Amplification Reagent 10 Vials
TSA Plus Cyanine 3/Cyanine 5 Kit	50–150 slides	NEL752001KT	1X Plus Amplification Diluent 30 mL Cyanine 3 Plus Amplification Reagent 2 Vials Cyanine 5 Plus Amplification Reagent 2 Vials
TSA Plus Cyanine 3/Fluorescein Kit	50–150 slides	NEL753001KT	1X Plus Amplification Diluent 30 mL Fluorescein Plus Amplification Reagent 2 Vials Cyanine 3 Plus Amplification Reagent 2 Vials
TSA Plus Cyanine 5/Fluorescein Kit	50–150 slides	NEL754001KT	1X Plus Amplification Diluent 30 mL Fluorescein Plus Amplification Reagent 2 Vials Cyanine 5 Plus Amplification Reagent 2 Vials
TSA Plus Fluorescein/TMR Kit	50–150 slides	NEL756001KT	1X Plus Amplification Diluent 30 mL Fluorescein Plus Amplification Reagent 2 Vials Tetramethylrhodamine Plus Amplification Reagent 2 Vials
TSA Plus Fluorescence Palette Kit (Fluorescein, TMR, Cyanine 3 and Cyanine 5)	10–35 slides	NEL760001KT	1X Plus Amplification Diluent 30 mL Fluorescein Plus Amplification Reagent 2 Vials Cyanine 3 Plus Amplification Reagent 2 Vials Tetramethylrhodamine Plus Amplification Reagent 2 Vials Cyanine 5 Plus Amplification Reagent 2 Vials

- The number of slides is determined by the reagent volume (approximately 100-300 µL) which is needed to completely cover the cell or tissue section on the slide.
- Each vial of tyramide dye is sufficient for 25 to 75 slides, except for vials from the Palette Kit which are good for 10-35 slides.

Storage and Stability

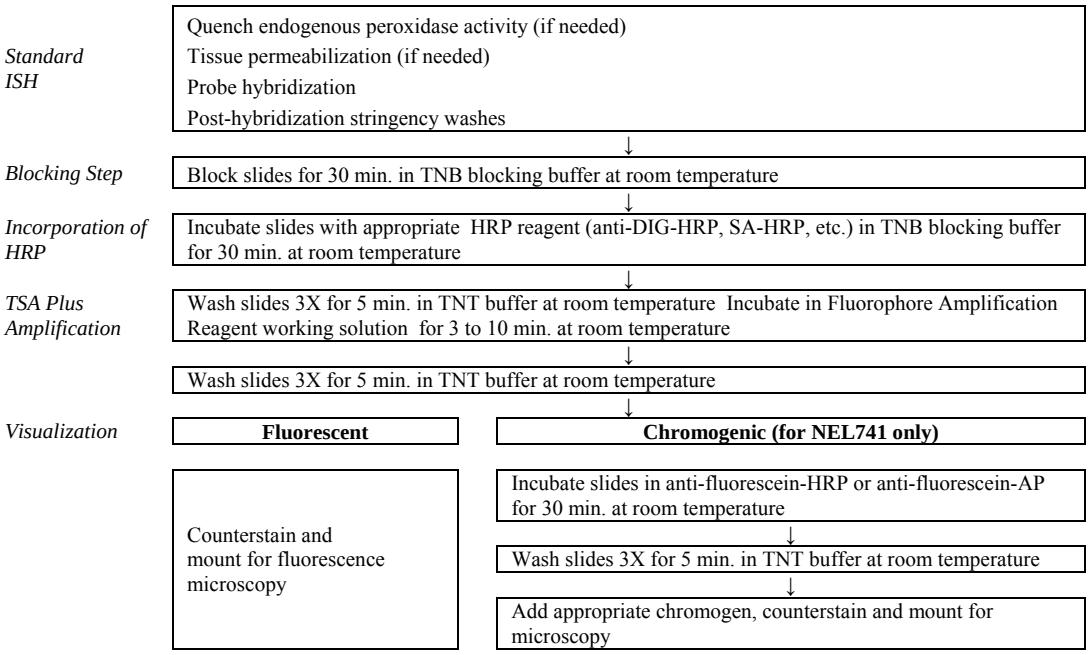
Upon receipt, TSA Plus Fluorescence kits should be stored at 4°C. The components in the kits are stable for a minimum of 3 months under proper storage conditions.

