MANUAL

TSA™ Research Reagents

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

TSA Plus DIG Kits

Material Provided

Kit contents: Each TSA Plus DIG kit contains the following 2 components necessary for signal amplification: DIG Amplification Reagent and 1X Plus Amplification Diluent.

Kit format:

	Format*	Catalog #	Kit Components
TSA Plus DIG (digoxigenin)	50-150 slides	NEL748001KT	1X Plus Amplification Diluent, 1 X 15 mL DIG Amplification Reagent (2 vials)
	250-750 slides	NEL748B001KT	1X Plus Amplification Diluent, 5 X 15 mL DIG Amplification Reagent (10 vials)
	Evaluation size (25-75 slides)	NEL748E001KT	1X Plus Amplification Diluent, 1 X 7.5 mL DIG Amplification Reagent (1 vial)

*The format of the kit is based on 100 - 300 µL per slide of Amplification Working Solution.

Product Information

Storage: Store kits in the dark at 4ºC.

Stability: The components in the kits are stable for a minimum of 3 months under proper storage conditions.

Application: TSA Plus DIG reagents are intended for high sensitivity detection in immunohistochemistry (IHC), immunocytochemistry (ICC) and *in situ* hybridization (ISH) experiments. Final detection may be fluorescent or chromogenic.

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Safety Note

All reagents are classified as nonhazardous. However, it is strongly recommended to wear disposable gloves and safety glasses while working. Thorough washing of hands after handling is also recommended.

Quality Control

We certify that QC results of these reagents meet our quality release criteria.

What is TSA Plus technology?

TSA Plus is based on Tyramide Signal Amplification (TSA™), a technology from PerkinElmer that generally provides 2-3 logs of improved sensitivity while allowing reduced consumption of primary antibodies or probes.

TSA Plus DIG uses horseradish peroxidase (HRP) to catalyze covalent deposition of digoxigenin (DIG) labels directly adjacent to the immobilized enzyme. The labeling reaction is quick (less than 10 minutes) and deposited labels can then be detected using anti-digoxigenin conjugates for fluorescent or bright field microscopy.

The use of TSA Plus reagents results in a significant increase in sensitivity over standard detection methods, while maintaining specificity and resolution. Moreover, TSA Plus reagents allow drastically reduced consumption of primary antibody or probe.

Reagents and Materials

Critical Reagents Required but not Supplied

- HRP-labeled reagent to drive the signal amplification reaction, for example:
    - SA-HRP for use with biotin-labeled probes or antibodies (cat. no. NEL750001EA)
    - Anti-digoxigenin-HRP for use with digoxigenin labeled probes or antibodies (cat. no. NEF832001EA)
    - Anti-fluorescein-HRP for use with fluorescein labeled probes or antibodies (cat. no. NEF710001EA)
    - HRP-labeled secondary antibody
        - Anti-rabbit IgG (goat) HRP (cat. no. NEF812001EA)
        - Anti-mouse IgG (goat) HRP (cat. no. NEF822001EA)
- DMSO (molecular biology or HPLC grade)
- Buffer components and detergents such as PBS, Triton-X100, Tween-20
- Blocking Reagent (Catalog number FP1012)
- Fluorophore or enzyme labeled anti-digoxigenin for detection of deposited DIG labels.

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Solutions to prepare

The following buffers and reagents are required for slide preparation and signal amplification

DIG Amplification Reagent Stock Solution
DIG Amplification Reagent is supplied as a solid. Each vial must be reconstituted with 150 µL DMSO to make the DIG Amplification Reagent Stock Solution. DIG Amplification Reagent Stock Solution is stable for at least 3 months when stored at 4ºC. (Note: DMSO freezes at 4ºC; therefore, thaw Stock Solution before each use).

TNT Wash Buffer
0.1 M TRIS-HCl, pH 7.5
0.15 M NaCl
0.05% Tween®20
Other wash buffers (such as PBS) may be used. Substitution of 0.3% Triton X-100 for the 0.05% Tween-20 is also possible. Users should validate the use of alternative wash buffers with their own systems.

