1. Introduction

Tyramide Signal Amplification (TSA) from APExBIO technology increases sensitivity by a factor of 100 and enables detection of low-abundance targets in fluorescent immunocytochemistry (ICC), immunohistochemistry (IHC) and *in situ* hybridization (ISH) application.

The TSA Fluorescence Kit uses horseradish peroxidase (HRP) to directly catalyze the covalent deposition of the fluorophore adjacent to the immobilized enzyme. The labeling process is so fast (less than ten minutes) and the deposition label can be viewed directly under standard or confocal microscopy. TSA fluorescein can also be used in combination with anti-fluorescein.

TSA technology is used for bright field microscopy of enzyme conjugates and suitable chromogenic substrates. The use of TSA reagents results in a significant increase in sensitivity compared to standard assays while maintaining stable specificity and resolution. In addition, TSA reagents can significantly reduce the consumption of primary antibodies or probes.

2. Materials

2.1. Material Provided

	Format	Catalog	Kit Components
TSA Fluorescein	100-300 sides	K1050	1X Amplification Diluent (30 mL) Fluorescein Tyramide (dry, dissolve in 60 µL DMSO) Blocking Reagent (6g)
	200-600 slides	K1050	1X Amplification Diluent (60 mL) Fluorescein Tyramide (2 tubes, dry, dissolve in 60 µL DMSO/tube) Blocking Reagent (12g)
TSA Cyanine 3	100-300 sides	K1051	1X Amplification Diluent (30 mL) Cyanine 3 Tyramide (dry, dissolve in 60 µL DMSO) Blocking Reagent (6g)
	200-600 slides	K1051	1X Amplification Diluent (60 mL) Cyanine 3 Tyramide (2 tubes, dry, dissolve in 60 µL DMSO/tube) Blocking Reagent (12g)
TSA Cyanine 5	100-300 sides	K1052	1X Amplification Diluent (30 mL) Cyanine 5 Tyramide (dry, dissolve in 60 µL DMSO) Blocking Reagent (6g)
	200-600 slides	K1052	1X Amplification Diluent (60 mL) Cyanine 5 Tyramide (2 tubes, dry, dissolve in 60 µL DMSO/tube) Blocking Reagent (12g)

Note! The format of the kit is based on 100 - 300 μL per slide of Fluorophore Tyramide Working Solution.

2.2. Product Information

Storage: Stored at 4℃.

Stability: The kit is stable for at least 3 months under suitable storage conditions.

Application: The TSA Fluorescence Kits is used for high sensitivity detection in immunohistochemistry (IHC), immunocytochemistry (ICC) and *in situ* hybridization (ISH) experiments. The final assay may be fluorescent or chromogenic (TSA Fluorescein Kit only).

2.3. Materials Not Supplied

Depending on your assay, additional HRP-labeled reagents may be required to drive the signal amplification reaction. Reagents are as follows：

Anti-digoxigenin-HRP for use with digoxigenin labeled probes or antibodies	
Anti-fluorescein-HRP for use with fluorescein labeled probes or antibodies	
HRP-conjugated anti-species secondary antibody	Anti-rabbit IgG (goat) HRP
	Anti-mouse IgG (goat) HRP
DMSO (molecular biology or HPLC grade)	
Buffer components and detergents such as PBS, Triton-X100, Tween-20	
H_2O_2 (molecular biology or HPLC grade)	

3. Protocols

3.1. Solutions to prepare

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

Fluorophore Tyramide Stock Solution

Fluorophore Tyramide Reagent is supplied as a solid. Each vial must be reconstituted with DMSO as indicated to make Fluorophore Tyramide Stock Solution. Fluorophore Tyramide 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). Here are recommended dissolution volumes for stock solutions.

	Catalog	Tyramide Stock Solution Preparation
TSA Fluorescein	K1050	Add 60 μL DMSO to reagent tube
TSA Cyanine 3	K1051	Add 60 μL DMSO to reagent tube
TSA Cyanine 5	K1052	Add 60 μL DMSO to reagent tube

Fluorophore Tyramide Working Solution

Before each procedure, dilute Fluorophore Tyramide Stock Solution 1:50--1:500 in 1X Amplification Diluent to make Fluorophore Tyramide Working Solution with 0.3% H_2O_2 (the dilution ratio can be justified for best amplification result). Approximately 100-300μL of Fluorophore Tyramide Working Solution is required per slide.