Critical Reagents Required But Not Supplied

- HRP-labeled reagent, for example
  - SA-HRP for use with biotin labeled probes or antibodies (PerkinElmer cat. no. NEL750001EA)
  - Anti-fluorescein-HRP for use with fluorescein labeled probes or antibodies (PerkinElmer cat. no. NEF710001EA)
  - HRP-labeled secondary antibody
    - Anti-rabbit IgG (goat) HRP (PerkinElmer cat. no. NEF812001EA)
    - Anti-mouse IgG (goat) HRP (PerkinElmer cat. no. NEF822001EA)
  - Anti-digoxigenin-HRP for use with DIG-labeled probes (for example Roche Anti-Digoxigenin-POD cat. no. 11 207 733 910)
- DMSO (molecular biology or HPLC grade)
- Buffer components
- Blocking reagent (PerkinElmer cat. no. FP1020)

II. PROTOCOL FOR ISH

A. Overview Protocol for TSA Plus Fluorescence for *In Situ* Hybridization



B. Suggested ISH Protocol

The following is a suggested protocol for using a TSA Plus Fluorescence Kit 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 Amplification Reagent working solution is added. Visualization is then done through the use of standard fluorescence microscopy. For TSA Plus Fluorescein systems (NEL741***), the fluorescence signal can be converted to a chromogenic signal. This is possible by following the fluorescein amplification 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 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 µ 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 users should apply it 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 regents) and an amplified negative control (i.e., no probe or non-specific probe + TSA reagents) slide. In addition to proving validity of results, control slides are often beneficial in determining the cause of non-specific background.

Reagent Titration

TSA usually requires lower probe and conjugate concentrations for optimal results when compared with standard unamplified methods.

	**Probe concentration must be optimized.** It should be assessed using the standard concentration used in unamplified procedures, and at reduced concentrations of 5, 10, and 20-fold - less in the hybridization mix. In many cases, a 10-fold reduction in probe concentration has been found to be optimal. **Failure to establish appropriate probe concentration can result in little to no signal development**
	Incubation with an appropriate HRP conjugate is required for the activation of the Fluorophore Amplification Reagent. 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 slides with HRP conjugate at 1:50, 1:100, 1:200, and 1:500.

Quenching Endogenous Peroxidase

Activation and covalent binding of the Fluorophore Amplification Reagent is 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 for quenching of endogenous peroxidases include 0.3% H_2O_2 to 3% H_2O_2 in either Methanol or PBS as diluent with incubation times of 10 to 60minutes.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. See Li *et al* from references section for more suggestions.

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 µL). 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 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/tsa.

C. Standard ISH Protocol

1. Preparation of Buffers and Reagents

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

Fluorophore Amplification Reagent Stock Solution

Fluorophore Amplification Reagent is supplied as a solid and may be difficult to see in the vial. For all kits except for catalog # NEL760, reconstitute by adding 150 µL/vial of DMSO (dimethyl sulfoxide -molecular biology or HPLC-grade) to make the Fluorophore Amplification Reagent Stock Solution. For the TSA Plus Fluorescence Palette System, reconstitute by adding 60 µL/vial of DMSO.

Fluorophore Amplification Reagent Stock Solution, when stored at 4° C, is stable for at least 3 months. (Note: DMSO should freeze at 4° C. Thaw the Stock Solution, before each use.)

Fluorophore Amplification Reagent Working Solution

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

TNT Wash Buffer

0.1 M TRIS-HCl, pH 7.5

0.15 M NaCl

0.05% Tween®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 alternative wash buffers with their own systems.

TNB Blocking Buffer

0.1 M TRIS-HCl, pH 7.5

0.15 M NaCl

0.5% Blocking Reagent (available separately, catalog number FP1020)

Add Blocking Reagent slowly in small increments to buffer while stirring. Heat gradually to 55°C with Continuous stirring to completely dissolve the Blocking Reagent. This should take no longer than 30-60 minutes. The solution will appear milky. Bring to room temperature before using. 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.