TNB Blocking Buffer
0.1 M TRIS-HCl, pH 7.5
0.15 M NaCl
0.5% Blocking Reagent (available separately, catalog number FP1020)
Add Blocking Reagent slowly in small increments to buffer while stirring. Heat gradually to 55°C with continuous stirring to completely dissolve the Blocking Reagent. (This should take no longer than 30-60 minutes. The solution will appear milky. Bring to room temperature before using. Aliquot and store at -20°C for long term use.

DIG Amplification Working Solution
Before each procedure, dilute DIG Amplification Reagent Stock Solution1:50 in 1X Plus Amplification Diluent to make DIG Amplification Working Solution. Approximately 100-300 µL of DIG Amplification Working Solution is required per slide. Discard any unused portion of DIG Amplification Working Solution.

Recommendations

- 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 tissue.
- 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.
- First time users should apply TSA Plus DIG to a proven system.

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Quenching Endogenous Peroxidase

Activation and covalent binding of TSA reagent is catalyzed by peroxidase. Endogenous peroxidase will be a source of background, and should be quenched if present. Users should establish the need for doing this and optimal methodology specific to the tissues or cells being stained. Options include:

- 0.3% H_2O_2 to 3% H_2O_2
- Methanol or PBS as diluent for H_2O_2
- 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 TNT or 1X PBS buffer for 5 minutes. See Li *et al* from references section for more suggestions. Titration of primary and secondary antibodies to find optimal concentration is critical for success with HCA ImagAmp. The presence of excess HRP will favor formation of dimers over label deposition, resulting in decreased signal and increased background.

TSA-ISH Optimization

Inadequate optimization of probe and HRP conjugate dilution may be a source of high background and reduced signal.

TSA is ideal for detection of targets that have weak signals using standard detection methods. If the signal is strong with standard detection methods, dilute the probe until the signal begins to disappear and then proceed with TSA optimization.

Probe Optimization

- Slide 1: use same probe dilution as standard method.
- Slide 2: 5-fold dilution from slide 1
- Slide 3: 2-fold dilution from slide 2 (further dilution may be necessary)
- Slide 4: negative control (probe omitted)

HRP Conjugate Dilution

- Streptavidin-HRP (catalog number NEL750001EA): 1:250-1:1000 dilution
- Anti-fluorescein-HRP (catalog number NEF710001EA): 1:100-1:500 dilution
- Anti-DNP-HRP (catalog number FP1129): 1:100-1:500 dilution
- Anti-digoxigenin-HRP (catalog number NEF832001EA): 1:100-1:1000 dilution
- HRP reagents from other manufacturers: Begin with recommended range for slide applications. Further dilution may be needed

