



RENAISSANCE®
TSA™ FLUORESCENCE SYSTEMS
TYRAMIDE SIGNAL AMPLIFICATION
For Fluorescence In situ Hybridization
and Immunohistochemistry

Fluorescein NEL701 100-300 slides*	Tetramethyl- Rhodamine NEL702 100-300 slides*	Coumarin NEL703 100-300 slides*	Cyanine 3 NEL704A 50-150 slides*
NEL701A 50-150 slides*			Cyanine 5 NEL705A 50-150 slides*

* 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	5
A.	Background Information	5
	What is (F) ISH?	5
	What is IHC?	5
	What is TSA?	5
	How does TSA Fluorescence Systems Signal Amplification work?	5
	What ISH and IHC Mediums are Compatible with the TSA Fluorescence Systems?	6
B.	The TSA Fluorescence Systems Intended Use	6
	Safety Note	7
C.	Components of TSA Fluorescence Systems Kits Storage and Stability	7
	Critical Reagents Required But Not Supplied	8
	Complementary Products	8
II.	PROTOCOL for ISH	9
A.	Overview Protocol for TSA Fluorescence Systems for In Situ Hybridization	9
B.	Suggested ISH Protocol First Time Users	10
	Controls	10
	Reagent Titration	11
	Quenching Endogenous Peroxidase	12
	Volumes	13
	Technical Support	13
C.	Standard ISH Protocol	13
	Preparation of Buffers and Reagents	13
	Procedural Notes	15
	Step by Step Protocol	15
III.	PROTOCOL FOR IHC	18
A.	Overview Protocol for TSA Fluorescence Systems Immunohistochemistry	18
B.	Suggested IHC Protocol First Time Users	19
	Controls	19
	Reagent Titration	20
	Quenching Endogenous Peroxidase	20
	Volumes	21
	Technical Support	21

C.	Standard IHC Protocol	21
	Preparation of Buffers and Reagents	21
	Procedural Notes	23
	Step by Step Protocol	23
IV.	TROUBLESHOOTING GUIDE	26
A.	In Situ Hybridization (ISH)	26
B.	Immunohistochemistry (IHC)	27
C.	Customer Technical Support Services	28
V.	REFERENCES	28

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?

TSA (Tyramide Signal Amplification) is a powerful, patented technology from PerkinElmer Life Sciences, Inc. 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™
Fluorescence Systems
Signal Amplification
work?

The TSA 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 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 Fluorescence Systems include: TSA Fluorescein System (NEL701/NEL701A), TSA Tetramethylrhodamine System (NEL702), TSA Coumarin System (NEL703), TSA Cyanine 3 System (NEL704A), and the TSA Cyanine 5 System (NEL705A).

What ISH and IHC
Media are
Compatible With
The TSA
Fluorescence Systems?

The TSA Fluorescence Systems have been successfully applied to the following media: formalin-fixed/paraffin-embedded sections, frozen sections, chromosome spreads, and cultured cells.

B. The TSA Fluorescence Systems

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

Fluorophore-labeled Tyramide (Amplification Reagent), 1X Amplification Diluent, Blocking Reagent and Streptavidin- Horseradish Peroxidase (SA-HRP). Fluorophore labels include Fluorescein (NEL701/701A), Tetramethylrhodamine (NEL 702), Coumarin (NEL 703), Cyanine 3 (NEL 704A), or Cyanine 5 (NEL705A).

The TSA 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. SA-HRP is included in the kit. Other 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 microtiter 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.

