PerkinElmer Life and Analytical Sciences, Inc.

RENAISSANCE®
TSA™ PLUS DNP (HRP OR AP) SYSTEM

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

For Chromogenic in Situ Hybridization
and Immunohistochemistry

NEL746A (AP) 50-150 slides*

NEL746B (AP) 25-75 slides*

NEL747A (HRP) 50-150 slides*

NEL747B (HRP) 25-75 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

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™ (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™ Plus DNP Signal Amplification work?

TSA Plus DNP technology uses HRP to catalyze the deposition of the dinitrophenyl (DNP) labeled 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 DNP labels immediately adjacent to the immobilized HRP enzyme. These labels can then be indirectly detected by standard techniques, with significant enhancement of the signal. Detection is accomplished through the use of an anti-DNP enzyme conjugate, followed by the appropriate chromogen. 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.

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

TSA Plus DNP has been successfully applied to the following media: formalin-fixed/paraffin-embedded sections, cryostat sections, and cultured cells.

B. The TSA Plus DNP System

The TSA Plus DNP (HRP or AP) System contains the following components necessary for signal amplification:

DNP Amplification Reagent, 1X Plus Amplification Diluent, Blocking Reagent and either Anti-DNP-Alkaline Phosphatase (AP) in NEL746A/746B

or

Anti-DNP-Horseradish Peroxidase (HRP) in NEL747A/747B.

TSA Plus DNP is compatible with a wide variety of standard ISH and IHC protocols. However, HRP must be available for the amplification to occur. Amplification is followed by standard chromogenic visualization techniques using various enzyme/chromogen options. PerkinElmer Life Sciences offers the HRP-activated chromogen DAB (NEL938) and the AP-activated chromogen BCIP/NBT (NEL937). Other chromogens are available from a variety of sources.

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 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 Plus DNP Kits

NEL746A 50-150 slides *

Reagent	Amount
Anti-DNP-AP	150 µL
Blocking Reagent	3 gm
1X Plus Amplification Diluent	15 mL
DNP Amplification Reagent	For 50-150 slides

NEL746B 25-75 slides *

Reagent	Amount
Anti-DNP-AP	75 µL
Blocking Reagent	1.5 gm
1X Plus Amplification Diluent	7.5 mL
DNP Amplification Reagent	For 25-75 slides

NEL747A 50-150 slides *

Reagent	Amount
Anti-DNP-HRP	150 µL
Blocking Reagent	3 gm
1X Plus Amplification Diluent	15 mL
DNP Amplification Reagent	For 50-150 slides

NEL747B 25-75 slides *

Reagent	Amount
Anti-DNP-HRP	75 µL
Blocking Reagent	1.5 gm
1X Plus Amplification Diluent	7.5 mL
DNP Amplification Reagent	For 25-75 slides

DNP Amplification Reagent is supplied as a solid and may have a slight yellow color.

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

Storage and Stability

Upon receipt, the TSA Plus DNP 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 (Anti-digoxigenin-HRP for use with DIG-labeled probes, SA-HRP for use with biotin-labeled probes or antibodies, HRP-labeled probe or antibody, etc.)
- DMSO (molecular biology or HPLC grade)
- Buffer components
- Chromogen (BCIP/NBT, DAB, AEC, etc.)