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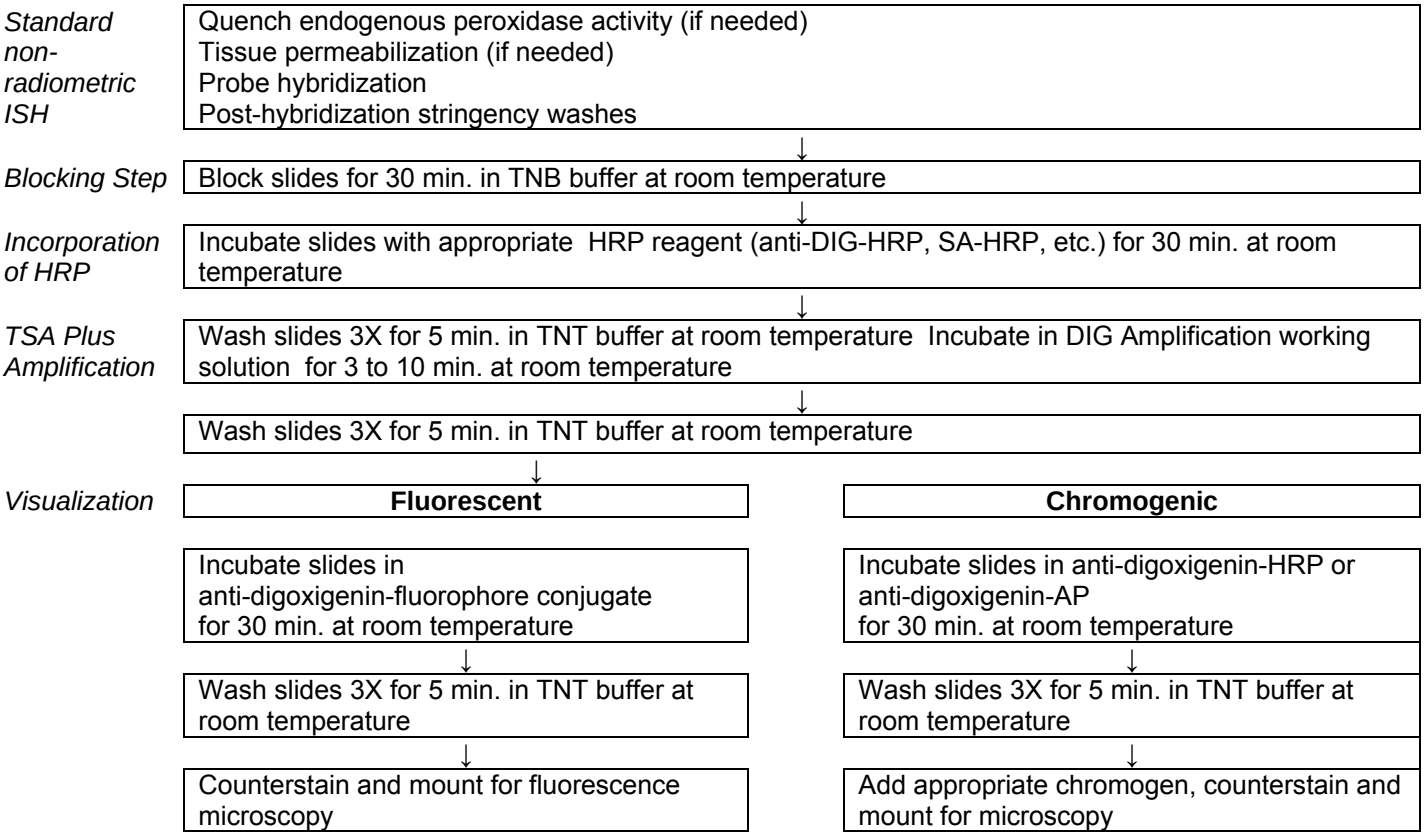
TSA-ISH Protocol Overview



Step by Step TSA-ISH Protocol

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

Step 2: Standard Non-radioactive ISH
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, DNP, or fluorescein-labeled probes) should be done using at concentration determined in optimization studies 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 30-60 minutes at room temperature or at 4ºC overnight.

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Step 4: Introduction of HRP
Incubate slides for 30 minutes at room temperature or at 4ºC overnight in a humidified chamber with appropriate HRP-labeled reagent using one of the following:

- For DIG-labeled probes, use 100-300 µL of anti-digoxigenin-HRP (catalog number NEF832001EA) diluted in TNB Buffer to concentration determined in optimization studies.
- For biotin-labeled probes, use 100-300 µL of SA-HRP (catalog number NEL750001EA) diluted in TNB Buffer to concentration determined in optimization studies.
- For fluorescein-labeled probes, use 100-300 µL of anti-fluorescein-HRP (catalog number NEF710001EA) diluted in TNB Buffer to concentration determined in optimization studies.
- For DNP-labeled probes, use 100-300 µL of anti-DNP-HRP (catalog number NEF710001EA) diluted in TNB Buffer to concentration determined in optimization studies.

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

Step 6: TSA Plus DIG Amplification
Pipette 100-300 µL of DIG Amplification Working Solution 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 DIG
DIG may be visualized for fluorescent or chromogenic detection.

For fluorescent detection, incubate slides with fluorophore labeled anti-DIG as directed by the manufacturer.

For chromogenic detection, incubate slides with HRP or AP labeled anti-DIG followed by detection with an appropriate chromogenic substrate.

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TSA-IHC Optimization

Inadequate optimization of primary antibody and HRP conjugate dilution may be a source of high background and reduced signal.

TSA is ideal for detection of targets that have weak signals with standard detection methods. It enables use of much more dilute antibody solutions for improved specificity. If the signal is strong with standard detection methods, dilute the primary antibody until the signal begins to disappear and then proceed with TSA optimization.