NEL701
(Fluorescein)

100–300 slides*

Reagent	Amount
Streptavidin-HRP	2 x 150 μ L
Blocking Reagent	10 gm
Amplification Diluent	30 mL
Fluorescein Tyramide	For 100–300 slides

NEL701A
(Fluorescein)

50–150 slides*

Reagent	Amount
Streptavidin-HRP	150 μ L
Blocking Reagent	3 gm
Amplification Diluent	15 mL
Fluorescein Tyramide	For 50–150 Slides

NEL702
(Tetramethylrhodamine)

100–300 slides*

Reagent	Amount
Streptavidin-HRP	2 x 150 μ L
Blocking Reagent	10 gm
Amplification Diluent	30 mL
Tetramethylrhodamine Tyramide	For 100–300 Slides

NEL703
(Coumarin)

100–300 slides*

Reagent	Amount
Streptavidin-HRP	2 x 150 μ L
Blocking Reagent	10 gm
Amplification Diluent	30 mL
Coumarin Tyramide	For 100–300 slides

NEL704A
(Cyanine 3)

50–150 slides*

NEL705A
(Cyanine 5)

50–150 slides*

Reagent	Amount
Streptavidin-HRP	150 μ L
Blocking Reagent	3 gm
Amplification Diluent	15 mL
Cyanine 3 Tyramide	For 50–150 Slides

Reagent	Amount
Streptavidin-HRP	150 μ L
Blocking Reagent	3 gm
Amplification Diluent	15 mL
Cyanine 5 Tyramide	For 50–150 slides

C. Components of TSA Fluorescence Systems Kits

* The number of slides is determined by the reagent volume (approximately 100–300 μ L) which is needed to completely cover the cells or tissue section on the slide.

Storage and Stability

Upon receipt, the TSA Fluorescence Systems kits should be stored at 4°C. The Blocking Reagent may be stored at room temperature if desired. 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: SA-HRP is supplied in kit. Alternatives including anti-digoxigenin-HRP for use with DIG-labeled probes, HRP-labeled probe or antibody, ABC reagent, etc. must be purchased separately.
- DMSO (molecular biology or HPLC grade)
- Buffer components

TSA Kits	Catalog Number
TSA Plus DNP (HRP or AP) System	NEL746A, NEL746B, NEL747A, NEL 747B
TSA Biotin System	NEL700, NEL700A

Reagent	Catalog Number
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®	NEL721
Streptavidin-Coumarin	NEL722

Complementary Products

Standard nonradiometric 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.



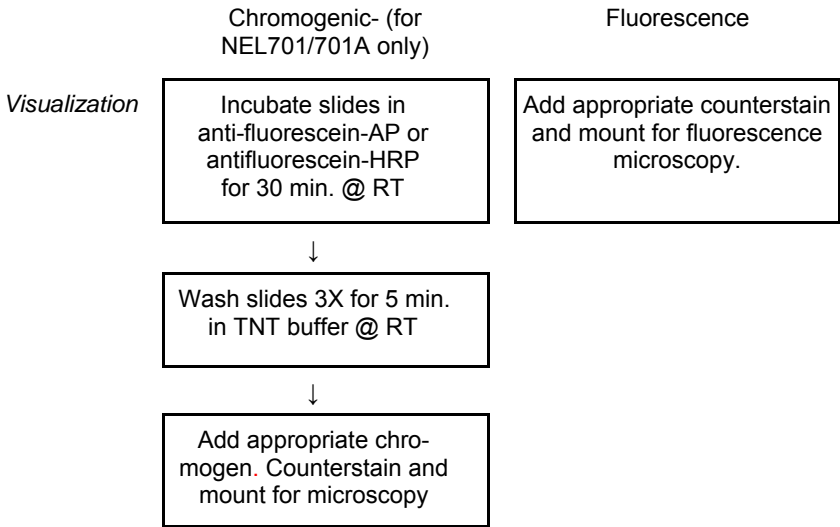
Wash slides 3X for 5 min. in TNT buffer @ RT

TSA 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



II. PROTOCOL FOR ISH

- A. Overview Protocol for TSA Fluorescence Systems for In Situ Hybridization
- B. Suggested ISH Protocol

The following is a suggested protocol for using TSA 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 kits (NEL 701/NEL701A), the fluorescence signal can be converted to a chromogenic signal. This is possible by following the Fluorescein tyramide step by an antifluorescein-enzyme conjugate. Signal is visualized by the addition of an appropriate chromogen. The kits are supplied with SA-HRP.