Complementary Products

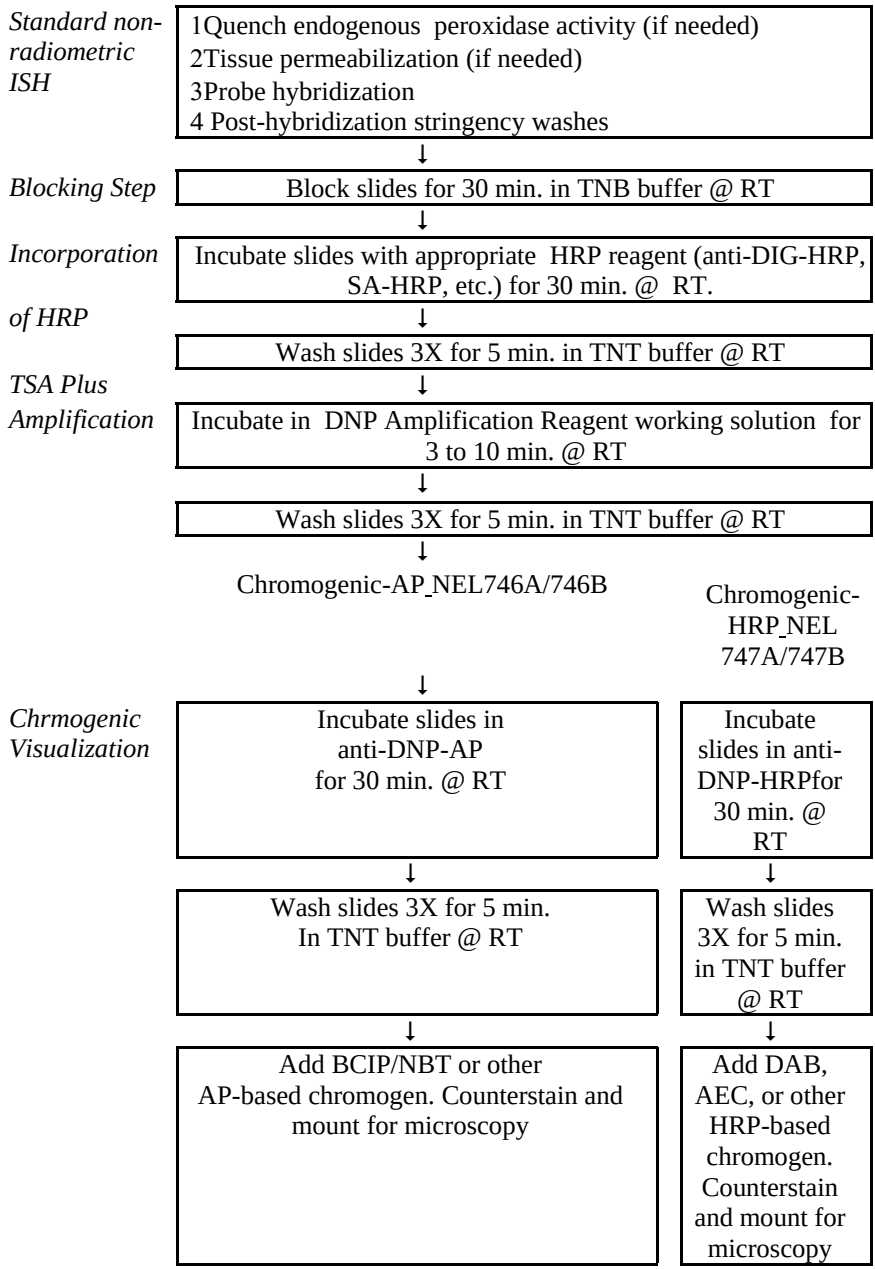
TSA Kits	Catalog Number
TSA Biotin System	NEL700, NEL700A
TSA Fluorescein System	NEL701, NEL701A
TSA Tetramethylrhodamine System	NEL702
TSA Coumarin System	NEL703
TSA Cyanine 3 System	NEL704A
TSA Cyanine 5 System	NEL705A

Reagent	Catalog Number
BCIP/NBT	NEL937
DAB	NEL938
Anti-fluorescein-HRP	NEF710
Streptavidin-HRP	NEL750
Streptavidin-AP	NEL751
Streptavidin-Fluorescein	NEL720
Streptavidin-Texas Red®	NEL721
Streptavidin-Coumarin	NEL722

Reagent	Catalog Number
BCIP/NBT	NEL937
DAB	NEL938
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 Plus DNP for In Situ Hybridization



B. Suggested ISH Protocol

The following is a suggested protocol for using TSA Plus DNP 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 DNP Amplification Reagent working solution is added. Visualization is done through the use of appropriate enzyme/chromogen combinations. The kits are supplied with either anti-DNP-AP (NEL746A, NEL746B) or anti-DNP-HRP (NEL747A, NEL747B) conjugates. Chromogenic substrates 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 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 DNP** 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 (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

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 5, 10, and 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. The first is required for the activation of the DNP amplification reagent. This must be an appropriate anti-hapten-HRP conjugate (see p.15 for suggestions) and 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 slides with HRP conjugate at 1:50, 1:100, 1:200, and 1:500.