This blocking buffer 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 the tissue section using a tissue but do not touch the tissue section.
- 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 in an 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 ISH 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 a concentration determined in optimization studies (see p. 6) 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 µL of TNB Blocking Buffer in a humidified chamber for 30-60 minutes at room temperature or at 4^0C overnight.

Step 4: Introduction of HRP

NOTE: Appropriate concentration for use of HRP labeled reagents should be established as per optimization studies suggested on p. 6.

Incubate slides for 30 minutes at room temperature or at 4^0C overnight. in a humidified chamber with appropriate HRP-labeled reagent using either:

a. DIG-labeled probes: 100-300 µL of antidigoxigenin-HRP (Roche anti-DIG-POD Cat. No. 11 207 733 910) diluted 1:100 in TNB Buffer.

or

b. Biotin-labeled probes: 100-300 µL of SA-HRP (PerkinElmer Cat. No. NEL750001EA) diluted 1:2000 in TNB Buffer.

or

c. Fluorescein-labeled probes: 100-300 µL of anti-fluorescein-HRP (PerkinElmer Cat. No. NEF710001EA) diluted 1:250 in TNB Buffer.

Step 5: Wash

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

Step 6: TSA Plus Fluorescence Amplification

Pipet 100-300 µL of the Fluorophore 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

a) Fluorescence Option

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

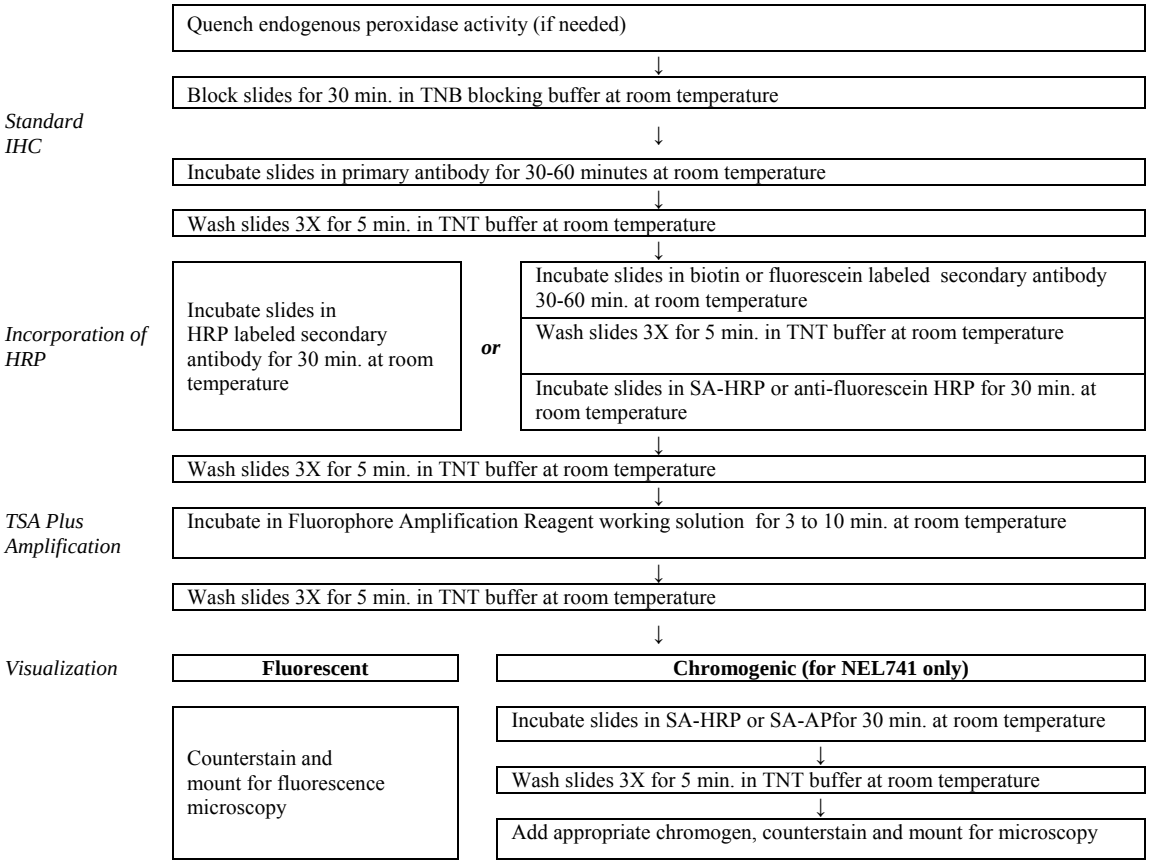
Fluorophore	**Excitation**	**Emission**
Fluorescein	494 nm	517 nm
Tetramethylrhodamine	550 nm	570 nm
Cyanine 3	550 nm	570 nm
Cyanine 5	648 nm	667 nm

