NEN® Life Science Products ***RENAISSANCE®***

TSA™ Biotin System

Tyramide **S**ignal **A**mplification
For Chromogenic and Fluorescence
in Situ Hybridization and Immunohistochemistry

NEL700 200-600slides*
NEL700A 50-150slides*

*** 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 1
- What is ISH? 1
- What is IHC? 1
- What is TSA? 1
- How does TSA™ Biotin System Signal Amplification work? 2
- What ISH and IHC Mediums are Compatible With the TSA Biotin System? 2

B. The TSA Biotin System 3
- Intended Use 3
- Safety Note 3

C. Components of TSA Biotin System Kits 4
- Storage and Stability 4
- Critical Reagents Required But Not Supplied 4
- Complementary Products 5

II. PROTOCOL FOR ISH

A. Overview Protocol for TSA Biotin System for In Situ Hybridization 6

B. Suggested ISH Protocol 7
- First Time Users 7
- Controls 8
- Reagent Titration 8
- Quenching Endogenous Peroxidase 10
- Volumes 10
- Technical Support 10

C. Standard ISH Protocol 11
- Preparation of Buffers and Reagents 11
- Procedural Notes 12
- Step by Step Protocol 13

III. PROTOCOL FOR IHC

A. Overview Protocol for TSA Biotin System Immunohistochemistry 16

B. Suggested IHC Protocol 17
- First Time Users 17
- Controls 17
- Reagent Titration 18
- Quenching Endogenous Peroxidase 19
- Volumes 19
- Technical Support 19

C. Standard IHC Protocol 20
- Preparation of Buffers and Reagents 20
- Procedural Notes 21
- Step by Step Protocol 22

IV. TROUBLESHOOTING GUIDE

A. In Situ Hybridization (ISH) 26

B. Immunohistochemistry (IHC) 27

C. Customer Technical Support Services 28

V. REFERENCES

I. INTRODUCTION

A. Background Information

What is 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, dye deposition for enzyme or hapten-labeled probes, and fluorescence for fluorophore-labeled probes.

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™ (**T**yramide **S**ignal **A**mplification) 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™ Biotin System Signal Amplification work?

The TSA Biotin System technology uses **HRP** to catalyze the deposition of **the biotin-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 biotin labels immediately adjacent to the immobilized HRP enzyme. These labels can then be indirectly detected by chromogenic or 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. Chromogenic visualization is accomplished through the use of a streptavidin-enzyme conjugate, followed by the appropriate chromogen. Fluorescence visualization is possible by the use of a streptavidin-fluorophore conjugate. This signal amplification technique may be applied to both ISH and IHC

What ISH and IHC Mediums are Compatible With the TSA Biotin System?

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

B. The TSA Biotin System

The **TSA Biotin System** contains the following components necessary for signal amplification:
Biotinyl Tyramide (Amplification Reagent), **1X Amplification Diluent**, **Blocking Reagent and Streptavidin- Horseradish Peroxidase (SA-HRP)**

The **TSA Biotin System** is 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 biotin tyramide amplification reagent. SA-HRP is included in the kit. Other HRP reagents must be purchased separately. Amplification is followed by chromogenic or fluorescence visualization techniques using various enzyme/chromogen or SA-fluorophore options. NEN Life Science Products offers the HRP-activated chromogen DAB (NEL938) and the AP-activated chromogen BCIP/NBT (NEL937). SA-Fluorescein (NEL720), SA-Texas Red® (NEL721), and SA-Coumarin (NEL722) are also available from NEN.

Intended Use

The intended use of this kit is to amplify signals generated by Horseradish Peroxidase in nonradioactive ISH and 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. Chromogenic substrates or Streptavidin-fluorophore conjugates used for visualization must be purchased separately.

