TSA DNP kits
Protocol-in-brief: immunohistochemistry

Preparation:

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

TNB Blocking Buffer:
- 0.1M Tris-HCl, pH 7.5
- 0.15 M NaCl
- 0.5% Blocking Reagent (w/v), supplied in kit or purchased separately
  - FP1020: 3 g. blocking reagent
  - FP1012: 20 g. blocking reagent

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 may take up to several hours. 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.

DNP Stock Solution

The DNP Amplification Reagent is supplied as a solid. Reconstitute as indicated in product manual. Use molecular biology grade or HPLC-grade DMSO. The DNP Amplification Reagent Stock Solution, when stored at 4 °C, is stable for at least 6 months. (DMSO freezes at 4 °C. Thaw the Stock Solution before each use.)

<table>
<thead>
<tr>
<th>Catalog number</th>
<th>Kit</th>
<th>Solvent</th>
<th>Reconstitution volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEL746A</td>
<td>TSA DNP (AP chromogen)</td>
<td>DMSO</td>
<td>0.3 mL</td>
</tr>
<tr>
<td>NEL746B</td>
<td>TSA DNP (AP chromogen)</td>
<td>DMSO</td>
<td>0.15 mL</td>
</tr>
<tr>
<td>NEL747A</td>
<td>TSA DNP (HRP chromogen)</td>
<td>DMSO</td>
<td>0.3 mL</td>
</tr>
<tr>
<td>NEL747B</td>
<td>TSA DNP (HRP chromogen)</td>
<td>DMSO</td>
<td>0.15 mL</td>
</tr>
</tbody>
</table>
DNP Amplification Reagent Working Solution

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

Reagent Titration

1. Primary antibody titration. The following test slides are recommended:
   Test slide 1: Primary or secondary antibody at manufacturer’s recommended dilution.
   Test slide 2: 5X dilution of slide #1 antibody concentration
   Test slide 3: 5X dilution of slide #2 antibody concentration
   Test slide 4: 5X dilution of slide #3 antibody 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 primary antibody often leads to better signal amplification.

2. Secondary Antibody and/or HRP Conjugate Titrations.
   Test 2-fold serial dilutions starting with the manufacturer’s recommended dilution. In cases where no signal and no background are seen, it may be necessary to use an increased concentration of reagent. For example, if the recommended starting dilution is 1:1000, run test slides using concentrations of 1:500, 1:1000 and 1:2000.

Quenching Endogenous Peroxidase

Amplification for all DNP kits is catalyzed by HRP. To minimize background, endogenous peroxidase activity must be quenched. Users should establish the need for doing this and optimal methodology specific to the tissues or cells being stained. For paraffin-embedded tissues, quenching can be done after dewaxing and alcohol rehydration but before the protease digestion step. After quenching wash with TNT or 1X PBS wash buffer for 5 minutes.

Recommended quenching solution:
0.3% - 3% H₂O₂ in PBS or methanol, 10-60 minute incubation

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.
TSA™ DNP Systems Immunohistochemistry (IHC) Protocol

1. Prepare tissues or cells using standard fixation and embedding techniques. Dewax and rehydrate slides according to standard procedures. 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 Blocking Buffer in a humidified chamber for 30 minutes at room temperature.

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.

4. Wash slides three times (5 minutes per wash) in fresh TNT Wash Buffer at room temperature.

Introduction of HRP (both the TSA DNP AP and TSA DNP HRP kits require amplification using an HRP)

5. Incubate slides with HRP by doing one of the following:
   
   a) Secondary-HRP conjugates
      
      100-300 µL of HRP labeled secondary antibody diluted in TNB Buffer
      (Use concentration determined in optimization studies – see Reagent Titration on page 2.)

   b) Biotinylated secondary antibodies
      
      i. 100-300 µL of biotinylated secondary antibody diluted in TNB Buffer. (Use concentration determined in optimization studies). Incubate 30-60 minutes in a humidified chamber.
      
      ii. Wash the slides for 3 - 5 minutes using TNT buffer at room temperature with agitation. Follow by 100-300 µL of SA-HRP diluted in TNB Buffer. (Use SA-HRP concentration determined in optimization studies – see Reagent Titration on page 2.) Incubate slides in a humidified chamber for 30 minutes at room temperature.

6. Wash slides three times (5 minutes per wash) in fresh TNT Wash Buffer at room temperature.

Amplification

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

8. Wash the slides three times (5 minutes per wash) in TNT Wash Buffer at room temperature with agitation.
Visualization

9. Follow desired chromogenic visualization option:

a) AP-Chromogenic Option (NEL746A/NEL746B)

- 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 Clearmount™ may be used for mounting.

b) HRP-Chromogenic Option (NEL747A/NEL747B)

- 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 chromogenic 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 Clearmount™ may be used for mounting DAB-stained slides. Use aqueous mounting media with AEC.