Other SA-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 0.01 M HCl or Proteinase K (1–10 µg/mL) in TRIS-HCl/0.05 M 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 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 Fluorescence Systems. This could be done by using hapten-labeled probes followed by an appropriate anti-hapten-HRP conjugate (see p.16 for suggestions). Streptavidin-HRP is supplied with the kit. Other 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% H₂O₂ to 3% H₂O₂
2. Methanol or PBS as diluent for H₂O₂.
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 μ 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 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/lifesciences.

C. Standard ISH Protocol

Preparation of Buffers and Reagents

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

Fluorophore Tyramide Fluorophore Tyramides (Amplification Reagents)

Catalog Number	Tyramide Label	Solvent	Amount Required
NEL701	Fluorescein	DMSO	0.6 mL
NEL701A	Fluorescein	DMSO	0.3 mL
NEL702	Tetramethylrhodamine	DMSO	0.6 mL
NEL703	Coumarin	DMSO	0.6 mL
NEL704A	Cyanine 3	Water	0.3 mL
NEL705A	Cyanine 5	Water	0.3 mL

(Amplification Reagent) are supplied as a solid and must be reconstituted
Stock Solution before use as directed in the following table.

Distilled, deionized water or DMSO (dimethyl sulfoxide -molecular biology or HPLC-grade) should be used. 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.)

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

Fluorophore Tyramide
(Amplification Reagent)
Working Solution

Before each procedure, dilute the Fluorophore Tyramide Working Solution 1:50 using 1X 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

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

portion of working solution.

Wash Buffer

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

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 supplied in this kit is optimal for use with the TSA kit reagents. The user should validate use of alternative blocking reagents.

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.

Step by Step Protocol

The following is a suggested protocol for the use of TSA Fluorescence Systems in a nonradioactive ISH protocol.

Slide Preparation

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

Standard Non-radioactive In Situ Hybridization of Technique

2. Follow standard non-radioactive in situ hybridization techniques. Include tissue permeabilization (if needed) and quenching 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. 12) followed by post-hybridization stringency washes.

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

Blocking Step

3. Incubate slides with 100–300 μL of TNB Buffer in a humidified chamber for 30 minutes at room temperature.

Introduction of HRP

4. 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 μL of anti-digoxigenin-HRP (Boehringer-Mannheim

anti-DIG-POD Cat. # 1-207-733) diluted 1:100 in TNB Buffer,

or

- b. Biotin-labeled probes: 100–300 µL of SA-HRP diluted 1:100 in TNB Buffer.

or

- c. Fluorescein-labeled probes: 100–300 µL of anti-fluorescein-HRP (Cat. # NEF710) diluted 1:250 in TNB Buffer.

NOTE: HRP-labeled reagents are available from a variety of vendors. Appropriate

Fluorophore	Excitation	Emission
Fluorescein	494 nm	517 nm
Tetramethylrhodamine	550 nm	570 nm
Coumarin	402 nm	443 nm
Cyanine 3	550 nm	570 nm
Cyanine 5	648 nm	667 nm

concentration for use should be established as per optimization studies suggested on p.12).

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

TSA Fluorescence
Systems Amplification

6. Pipet 100–300 µL of the Fluorophore Tyramide (Amplification Reagent) Working Solution (p. 14) onto each slide. Incubate the slides at room temperature for 3 to 10 minutes.