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 Biotin System Kits

NEL700 **200 - 600 slides ***

Reagent	Amount
Streptavidin-HRP	1.2 mL
Blocking Reagent	10 gm
1X Amplification Diluent	60 mL
Biotinyl Tyramide	For 200-600 slides

NEL700A **50-150 slides ***

Reagent	Amount
Streptavidin-HRP	2x 150 µL
Blocking Reagent	3 gm
1X Amplification Diluent	15 mL
Biotinyl Tyramide	For 50-150 slides

The Biotinyl Tyramide (Amplification Reagent) is supplied as a solid and may not be visible in the vial.

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

Storage and Stability

Upon receipt, the **TSA Biotin System** kit 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 6 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
- Chromogenic visualization: Chromogen (BCIP/NBT, DAB, AEC, etc.)
- Fluorescence visualization: SA-Fluorophore conjugate

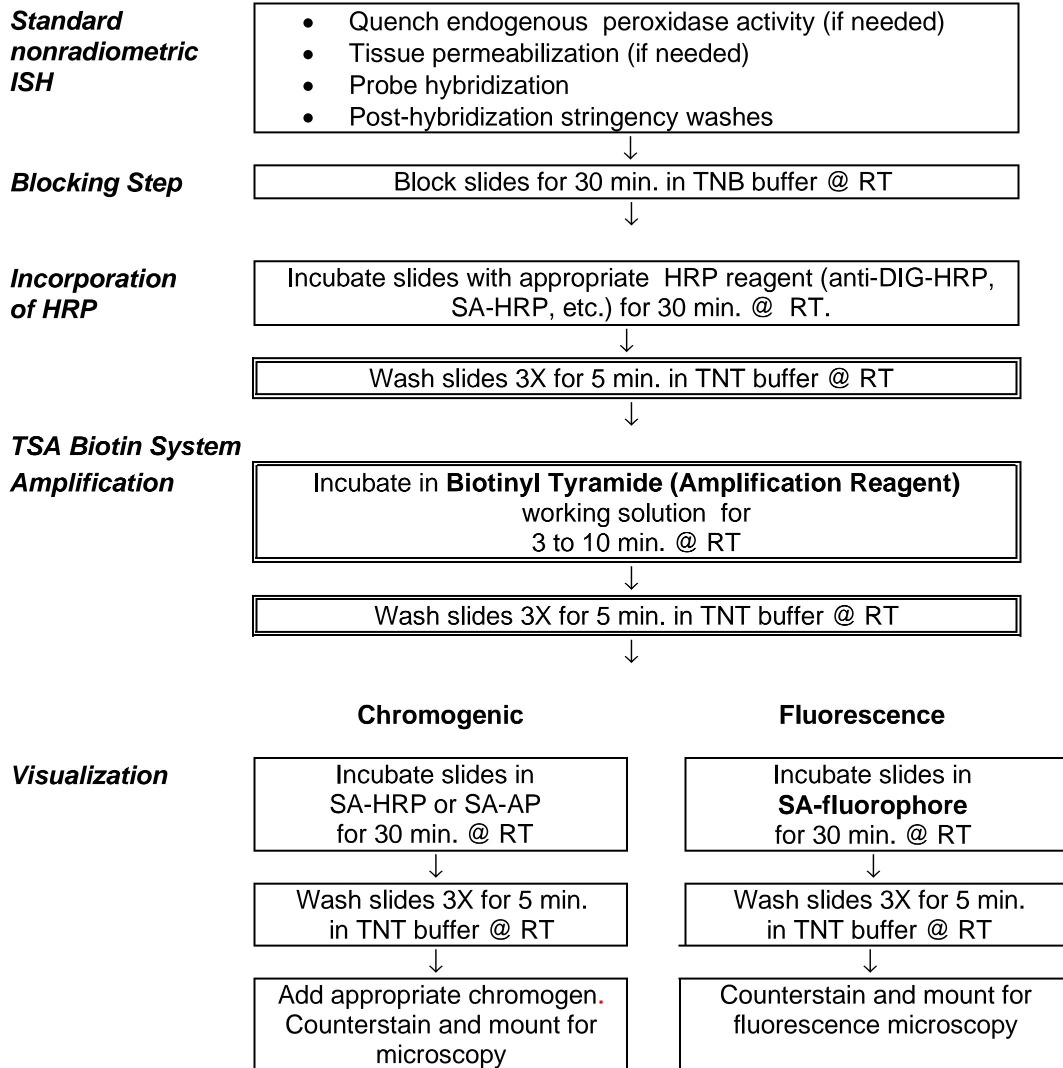
Complementary Products

TSA Kits	Catalog Number
TSA Plus DNP (HRP or AP) System	NEL746A,NEL746B, NEL747A, NEL 747B
TSA Fluorescein System	NEL701, NEL701A
TSA Tetramethylrhodamine System	NEL702
TSA Coumarin System	NEL703
TSA Cyanine 3 System	NEL704A
TSA Cyanine 5 System	NEL705A

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

II. PROTOCOL FOR ISH

A. Overview Protocol for TSA Biotin System for In Situ Hybridization



B. Suggested ISH Protocol

The following is a suggested protocol for using the **TSA Biotin System** 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 **Biotinyl Tyramide (Amplification Reagent)** working solution is added. Visualization is then done through the use of appropriate enzyme/chromogen combinations or through the use of a SA-fluorophore conjugate. The kits are supplied with SA-HRP. Other SA-enzyme conjugates, chromogenic substrates, or SA-fluorophore conjugates must be bought 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 µg/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 Biotin System 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 regents) 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, 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. In general, a 10-fold reduction in probe concentration has most often been found to be optimal. **Failure to establish appropriate probe concentration can result in little to no signal development.**

2. **Titration of enzyme conjugates:**