Note! The H_2O_2 must be added freshly before use.

Discard any unused portion of Fluorophore Tyramide Working Solution.

TNT Wash Buffer

0.15 M NaCl

0.1 M TRIS-HCl, pH 7.5

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

0.15 M NaCl

0.1 M TRIS-HCl, pH 7.5

0.5% Blocking Reagent

Add Blocking Reagent slowly in small increments to buffer while stirring. Heating gradually to 55°C with continuous stirring 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.

Recommendations:

(1) The first time users should apply TSA to a validated system.

(2) It is recommended to humidify the chamber (i.e., a damp paper in the covered box) in all incubation steps.

(3) Keep the slides wet between the steps.

(4) If the signal is excessive, further dilute the primary antibody, probe or HRP conjugate. The TSA kit is designed for a 1:50 dilution of tyramide reagent for best results.

(5) 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.

(6) Make sure to cover the sections or cells completely with a sufficient amount of each reagent.

(7) Optional: The use of coverslips can reduce reagent evaporation, especially in steps that require long incubations at elevated temperatures (i.e., probe hybridization). However, care must be taken to prevent damage when removing tissue or cell.

(8) If your assay includes streptavidin conjugates, check for endogenous biotin which may be a source of non-specific background.

3.2. Quenching Endogenous Peroxidase

Activation and covalent binding of the TSA reagent is catalyzed by peroxidase. Endogenous peroxidase will be the source of its background and should be quenched if present. For specially stained tissues or cells, the user should determine the necessity and best practice for doing so. The available options are as follows：

- 0.3% H_2O_2 to 3% H_2O_2 in PBS, incubation for 10 to 60 minutes
- 0.3% H_2O_2 to 3% H_2O_2 in Methanol, incubation for 10 to 60 minutes

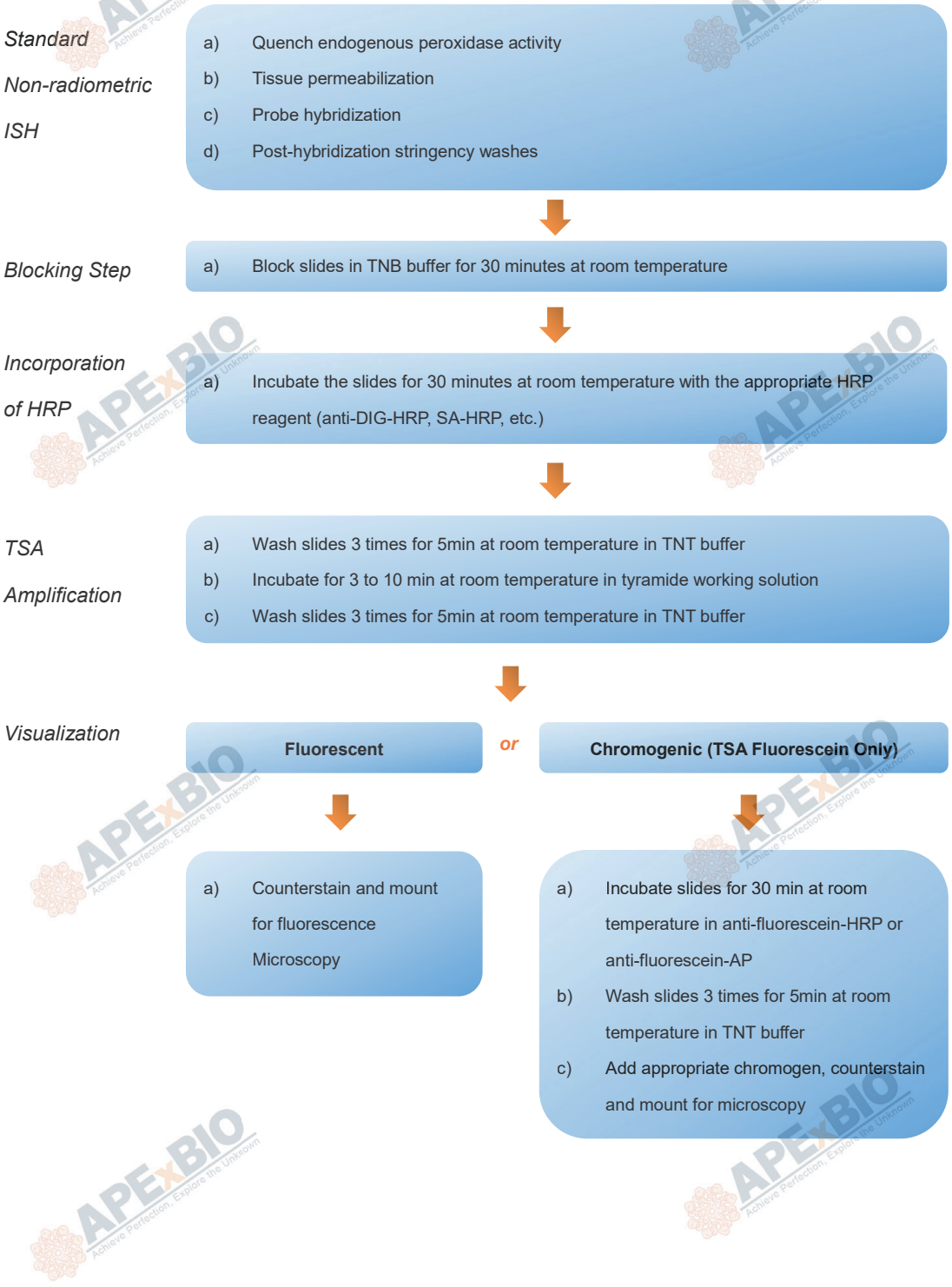
For paraffin-embedded tissues, quenching is performed after dewaxing and alcohol rehydration but prior to the protease digestion step. For frozen tissue or cell preps, quenching is performed after fixation and prior to the protease digestion step. After quenching, wash with TNT or 1X PBS buffer for 5 minutes.