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

Visualization of
Deposited
Fluorophores

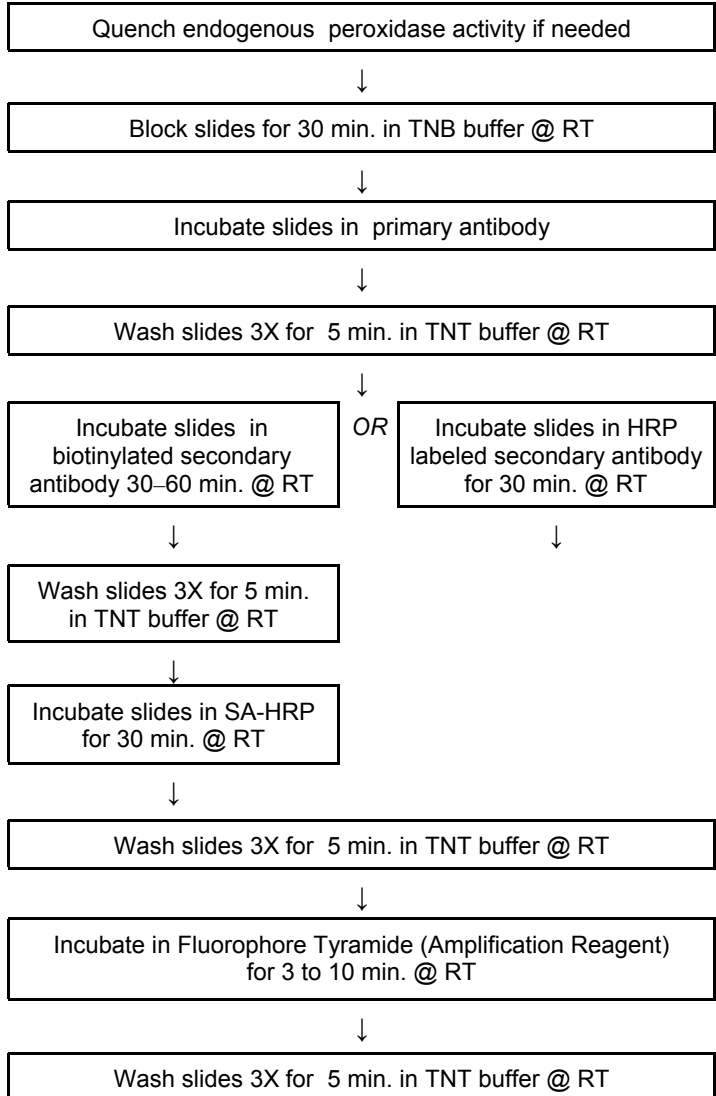
Follow desired fluorescence or chromogenic visualization option:

a) Fluorescence Option

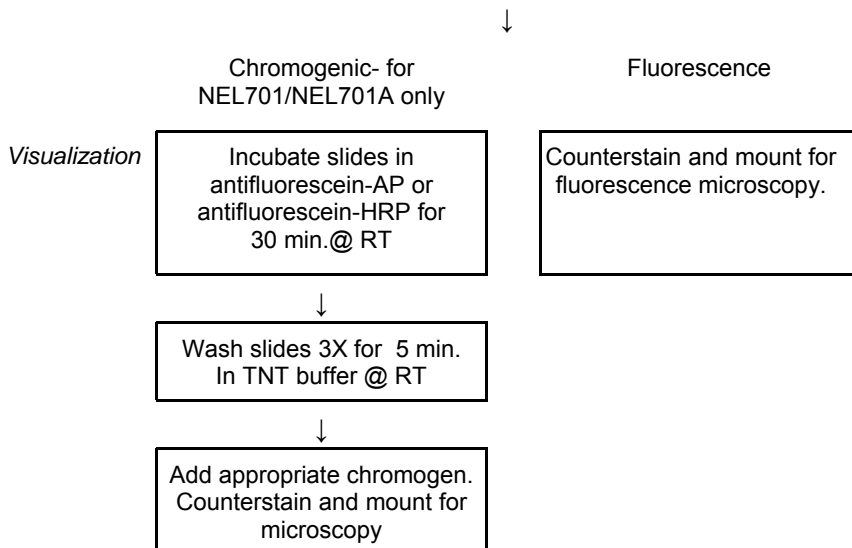
- Steps 8. Counterstain if appropriate. Slides are now ready for mounting and for fluorescence microscopy evaluation.