The second enzymatic reaction in the protocol, utilizing the anti-DNP-AP or anti-DNP-HRP included in the kit, is used to activate the chromogen. Optimal results may require the titration of one or both enzyme conjugate reagents used.

Quenching Endogenous Peroxidase

Activation and covalent binding of the DNP 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 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.

C. Standard ISH Protocol

1. Preparation of Buffers and Reagents

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

DNP Amplification Reagent Stock Solution

DNP Amplification Reagent is supplied as a solid (which may have a light yellow color.) Reconstitute by adding 0.3 mL (for NEL746A/ NEL747A) or 0.15 mL (for NEL746B/747B) of DMSO (dimethyl sulfoxide -molecular biology or HPLC-grade) to make the DNP Stock Solution. The stock solution may also exhibit a light yellow color. DNP Stock Solution, when stored at 4 C, is stable for at least six 6 months. (Note: DMSO may freeze at 4 C. Thaw the Stock Solution, if necessary, before each use.)

DNP Amplification Reagent Working Solution

Before each procedure, dilute the DNP Stock Solution 1:50 using 1X Plus Amplification Diluent to make the DNP Working Solution. Approximately 100-300 µL of DNP Working Solution is required per slide. Discard any unused portion of DNP 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 more than 24 hours at room temperature.

The Blocking Reagent supplied in this kit is optimal for use with the TSA Plus DNP kit reagents. Use of alternative blocking reagents should be validated by the user.

2. Procedural Notes

- Do not let slides dry out between steps.
- A humidified chamber is recommended for all incubation steps (i.e., a damp paper towel in a covered box).

- Drain off as much of the incubation solutions as possible, before the addition of the next solution, to prevent reagent dilution and uneven staining. Blot area around, but not on, tissue section using a labwipe.
- Be sure to use enough volume of each reagent to completely cover sections or cells.
- Optional: Use of coverslips may reduce reagent evaporation, especially during steps which require long incubation at elevated temperatures (such as probe hybridization). However care must be taken upon removal to prevent damage to tissues or cells.

3. Step by Step Protocol

The following is a suggested protocol for the use of TSA Plus DNP in a nonradioactive ISH protocol.

Step 1: Slide Preparation

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

Step 2: Standard Non-radioactive In Situ Hybridization Technique

Follow standard non-radioactive in situ hybridization techniques. Include tissue permeabilization (if needed) and quenching of endogenous peroxidase activity (if needed). Probe hybridization (with digoxigenin, biotin, or fluorescein-labeled probes) should be done using concentration determined in

optimization studies (see p. 9) followed by post-hybridization stringency washes.

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

Step 3: Blocking Step

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

Step 4: Introduction of HRP

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

a. DIG-labeled probes: 100-300 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 (PerkinElmer Cat. # NEL750) diluted 1:2000 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).

Step 5: Wash

Wash the slides 3X for 5 minutes each in

TNT Buffer at room temperature with agitation.

Step 6: TSA Plus DNP Amplification

Pipet 100-300 µL of the DNP 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 DNP

Follow the applicable chromogenic visualization option:

a.) AP-Chromogenic Option (NEL 746A/ NEL 746B)

Steps

- Add 100-300 µL of anti-DNP-AP (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.

- Wash the slides 3X for 5 minutes each in TNT Buffer at room temperature with agitation.
- 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.
- Counterstain if desired. Nuclear Fast Red is an effective counterstain for BCIP/NBT. Histomount™ and Clear-mount™ may be used for mounting.

or

b.) HRP-Chromogenic Option (NEL747A/ NEL747B)