   Two enzyme conjugate steps are used in this procedure for chromogenic visualization. The first is required for the activation of the biotinyl tyramide amplification reagent. This must be an appropriate anti-hapten-HRP conjugate (see p.13 for suggestions). Streptavidin-HRP is supplied with the kit. Other HRP reagents 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 is 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.

The second enzymatic reaction in the protocol, is used to activate the chromogen. The SA-HRP supplied in the kit is suitable for the activation of HRP-based chromogens such as DAB or AEC. Streptavidin-alkaline phosphatase, for activation of AP-based chromgens such as BCIP/NBT, must be purchased separately. **Optimal results may require the titration of one or both enzyme conjugate reagents used.**

Alternatively, for fluorescence visualization the second enzyme conjugate step can be replaced by a streptavidin-fluorophore conjugate.

Quenching Endogenous Peroxidase

Activation and covalent binding of the **Biotinyl Tyramide (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 include:

1. 0.3% H_2O_2 to 3% H_2O_2
2. Methanol or PBS as diluent for H_2O_2.
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 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 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.nenlifesci.com.

C. Standard ISH Protocol

Preparation of Buffers and Reagents

The following buffers and reagents are required for slide preparation and **TSA Biotin System** amplification.

Biotinyl Tyramide (Amplification Reagent) Stock Solution

Biotinyl Tyramide (Amplification Reagent) is supplied as a solid (which may not be visible in the vial.) Reconstitute by adding 1.2 mL (for NEL 700) or 0.3 mL (for NEL700A) of DMSO (dimethyl sulfoxide -molecular biology or HPLC-grade) to make the Biotinyl Tyramide Stock Solution. The Biotinyl Tyramide Stock Solution, when stored at 4° C, is stable for at least six months. (Note: DMSO freezes at 4° C. Thaw the Stock Solution before each use.)

Biotinyl Tyramide (Amplification Reagent) Working Solution

Before each procedure, dilute the Biotinyl Tyramide Stock Solution 1:50 using **1X Amplification Diluent** to make the Biotinyl Tyramide Working Solution. Approximately 100-300 µL of Biotinyl 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®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.

Blocking Buffer

TNB Blocking Buffer
0.1 M TRIS-HCl, pH 7.5
0.15 M NaCl
0.5% **Blocking Reagent** (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** supplied in this kit is optimal for use with the **TSA** kit reagents. Use of alternative blocking reagents should be validated by the user.