The following is a table of excitation and emission wavelengths for Fluorescein, Tetramethylrhodamine, Coumarin, Cyanine 3, and Cyanine 5:

Standard IHC Technique



TSA Amplification



b) Chromogenic Option for TSA Fluorescein (NEL701/NEL701A) only

- | | |
|-------|--|
| Steps | <ol style="list-style-type: none"> 8. 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. 8.) 9. Incubate the slides in a humid chamber at room temperature for 30 minutes. 10. Wash the slides 3X for 5 minutes each in TNT Buffer at room temperature with agitation. 11. 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). 12. 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 Fluorescence Systems Immunohistochemistry
- B. Suggested IHC Protocol

The following is a suggested protocol for using TSA 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 kits (NEL701/NEL701A), 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. The kits are supplied with SA-HRP. Other SA-enzyme conjugates and chromogenic substrates must be purchased separately.

First Time Users	First time users should apply the TSA 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:

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.

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.

Quenching Endogenous Peroxidase

Activation and covalent binding of the Fluorophore Tyramides (Amplification Reagents) 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 include:

1. 0.3% H₂O₂ to 3% H₂O₂
2. Methanol or PBS as diluent for H₂O₂.
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 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

Catalog Number	Tyramide Label	Solvent	Amount Required
NEL701	Fluorescein	DMSO	0.6 mL
NEL701A	Fluorescein	DMSO	0.3 mL
NEL702	Tetramethylrhodamine	DMSO	0.6 mL
NEL703	Coumarin	DMSO	0.6 mL
NEL704A	Cyanine 3	Water	0.3 mL
NEL705A	Cyanine 5	Water	0.3 mL

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

TNT Wash Buffer
0.1 M TRIS-HCl, pH 7.5
0.15 M NaCl
0.05% Tween 20

through our web site at www.perkinelmer.com/lifesciences

C. Standard IHC Protocol

Preparation of Buffers and Reagents

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

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

amplification.

Fluorophore Tyramide
(Amplification Reagent)
Stock Solution

Fluorophore Tyramides (Amplification Reagents) are supplied as a solid and must be reconstituted before use as directed in the following table. Distilled, deionized water or DMSO (dimethyl sulfoxide -molecular biology or HPLC-grade) should be used. 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 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

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

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 supplied in this kit is optimal for use with the TSA kit reagents provided. The user should validate use of alternative blocking reagents.

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.

Step by Step Protocol The following is a suggested protocol for the use of TSA Fluorescence Systems in IHC applications.

Slide Preparation 1. 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.

Blocking Step 2. Incubate slides with 100–300 μ L of TNB Buffer in a humidified chamber for 30 minutes at room temperature. (Note: PBS may be substituted for the TRIS-NaCl buffer.)

Primary Antibody Incubation 3. Drain off the TNB Buffer and apply 100–300 μ L of the primary antibody, diluted in TNB Buffer. Incubate the primary antibody preparation per

Fluorophore	Excitation	Emission
Fluorescein	494 nm	517 nm
Tetramethylrhodamine	550 nm	570 nm
Coumarin	402 nm	443 nm
Cyanine 3	550 nm	570 nm
Cyanine 5	648 nm	667 nm

the manufacturer's instructions regarding incubation time and temperature requirements. (Use concentration determined in optimization studies – see p.20).

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

Introduction of HRP 5. Incubate slides with HRP by doing one of the following:

a. 100–300 μ L of HRP labeled secondary antibody diluted in TNB Buffer (Use concentration determined in optimization studies – see p.20)

or

- b. 100–300 μL of biotinylated secondary antibody diluted in TNB Buffer. (Use concentration determined in optimization studies- see p.20) 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 1:100 in TNB Buffer. Incubate slides in a humidified chamber for 30 minutes at room temperature.
6. Wash the slides 3X for 5 minutes each in TNT Buffer at room temperature with agitation.

TSA Fluorescence
Systems Amplification

7. Pipet 100–300 μL of the Fluorophore Tyramide (Amplification Reagent) Working Solution (p. 22) onto each slide. Incubate the slides at room temperature for 3 to 10 minutes.
8. Wash the slides 3X for 5 minutes each in TNT Buffer at room temperature with agitation.