Steps

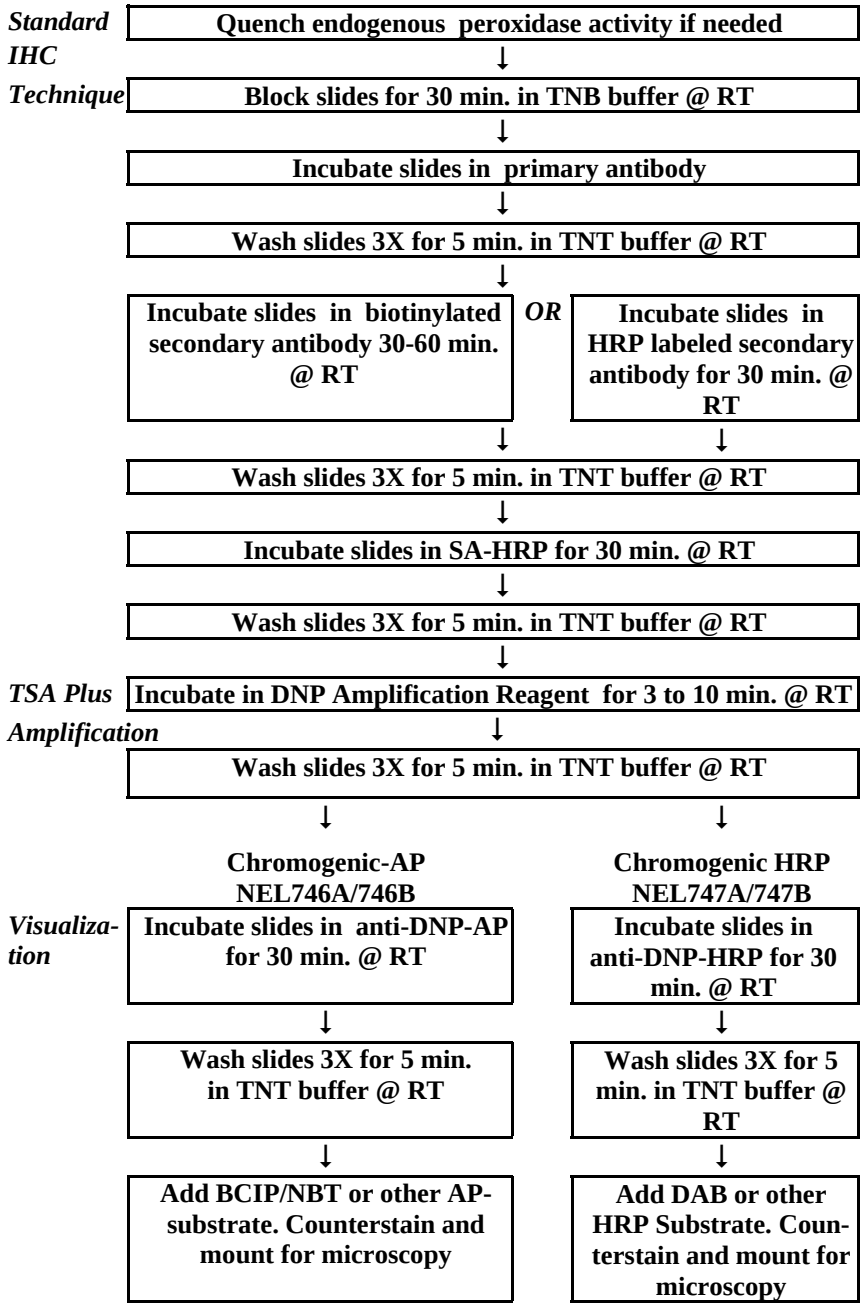
- Add 100-300 µL of anti-DNP-HRP (provided in kit) diluted 1:100 in TNB Buffer to each slide. Incubate the slides in a humidified chamber at room temperature for 30 minutes.
- Wash the slides 3X for 5 minutes each in TNT Buffer at room temperature with agitation.
- Visualize with standard HRP chro-mogenic substrates such as DAB (diaminobenzidine) or AEC

(aminoethyl carbazole). Incubate 5 minutes in the dark.

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

III. PROTOCOL FOR IHC

A. Overview Protocol for TSA Plus DNP Immunohistochemistry



B. Suggested IHC Protocol

The following is a suggested protocol for using TSA Plus DNP 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 DNP Amplification Reagent Working Solution is added. Visualization is done through the use of appropriate enzyme/chromogen combinations. The kits are supplied with either anti-DNP-AP (NEL746A, NEL746B) or anti-DNP-HRP (NEL747A, NEL747B) conjugates. Chromogenic substrates must be bought separately.

First Time Users

First time users should apply TSA DNP Plus 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. 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 **DNP 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 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.