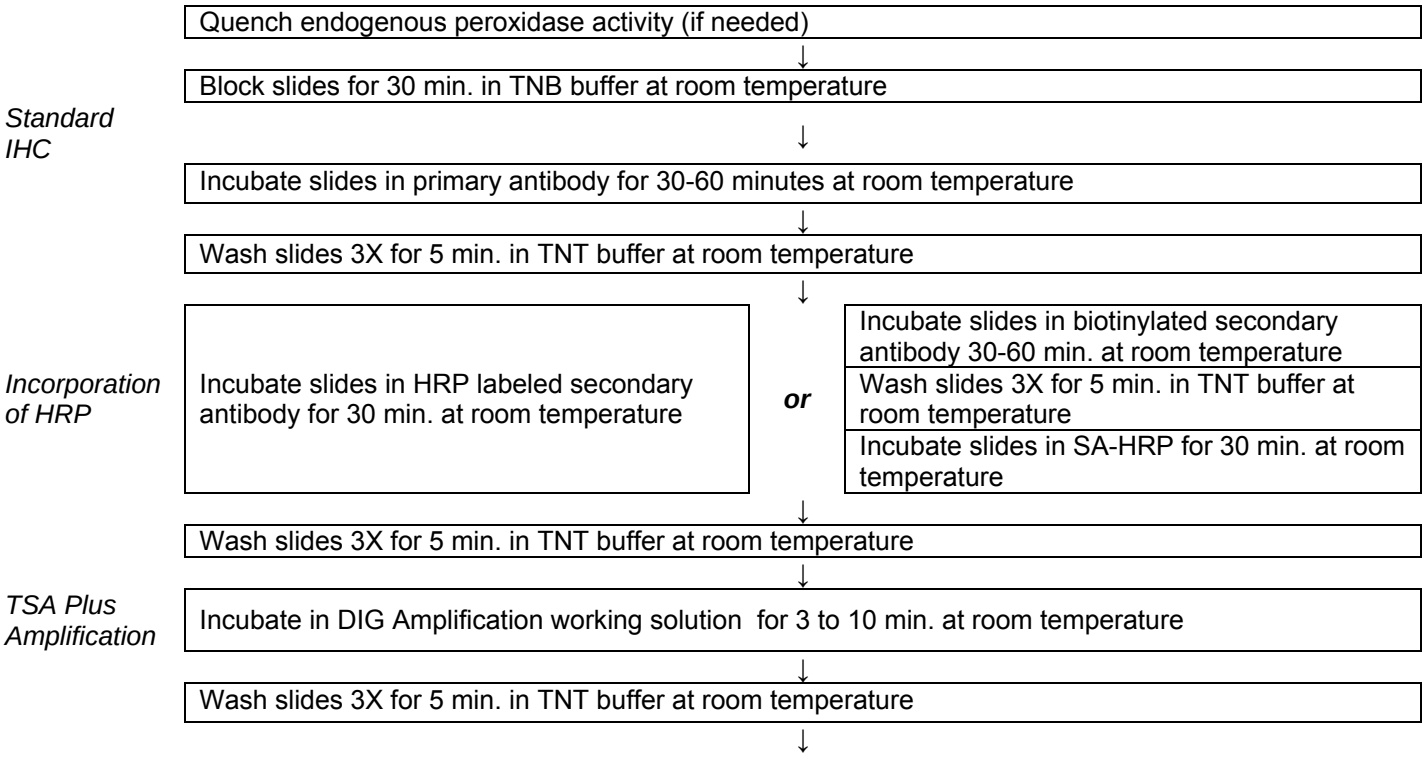
Primary Antibody Optimization

- Slide 1: use same probe dilution as standard method.
- Slide 2: 5-fold dilution from slide 1
- Slide 3: 5-fold dilution from slide 2
- Slide 4: 5-fold dilution from slide 3 (further dilution may be necessary)
- Slide 4: negative control (primary antibody omitted)

HRP Conjugate Dilution

- Anti-mouse-HRP (catalog number NEF822001EA): 1:500 to 1:2000 dilution
- Anti-rabbit-HRP (catalog number NEF812001EA): 1:500 to 1:2000 dilution
- Streptavidin-HRP (catalog number NEL750001EA): 1:1250-1:2500 dilution
- Anti-fluorescein-HRP (catalog number NEF710001EA): 1:100-1:500 dilution
- HRP reagents from other manufacturers: Begin with recommended range for slide applications. Further dilution may be needed