3.3. TSA-ISH Optimization

Inadequate optimization of probe and HRP conjugate dilution may be a source of high background and low signal. The TSA is ideal for detecting weaker targets using standard detection methods. If a strong signal is detected using standard detection methods, dilute the probe until the signal begins to disappear and then perform TSA

optimization.

3.4. TSA-ISH Protocol Overview



3.5. TSA-ISH Protocol

1: Slide Preparation

Tissue or cells for TSA detection are prepared using standard fixation and embedding techniques. Dewax and rehydrate using standard procedures.

2: Standard Non-radioactive ISH

Follow standard non-radioactive in situ hybridization techniques. Include tissue permeabilization and quenching of endogenous peroxidase activity. Probe hybridization (with digoxin, biotin, DNP or fluorescein-labeled probes) should be carried out using the concentrations determined in the optimization study, followed by stringent washing.

Note: An Unscaled control slide and magnified negative control slide should always be taken for each experiment.

3: Blocking Step

Tissue sections were covered with TNB buffer (or other effective blocking buffer) and the slides will be incubated in a humidified chamber for 30-60 minutes at room temperature or overnight at 4°C.

4: Introduction of HRP

Incubate the slides in a humidified chamber with the appropriate HRP label for 30 minutes at room temperature or overnight at 4°C. Tissue sections are covered with sufficient reagent volume, typically 100-300µL per slide.

- For fluorescein-labeled probes, anti-fluorescein-HRP diluted in TNB buffer (or other effective blocking buffer) to the concentration determined in the optimization study should be used
- For DNP-labeled probes, anti-fluorescein-HRP diluted in TNB buffer (or other effective blocking buffer) to the concentration determined in the optimization study should be used
- For DIG-labeled probes, anti-digoxigenin-HRP diluted in TNB buffer (or other effective blocking buffer) to the concentration determined in the optimization study should be used
- For biotin-labeled probes, SA-HRP diluted in TNB buffer (or other effective blocking buffer) to the concentration determined in the optimization study should be used

5: Wash

Wash the slides 3 times in TNT buffer (or other validated wash buffer) for 5 minutes at room temperature.

6: Amplification

Add fluorophore Tyramide working solution onto each slide using a pipette. Ensure enough working solution to completely cover the tissue sections, usually 100-300μL per slide. Incubate slides for 3 to 10 minutes at room temperature.

7: Wash

Wash the slides 3 times in TNT buffer (or other validated wash buffer) for 5 minutes at room temperature.

8: Visualization of Deposited Fluorophores

For fluorescent detection, counterstain and mount for fluorescence microscopy.