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

- Add approximately 100 µL 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.

III. PROTOCOL FOR IHC

A. Overview Protocol for TSA Plus Fluorescence Immunohistochemistry



B. Suggested IHC Protocol

The following is a ***suggested*** protocol for using TSA Plus Fluorescence 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 hapten labeled secondary antibody followed by an appropriate HRP conjugate. Once HRP is introduced, Fluorophore Amplification Reagent Working Solution is added. Visualization is done through the use of appropriate labeled streptavidin for fluorescent or chromogenic detection.

First Time Users

First time users should apply TSA Plus Fluorescence kits 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 Plus** reagents) and one unamplified control slide (include all reagents except **TSA-Plus**). 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.

	**Failure to establish appropriate primary antibody concentration can result in little to no signal development.** The following test slides are recommended: **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. **Test slide 5:** Unamplified control.
	Incubation with an appropriate HRP conjugate is required for the activation of the Fluorophore Amplification Reagent. Appropriate HRP conjugate concentrations may be assessed in the same manner as above. Too much HRP reagent will result in increased background and reduced signal.

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.** In many cases, the optimal dilution of the primary antibody for TSA detection is >1000-fold less than that used for standard detection.

Quenching Endogenous Peroxidase

Activation and covalent binding of the Fluorophore Amplification Reagent is 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 for quenching of endogenous peroxidases include 0.3% H_2O_2 to 3% H_2O_2 in either Methanol or PBS as diluent with incubation times of 10 to 60minutes.

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. See Li *et al* from the references for more suggestions.

After quenching wash with TNT buffer for 5 minutes.

Volumes

The protocol is written for minimal volumes of reagent (e.g., 100-300 µL). 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 PerkinElmer 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/tsa.

C. Standard IHC Protocol

1. Preparation of Buffers and Reagents

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

Fluorophore Amplification Reagent Stock Solution

Fluorophore Amplification Reagent is supplied as a solid. Reconstitute by adding 150 µL/vial of DMSO (dimethyl sulfoxide-molecular biology or HPLC-grade) to make the Fluorophore Amplification Reagent Stock Solution. Fluorophore Amplification Reagent Stock Solution, when stored at 4° C, is stable for at least 3 months. (Note: DMSO should freeze at 4° C. Thaw the Stock Solution before each use.)

Fluorophore Amplification Reagent Working Solution

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

portion of Fluorophore working solution.

TNT Wash Buffer

0.1 M TRIS-HCl, pH 7.5

0.15 M NaCl

0.05% Tween® 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.

TNB Blocking Buffer

0.1 M TRIS-HCl, pH 7.5

0.15 M NaCl

0.5% Blocking Reagent (available separately, catalog number FP1020)

Add Blocking Reagent slowly in small increments to buffer while stirring. Heat gradually to 55°C with continuous stirring to completely dissolve the Blocking Reagent. (This should take no longer than 30-60 minutes.) The solution will look milky. . Bring to room temperature before using. 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.

This blocking buffer 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 tissue.
- 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 in IHC applications.

Step 1: Slide Preparation

Prepare tissues or cells for detection with TSA Plus Fluorescence 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 µL of TNB Blocking Buffer in a humidified chamber for 30-60 minutes at room temperature or at 4^0C overnight. (Note: PBS may be substituted for the TN buffer.)

Step 3: Primary Antibody Incubation

Drain off the TNB Buffer and apply 100-300 µL 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. 10).