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 that 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 Biotin System** 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 Technique

(2) 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. 8) 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 diluted1:100 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 concentration for use should be established as per optimization studies suggested on p.9).

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

TSA Biotin System Amplification

(6) Pipet 100-300 µL of the Biotinyl Tyramide (Amplification Reagent) Working Solution (p. 11) 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 Biotin

Follow desired chromogenic or fluorescence visualization option:

a.) HRP-Chromogenic Option

Steps

(8) Add 100-300 µL of **SA-HRP** (provided in kit) diluted 1:100 in TNB Buffer to each slide. Incubate the slides in a humidified chamber at room temperature for 30 minutes.

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

(10) Visualize with standard HRP chromogenic substrates such as DAB (diaminobenzidine) or AEC (aminoethyl carbazole). Incubate 5 minutes in the dark. Wash with distilled water after desired signal intensity is achieved.

(11) Counterstain if desired. Hematoxylin is an effective counterstain for DAB and AEC. Histomount™ and Clearmount™ may be used for mounting DAB-stained slides. Use aqueous mounting media with AEC.

OR-

b.) AP Chromogenic Option

Steps

(8) Add 100-300 µL of SA-AP (purchased separately) diluted in TNB Buffer to each slide. (Use concentration established in reagent titration studies, see p. 9). Incubate the slides in a humidified chamber at room temperature for 30 minutes.

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

(10) Visualize with 100-300 µL standard alkaline phosphatase chromogenic substrates such as BCIP/NBT (5-bromo-4-chloro-indolyl phosphate/ nitroblue tetrazolium). Incubate slides 10 minutes in the dark. Examine slides for signal strength. If a darker signal is desired, incubate slides an additional 10-30 minutes. Wash with distilled water after desired signal intensity is achieved.

(11) Counterstain if desired. Nuclear Fast Red is an effective counterstain for BCIP/NBT. Histomount™ and Clearmount™ may be used for mounting.