For the wavelength of the excitation and emission maximum of the fluorophore, refer to the following：

Fluorophore Excitation and Emission Maxima

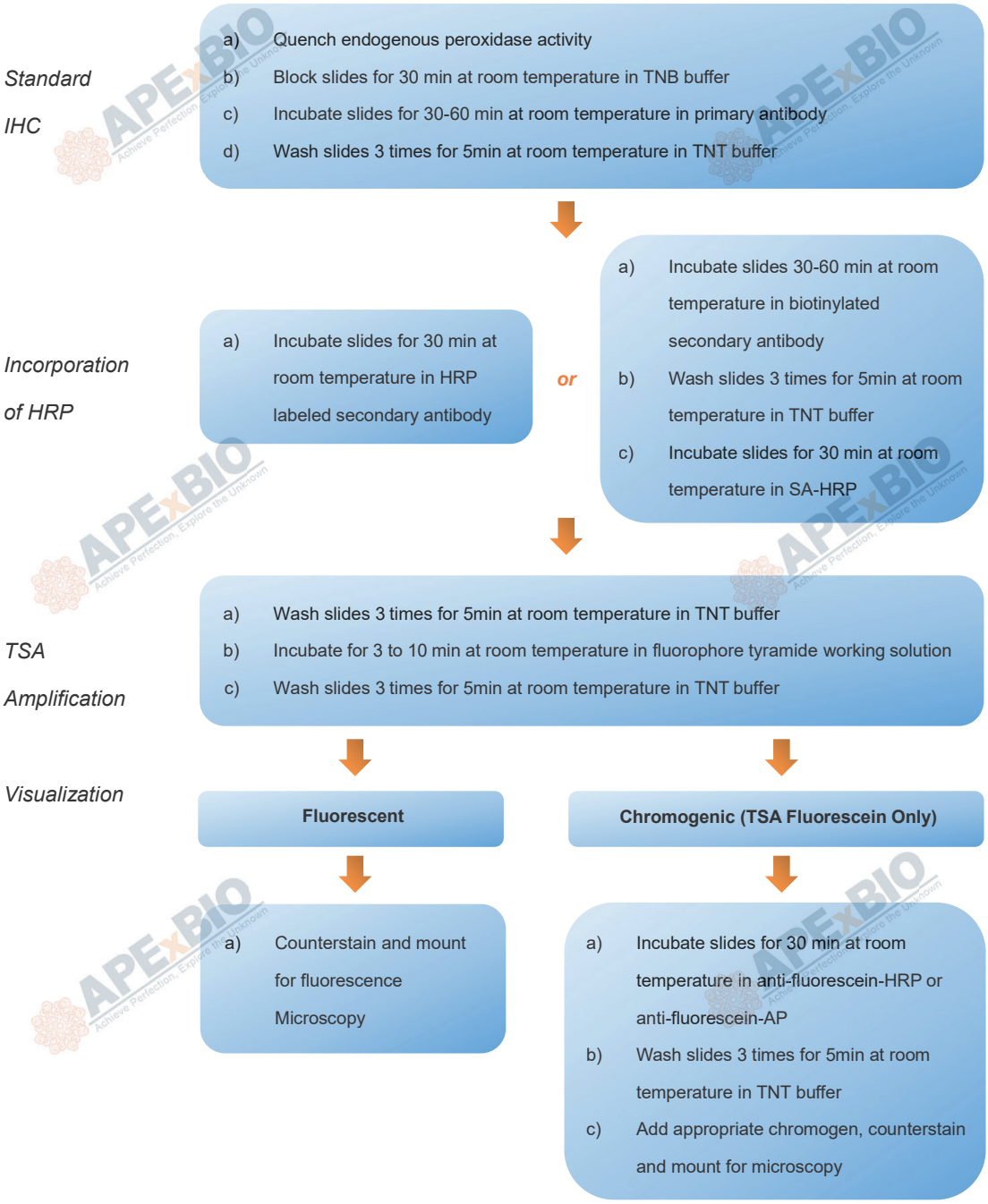
Fluorophore	Excitation	Emission
Fluorescein	494nm	517nm
Cyanine 3	550nm	570nm
Cyanine 5	648nm	667nm

For the chromogenic detection of TSA fluorescein, incubate the slide with anti-fluorescein-HRP or anti-fluorescein-AP and detect with a suitable chromogenic substrate.

3.6. TSA-IHC Optimization

Inadequate optimization of primary antibody and HRP conjugate dilution may be a source of high background and low signal. The TSA is ideal for detecting weaker targets using standard detection methods. Use more diluted antibody solution to increase specificity. 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.