C. Standard IHC Protocol

1. Preparation of Buffers and Reagents

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

DNP Amplification Reagent Stock Solution

DNP Amplification Reagent is supplied as a solid (which may have a slight yellow color). Reconstitute by adding 0.3 mL (for NEL746A/ NEL747A) or 0.15 mL (for NEL746B/747B) of dimethyl sulfoxide (DMSO-molecular biology or HPLC-grade) to make the DNP Stock Solution. The stock solu-

tion may also exhibit a light yellow color. DNP Stock Solution, when stored at 4 C, is stable for at least six 6 months. (Note: DMSO may freeze at 4 C. Thaw the Stock Solution, if necessary, before each use.)

DNP Amplification Reagent Working Solution

Before each procedure, dilute the DNP Stock Solution 1:50 using 1X Plus Amplification Diluent to make the DNP Working Solution. Approximately 100-300 µL of DNP Working Solution is required per slide. Discard any unused portion of DNP 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 60ºC 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 -20ºC 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.

2. Procedural Notes

- Do not let slides dry out between steps.
- A humidified chamber is recommended for all incubation steps (i.e., a damp paper towel in a covered box).
- Drain off as much of the incubation solutions as possible, before the addition of the next solution, to prevent dilution and uneven staining. Blot area around, but not on, tissue section using a labwipe.

- Be sure to use enough volume of solutions to cover sections or cells.
- Optional: Use of coverslips may reduce reagent evaporation. However care must be taken upon removal to prevent damage to tissues or cells.

3. Step by Step Protocol

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

Step 1: Slide Preparation

Prepare tissues or cells for detection with **TSA Plus DNP** 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 Buffer in a humidified chamber for 30 minutes at room temperature. (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 manu-

facturer's instructions regarding incubation time and temperature requirements. Use concentration determined in optimization studies(see p.19).

Step 4: Wash

Wash the slides 3X for 5 minutes each in

TNT Buffer at room temperature with agitation.

Step 5: Introduction of HRP

Incubate slides with HRP by doing one of the following:

a) 100-300 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. # NEL750. 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 minutes at room temperature.

Step 6: Wash

Wash the slides 3X for 5 minutes each in

TNT Buffer at room temperature with agitation.

Step 7: TSA Plus DNP Amplification

Pipet 100-300 µL of the DNP Amplification Reagent Working Solution (p. 21) onto each slide. Incubate the slides at room temperature for 3 to 10 minutes.

Step 8: Wash

Wash the slides 3X for 5 minutes each in

TNT Buffer at room temperature with agitation.

Step 9: Visualization of Deposited DNP

Follow the applicable chromogenic visualization option:

a.) AP-Chromogenic Option (NEL746A/ NEL746B)

Steps

- Add 100-300 µL of anti-DNP-AP (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.
- Wash the slides 3X for 5 minutes each in TNT Buffer at room temperature with agitation.

- Visualize with 100-300 µL standard alkaline phosphatase chromogenic substrates such as BCIP/NBT (5-bromo-4-chloro-indolyl phosphate/ nitroblue tetrazolium). Incubate slides ten minutes in the dark. Examine slides for signal strength. If darker signal is desired, incubate slides for up to an additional 10-30 minutes.
- Counterstain if desired. Nuclear Fast Red is an effective counterstain for BCIP/NBT. Histomount™ and Clear-mount™ may be used for mounting.

or

b.) HRP-Chromogenic Option (NEL747A/ NEL747B)

Steps

- 100-300 µL of anti-DNP-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.
- Wash the slides 3X for 5 minutes each in TNT Buffer at room temperature with agitation.
- Visualize with standard HRP chro-mogenic substrates such as DAB (diaminobenzidine) or AEC

(aminoethyl carbazole). Incubate 5 minutes in the dark.

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

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 DNP 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 DNP Amplification Reagent incubation time. • Decrease concentration of anti-DNP-enzyme conjugate used for 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 DNP 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 DNP 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.:	PerkinElmer Technical Support Department at 1 (800) 762-4000.
Outside the U.S.:	Contact your local PerkinElmer sales office or distributor.
Web site is:	http://www.perkinelmer.com

V. REFERENCES

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

Patents

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