TSA-IHC Protocol Overview



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Visualization	**Fluorescent**	**Chromogenic**
	Incubate slides in anti-DIG-fluorophore for 30 min. at room temperature	Incubate slides in anti-DIG-HRP or anti-DIG-AP for 30 min. at room temperature
	↓	↓
	Wash slides 3X for 5 min. in TNT buffer at room temperature	Wash slides 3X for 5 min. in TNT buffer at room temperature
	↓	↓
	Counterstain and mount for fluorescence microscopy	Add appropriate chromogen, counterstain and mount for microscopy

Step by Step TSA-IHC Protocol

Step 1: Slide Preparation
Prepare tissues or cells for detection with TSA Plus DIG 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 30-60 minutes at room temperature or at 4ºC overnight. (Note: PBS may be substituted for TN buffer.)

Step 3: Primary Antibody Incubation
Drain off the TNB Buffer and apply 100-300 µL of 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.

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:

- 100-300 µL of HRP labeled secondary antibody diluted in TNB Buffer.
- 100-300 µL of biotinylated secondary antibody diluted in TNB Buffer. Incubate 30-60 minutes in a humidified chamber. Wash the slides for 3 X 5 minutes TNT buffer at room temperature with agitation. Follow by 100-300 µL of SA-HRP diluted in TNB Buffer. Use SA-HRP at 1:2000 if using PerkinElmer Cat. # NEL750001EA. When using alternative suppliers, reagents should be optimized for use with TSA starting with manufacturer's recommended dilu¬tions. Incubate slides in a humidified chamber for 30-60 minutes at room temperature or at 4ºC overnight.

Step 6: Wash
Wash the slides 3X for 5 minutes each in TNT Buffer at room temperature with agitation.

Step 7: TSA Plus DIG Amplification
Pipette 100-300 µL of DIG Amplification Working Solution onto each slide. Incubate the slides at room temperature for 3 to 10 minutes.

Step 8: Wash
Wash the slides 3X for 5 minutes each in TNT Buffer at room temperature with agitation.

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Step 9: Visualization of Deposited DIG
DIG may be visualized for fluorescent or chromogenic detection.

For fluorescent detection, incubate slides with fluorophore labeled anti-DIG as directed by the manufacturer.

For chromogenic detection, incubate slides with HRP or AP labeled anti-DIG followed by detection with an appropriate chromogenic substrate.

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Troubleshooting

Technical Support Resources

- **Assay Support Knowledge Base:** **www.perkinelmer.com/askTSA**
- **Email:** **global.techsupport@perkinelmer.com**
- **Telephone**
  - **USA toll-free** **800-762-4000**
  - **EU toll-free** **00800 33 29 0000**
  - **Finland toll-free** **999 800 33 29 0000**
  - **China toll-free** **800 820 5046**

ISH Troubleshooting

PROBLEM	REMEDY
Low Signal	• Optimize probe concentration. • Titer HRP conjugate to determine optimum concentration for signal amplification. • Add tissue permeabilization step to facilitate penetration of reagents. • Lengthen incubation time for DIG Amplification Working Solution.
Excess Signal	• Decrease concentration of HRP conjugate introduced prior to amplification. • Decrease probe concentration. • Decrease DIG Amplification Reagent incubation time. • Decrease concentration of anti-DIG-enzyme conjugate used for visualization.
High Background	• Decrease concentration of HRP conjugate. • Decrease probe concentration. • Shorten chromogenic development time. • Lengthen endogenous peroxide quenching step. • Filter buffers. • Increase number and/or length of washes. • Nonqualified or contaminated blocking reagent used. Use PerkinElmer Blocking Reagent (FP1020).

IHC Troubleshooting

PROBLEM	REMEDY
Low Signal	• Titer primary and/or secondary antibodies to determine optimum concentration for signal amplification • Lengthen incubation time for DIG Amplification Working Solution. • Use antigen retrieval techniques to unmask the target.
Excess Signal	• Decrease concentration of primary and/or secondary antibody or HRP conjugates. • Decrease DIG Amplification Working Solution incubation time. • Decrease concentration of anti-DIG 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 chromogenic development time. • Nonqualified or contaminated blocking reagent used. Use PerkinElmer Blocking Reagent (FP1020).

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TSA reagents and related methods are covered by U.S. Patents 5,863,748, 6,372,937, 6,593,100, and 6,617,125, related patent applications, and international equivalents thereof.

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