3.7. TSA-IHC Protocol Overview



3.8. TSA-IHC Protocol

1: Slide Preparation

Tissue or cells for TSA detection are prepared using standard fixation and embedding techniques. Dewax and rehydrate using standard procedures. Endogenous peroxidase activity is quenched.

Note: An Unscaled control slide and magnified negative control slide should always be taken for each experiment.

2: Blocking Step

Cover the tissue sections with TNB buffer (or other effective blocking buffer) and the slides can be incubated in a humidified chamber for 30-60 minutes at room temperature or overnight at 4°C.

3: Primary Antibody Incubation

Drain the blocking buffer, apply a primary antibody, and dilute in TNB buffer (or other effective blocking buffer). Incubate primary antibody preps according to the manufacturer's instructions for incubation time and temperature requirements. Add sufficient volume (generally 100-300 µL) to cover tissue sections to at the concentration in the optimization studies determined.

4: Wash

Wash the slides 3 times in TNT buffer (or other validated wash buffer) for 5 minutes at room temperature.

5: Introduction of HRP

Incubate the slides in a humidified chamber with the appropriate HRP label for 30 minutes at room temperature or overnight at 4°C. Tissue sections are covered with sufficient reagent volume, typically 100-300µL per slide. Available options as follows:

- HRP labeled secondary antibody diluted in TNB Buffer (or other validated blocking buffer)
- 100-300 µL of biotinylated secondary antibody diluted in TNB Buffer (or other validated blocking 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.
- When using an alternate supplier, optimize the reagents for use with TSA, starting with the manufacturer's recommended dilution. Incubate slides in a humidified chamber for 30-60 minutes at room temperature or overnight at 4 °C.

6: Wash

Wash the slides 3 times in TNT buffer (or other validated wash buffer) for 5 minutes at room temperature.

7: Amplification

Add fluorophore Tyramide working solution onto each slide using a pipette. Ensure enough working solution to completely cover the tissue sections, usually 100-300μL per slide. Incubate slides for 3 to 10 minutes at room temperature.

8: Wash

Wash the slides 3 times in TNT buffer (or other validated wash buffer) for 5 minutes at room temperature.

9: Visualization of Deposited Fluorophores

For fluorescent detection, counterstain and mount for fluorescence microscopy.

For the wavelength of the excitation and emission maximum of the fluorophore, refer to the following：

Fluorophore Excitation and Emission Maxima

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

For the chromogenic detection of TSA fluorescein, incubate the slide with anti-fluorescein-HRP or anti-fluorescein-AP and detect with a suitable chromogenic substrate.

4. Note

4.1. ISH Troubleshooting

Low Signal：

- Consider TSA Plus reagents for higher signal
- Optimize probe concentration
- Add tissue permeabilization step to facilitate penetration of reagents
- Titer HRP conjugate to determine optimum concentration for signal amplification

- Lengthen incubation time for Fluorophore Tyramide Working Solution

Excess Signal:

- Decrease probe concentration
- Decrease Fluorophore Tyramide Working Solution incubation time
- Decrease concentration of HRP conjugate introduced prior to amplification
- Decrease concentration of anti-fluorescein-enzyme conjugate used for chromogenic visualization

High Background:

- Decrease concentration of HRP conjugate
- Decrease probe concentration
- Filter buffers
- Check for endogenous biotin (if using streptavidin conjugates)
- Shorten chromogenic development time
- Lengthen endogenous peroxidase quenching step
- Increase number and/or length of washes
- Nonqualified or contaminated blocking reagent used

4.2. IHC Troubleshooting

Low Signal:

- Lengthen incubation time for Fluorophore Tyramide Working Solution
- Use antigen retrieval techniques to unmask the target
- Titer primary and/or secondary antibodies to determine optimum concentration for signal amplification
- Consider TSA Plus reagents for higher signal

Excess Signal:

- Decrease Fluorophore Tyramide Working Solution incubation time

- Decrease concentration of anti-fluorescein-enzyme conjugate used for chromogenic visualization
- Decrease concentration of primary and/or secondary antibody or HRP conjugates

High Background：

- Check for endogenous biotin (if using streptavidin conjugates)
- Filter buffers
- Decrease concentration of primary and/or secondary antibody or HRP conjugates
- Increase number and/or length of washes. Shorten chromogenic development time
- Nonqualified or contaminated blocking reagent used.
- Lengthen endogenous peroxidase quenching step