Visualization of
Deposited Fluorophores

Follow desired fluorescence or chromogenic visualization option:

a. Fluorescence Option

Steps

9. 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, Cyanine 3, and Cyanine 5:

b. Chromogenic Option for TSA Fluorescein (NEL701/NEL701A) only

Steps

9. 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. 8.)
10. Incubate the slides in a humid chamber at room temperature for 30 minutes.

11. Wash the slides 3X for 5 minutes each in TNT Buffer at room temperature with agitation.
12. 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).
13. 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 visualization to determine optimum concentration for signal amplification.
- Increase concentration of Fluorophore Tyramide (Amplification Reagent) solution and/or lengthen incubation time.
- Add tissue permeabilization step to facilitate penetration of reagents.

Excess Signal

- Decrease concentration of HRP conjugate.
- Decrease probe concentration.
- Decrease Fluorophore Tyramide (Amplification Reagent) incubation time.
- Decrease concentration of enzyme conjugate used for chromogenic visualization.

High Background

- Decrease concentration of HRP conjugate.
- Decrease probe concentration.

- Shorten chromogen developing time.
- Lengthen endogenous peroxide quenching step.

In the U.S.: PerkinElmer Technical Support
Department at 1 (800) 551-2121.

Outside the U.S.: Contact your local PerkinElmer sales
office or distributor.

Web site is: www.perkinelmer.com/lifesciences

- 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 Blocking Reagent supplied in kit.

B. Immunohistochemistry (IHC)

PROBLEM

Low Signal

REMEDY

- Titer primary and/or secondary antibodies to determine optimum concentration for signal amplification
- Increase concentration of Fluorophore Tyramide (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 Tyramide (Amplification Reagent) incubation time.
- Decrease concentration of DNP conjugates used for visualization.

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 chromogen development time.
- Nonqualified or contaminated blocking reagent used. Use Blocking Reagent supplied in kit.

C. Customer Technical Support Services

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

V. REFERENCES

A complete updated reference list is available upon request from Customer Technical Support as well as through the PerkinElmer web site at www.perkinelmer.com/lifesciences

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.

For Research Use Only

This product is distributed and sold for research purposes only by the end-user in the research market, and, to that extent, by purchasing this product the end-user is granted a limited license to use this product for research only. This product is not intended for diagnostic or therapeutic use and no license or right is granted to use this product for diagnostic or therapeutic purposes. Purchase does not include or carry any right or license to use develop or otherwise exploit this product commercially. Any commercial use, development or exploitation of this product without the express prior written authorization of PerkinElmer is strictly prohibited and constitutes an infringement of the intellectual property rights of PerkinElmer under the aforementioned patents.

Trademarks

Renaissance[®] and TSA[™] are trademarks of PerkinElmer.

Histomount[™] and Clearmount[™] are trademarks of Zymed Laboratories Inc.

TWEEN[®]20 is a registered trademark of ICI Americas, Inc.

Texas Red[®] is a registered trademark of Molecular Probes, Inc.

Manufactured by:

PerkinElmer Life Sciences, Inc.
549 Albany Street
Boston, MA USA 02118
Toll-Free 1 800 551-2121
International 617 482-9595
www.perkinelmer.com/lifesciences

Notes

Notes

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
Shelton, CT 06484-4794 USA
Phone: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com



For a complete listing of our global offices, visit www.perkinelmer.com/lasoffices

©2007 PerkinElmer, Inc. All rights reserved. The PerkinElmer logo and design are registered trademarks of PerkinElmer, Inc. TSA is a trademark and Renaissance is a registered trademarks of PerkinElmer, Inc. or its subsidiaries, in the United States and other countries. All other trademarks not owned by PerkinElmer, Inc. or its subsidiaries that are depicted herein are the property of their respective owners. PerkinElmer reserves the right to change this document at any time without notice and disclaims liability for editorial, pictorial or typographical errors.

PC2633-0403