Step 4: Wash

Wash the slides 3X for 5 minutes each in

TNT Buffer at room temperature with agitation.

Step 5: Introduction of HRP

NOTE: HRP-labeled reagents:. Appropriate concentration for use should be established as per optimization studies suggested on p. 10.

Incubate slides with HRP by doing one of the following:

a) 100-300 µL of HRP labeled secondary antibody diluted in TNB Buffer.

or

b) 100-300 µL of biotinylated secondary antibody diluted in TNB Buffer. 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 µL of SA-HRP diluted in TNB Buffer. Use SA-HRP at 1:2000 if using PerkinElmer Cat. # NEL750001EA. When using alternative suppliers, reagents should be optimized for use with TSA starting with manufacturer's recommended dilutions. Incubate slides in a humidified chamber for 30-60 minutes at room temperature or at 4^0C overnight.

Step 6: Wash

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

Step 7: TSA Plus Fluorescence Amplification

Pipette 100-300 µL of the Fluorophore Amplification Working Solution 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

a) Fluorescence Option

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

Fluorophore	Excitation	Emission
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*) only**

- Add approximately 100 µL 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
Low Signal	• Titer HRP conjugate used for amplification to determine optimum concentration for signal amplification. • Increase concentration of Fluorophore Amplification Reagent solution and/or lengthen incubation time. • Add tissue permeabilization step to facilitate penetration of reagents.
Excess Signal	• Decrease concentration of HRP conjugate introduced prior to amplification. • Decrease probe concentration. • Decrease Fluorophore Amplification Reagent incubation time.
High Background	• Decrease concentration of HRP conjugate in amplification step. • Decrease probe concentration. • Lengthen endogenous peroxide quenching step. • Samples may contain endogenous biotin. Switch to fluorescein or digoxigenin labeled probes. • Filter buffers. • Increase number and/or length of washes. • Nonqualified or contaminated blocking reagent used. Use PerkinElmer Blocking Reagent (FP1020).

B. Immunohistochemistry (IHC)

PROBLEM	REMEDY
Low Signal	• Titer primary and/or secondary antibodies to determine optimum concentration for signal amplification • Increase concentration of Fluorophore Amplification Reagent solution and/or increase incubation time. • In some cases it may be necessary to utilize antigen retrieval techniques to unmask the target.
Excess Signal	• Decrease concentration of primary and/or secondary antibody or HRP conjugates. • Decrease Fluorophore Amplification Reagent incubation time.
High Background	• Filter buffers • Decrease concentration of primary and/or secondary antibody or HRP conjugates. • Lengthen endogenous peroxide quenching step. • Increase number and/or length of washes. Shorten chromogenic development time. • Nonqualified or contaminated blocking reagent used. Use PerkinElmer Blocking Reagent (FP1020).

C. Customer Technical Support Services

For Further Technical Information, or, to Place an Order Contact:

Web site: http://www.perkinelmer.com/

Email: global.techsupport@perkinelmer.com

United States	PerkinElmer Technical Support Department at (800) 762-4000.
Europe	PerkinElmer Technical Support Department at Europe 00800-33290000
Finland	PerkinElmer Technical Support Department at 999-800-33290000
Outside the U.S/Europe	Contact your local PerkinElmer sales office or distributor.