-OR-

c.) Fluorescence Visualization Option

Steps

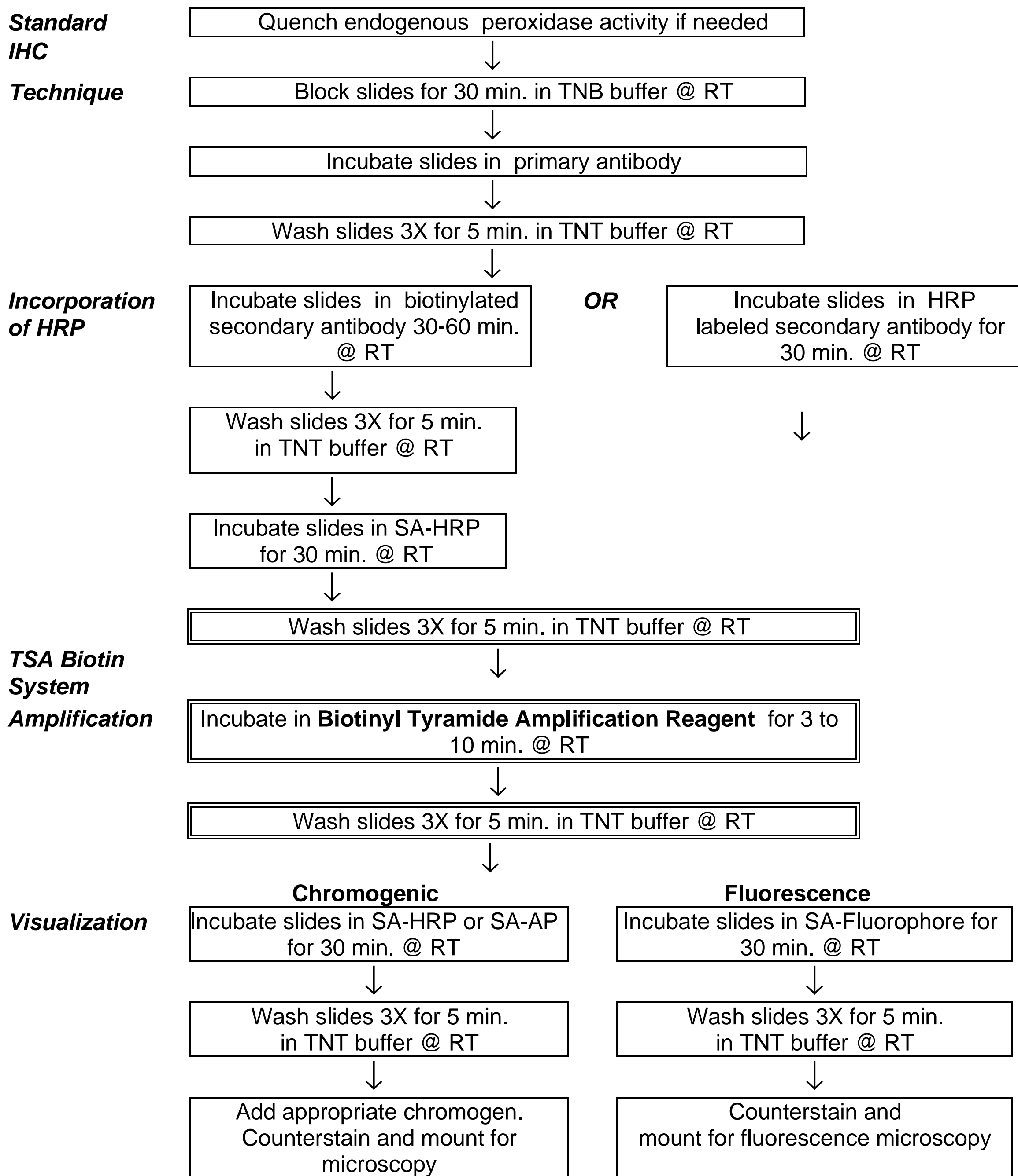
(8) Add 100-300 µL of a **Streptavidin-Fluorophore conjugate** (purchased separately) diluted in TNB Buffer to each slide. Use manufacturer's recommended dilution. Incubate the slides in a humidified chamber at room temperature for 30 minutes. SA-Fluorescein (NEL720), SA-Texas Red® (NEL721), and SA-Coumarin (NEL 722) are available from NEN and can be used at a 1:500 dilution.

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

(10) Counterstain if desired. Mount for fluorescence microscopy.

III. PROTOCOL FOR IHC

A. Overview Protocol for TSA Biotin System Immunohistochemistry



B. Suggested IHC Protocol

The following is a **suggested** protocol for using **TSA Biotin System** 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 **Biotinyl Tyramide (Amplification Reagent) Working Solution** is added. Visualization is then done through the use of appropriate enzyme/chromogen combinations or through the use of a SA-Fluorophore conjugate. Chromogenic substrates or SA-fluorophore conjugates must be bought separately**.**

First Time Users

First time users should apply the TSA Biotin System 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 Biotin System 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 **Biotinyl Tyramide 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 include:

1. 0.3% H_2O_2 to 3% H_2O_2
2. Methanol or PBS as diluent for H_2O_2.
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 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.nenlifesci.com.

C. Standard IHC Protocol

Preparation of Buffers and Reagents

The following buffers and reagents are required for slide preparation and **TSA Biotin System** amplification.

Biotinyl Tyramide (Amplification Reagent) Stock Solution

Biotinyl Tyramide (Amplification Reagent) is supplied as a solid (which may not be visible in the vial). Reconstitute by adding 1.2 mL (for NEL700) or 0.3 mL (for NEL700A) of DMSO (dimethyl sulfoxide molecular biology or HPLC-grade) to make the Biotinyl Tyramide Stock Solution. The Biotinyl Tyramide Stock Solution, when stored at 4° C, is stable for at least six months. (Note: DMSO freezes at 4° C. Thaw the Stock Solution before each use.)

Biotinyl Tyramide (Amplification Reagent) Working Solution

Before each procedure, dilute the Biotinyl Tyramide Stock Solution 1:50 using **1X Amplification Diluent** to make the Biotinyl Tyramide Working Solution. Approximately 100-300 µL of Biotinyl 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®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** (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 100mL 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. Use of alternative blocking reagents should be validated by the user.

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 Biotin System** 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 μL 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 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.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 µL of HRP labeled secondary antibody diluted in TNB Buffer (Use concentration determined in optimization studies- see p.18)

or

b) 100-300 µL of biotinylated secondary antibody diluted in TNB Buffer. (Use concentration determined in optimization studies- see p.18) 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 Biotin System Amplification

(7) Pipet 100-300 µL of the **Biotinyl Tyramide (Amplification Reagent) Working Solution** (p. 20) 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 Biotin

Follow the desired chromogenic or fluorescence visualization option:

a.) HRP-Chromogenic Option

Steps

(9) Add 100-300 μL of **SA-HRP** (provided in kit) diluted 1:100 in TNB Buffer to each slide. Incubate the slides in a humidified 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 chromogenic substrates such as DAB (diaminobenzidine) or AEC (aminoethyl carbazole). Incubate 5 minutes in the dark. Wash with distilled water after desired signal intensity is achieved.

(12) Counterstain if desired. Hematoxylin is an effective counterstain for DAB and AEC. Histomount™ and Clearmount™ may be used for mounting DAB-stained slides. Use aqueous mounting media with AEC.

-OR-

b.) AP Chromogenic Option

Steps

(9) Add 100-300 µL of SA-AP (purchased separately) diluted in TNB Buffer to each slide. (Use concentration established in reagent titration studies-see p.18.) Incubate the slides in a humidified 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 100-300 µL standard alkaline phosphatase chromogenic substrates such as BCIP/NBT (5-bromo-4-chloro-indolyl phosphate/ nitroblue tetrazolium). Incubate slides 10 minutes in the dark. Examine slides for signal strength. If a darker signal is desired, incubate slides an additional 10-30 minutes. Wash with distilled water after desired signal intensity is achieved.

(12) Counterstain if desired. Nuclear Fast Red is an effective counterstain for BCIP/NBT. Histomount™ and Clearmount™ may be used for mounting.

-OR-

c.) Fluorescence Visualization Option

Steps

(9) Add 100-300 µL of **a Streptavidin-Fluorophore conjugate** (purchased separately) diluted in TNB Buffer to each slide. Use manufacturer's recommended dilution. Incubate the slides in a humidified chamber at room temperature for 30 minutes. SA-Fluorescein (NEL720), SA-Texas Red® (NEL721), and SA-Coumarin (NEL 722) are available from NEN and can be used at a 1:500 dilution.

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

(11) Counterstain if desired. Mount for fluorescence microscopy.

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 **Biotinyl 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 **Biotinyl 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. • 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	REMEDY
Low Signal	• Titer primary and/or secondary antibodies to determine optimum concentration for signal amplification • Increase concentration of **Biotinyl 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 **Biotinyl 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 developing 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:

In the U.S.:	NEN Life Science Products Technical Support Department at 1 (800) 551-2121.
Outside the U.S.:	Contact your local NEN Life Science Products sales office or distributor.
Web site is:	http://www.nenlifesci.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.nenlifesci.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.

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 NEN LIFE SCIENCE PRODUCTS, INC. is strictly prohibited and constitutes an infringement of the intellectual property rights of NEN LIFE SCIENCE PRODUCTS, INC. under the aforementioned patents.

Trademarks

Renaissance® and TSA™ are trademarks of NEN Life Science Products, Inc.

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:

NEN® Life Science Products, Inc.
549 Albany Street
Boston, MA USA 02118

Toll-Free 1 800 551-2121
International 617 482-9595

PC2632-0300