V. Ordering information

Product	No. of Slides	Product No.
TSA Plus Fluorescence Kits		
TSA Plus Fluorescein Kit	50–150	NEL741001KT
	250–750	NEL741B001KT
TSA Plus TMR Kit	50–150	NEL742001KT
	250–750	NEL742B001KT
TSA Plus Cyanine 3 Kit	50–150	NEL744001KT
	250–750	NEL744B001KT
TSA Plus Cyanine 5 Kit	50–150	NEL745001KT
	250–750	NEL745B001KT
TSA Plus Multi-Fluor Combination Kits		
TSA Plus Cyanine 3/Cyanine 5 Kit	50–150	NEL752001KT
TSA Plus Cyanine 3/Fluorescein Kit	50–150	NEL753001KT
TSA Plus Cyanine 5/Fluorescein Kit	50–150	NEL754001KT
TSA Plus Fluorescein/TMR Kit	50–150	NEL756001KT
TSA Plus Fluorescence Palette Kit (contains one each of Fluorescein, TMR, Cyanine 3 and Cyanine 5)	10–35	NEL760001KT
TSA Plus Biotin Kits		
TSA Plus Biotin Kit	50–150	NEL749A001KT
	250–750	NEL749B001KT
TSA Plus DNP Kits		
TSA Plus DNP (AP) System	25–75	NEL746B001KT
	50–150	NEL746A001KT
TSA Plus DNP (HRP) System	25–75	NEL747B001KT
	50–150	NEL747A001KT
TSA Fluorescence Systems		
TSA Fluorescein System	50–150	NEL701A001KT
	100–300	NEL701001KT
TSA TMR System	100–300	NEL702001001KT
TSA Coumarin System	100–300	NEL703001KT
TSA Cyanine 3 System	50–150	NEL704A001KT
TSA Cyanine 5 System	50–150	NEL705A001KT
TSA Biotin Systems		
TSA Biotin System	50–150	NEL700A001KT
	200–600	NEL700001KT
TSA Reagent Packs		
TSA Biotin Tyramide Reagent Pack	200–600	SAT700001EA
	1,000–3,000	SAT700B001EA
TSA Fluorescein Tyramide Reagent Pack	100–300	SAT701001EA
	500–1,500	SAT701B001EA
TSA TMR Tyramide Reagent Pack	100–300	SAT702001EA
TSA Cyanine 3 Tyramide Reagent Pack	50–150	SAT704A001EA
	250–750	SAT704B001EA
TSA Cyanine 5 Tyramide Reagent Pack	50–150	SAT705A001EA

Complementary Products		
TSA Blocking Reagent		FP1020
Horseradish Peroxidase Reagents		
Anti-rabbit IgG (goat) HRP		NEF812001EA
Anti-mouse IgG (goat) HRP		NEF822001EA
Anti-human IgG (goat)* HRP		NEF802001EA
Anti-DNP-HRP		FP1129
Antifluorescein-HRP		NEF710001EA
Streptavidin-HRP		NEL750001EA
Biotin Conjugates		
Anti-rabbit IgG (goat) biotin		NEF813001EA
Anti-mouse IgG (goat) biotin		NEF823001EA
Anti-human IgG (goat) biotin		NEF803001EA
Labeled Streptavidin		
Streptavidin Fluorescein		NEL720001EA
Streptavidin Texas Red®		NEL721001EA
Streptavidin Coumarin		NEL722001EA
Streptavidin-HRP		NEL750001EA
Streptavidin-AP		NEL751001EA
Chromogens		
BCIP/NBT Substrate		NEL937001PK
DAB Substrate		NEL938001EA
Alkaline Phosphatase Reagents		
Anti-Mouse IgG (Goat), AP-Labeled		NEF814001EA
Anti-Rabbit IgG (Goat), AP-Labeled		NEF824001EA
Streptavidin- AP Conjugate		NEL751001EA
Antifluorescein-AP Conjugate		NEF709001PK
Anti-DNP-AP		FP1131
Hapten Labeled Deoxynucleotides (for labeling of ISH probes)		
3-Amino-3-Deoxydigoxigenin-9-dCTP		NEL562001EA
Biotin-11-dATP		NEL540001EA
Biotin-11-dCTP		NEL538001EA
Biotin-11-dGTP		NEL541001EA
Biotin-11-dUTP		NEL539001EA
DNP-11-dUTP		NEL551001EA
Fluorescein-12-dATP		NEL465001EA
Fluorescein-12-dCTP		NEL424001EA
Fluorescein-12-dGTP		NEL429001EA
Fluorescein-12-dUTP		NEL413001EA
Hapten Labeled Ribonucleotides (for labeling of ISH probes)		
Biotin-11-ATP		NEL544001EA
Biotin-11-CTP		NEL542001EA
Biotin-11-GTP		NEL545001EA
Biotin-11-UTP		NEL543001EA
Fluorescein-12-ATP		NEL439001EA
Fluorescein-12-CTP		NEL434001EA
Fluorescein-12-GTP		NEL496001EA
Fluorescein-12-UTP		NEL414001EA

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