



PolyStain TS Kit - for Mouse, Rabbit and Rat antibody on Rodent tissue

(DAB/Permanent Red/Ni-DAB)

NB-23-00139-1 (24 ml)

NB-23-00139-2 (72 ml)

NB-23-00139-3 (240 ml)

***PolyStain TS Kit - for Mouse, Rabbit and Rat antibody on Rodent tissue
(DAB/Permanent Red/Ni-DAB)***

NB-23-00139-1; NB-23-00139-2; NB-23-00139-3

Storage: 2- 8°C

INTENDED USE:

PolyStain TS Kit - for Mouse, Rabbit and Rat antibody is designed to use with user supplied mouse, rat and rabbit primary antibodies to detect three distinct antigens on mouse tissue, cell sample or human tissue.

This kit has been tested on paraffin embedded tissue, which can be used on frozen tissue or cell smears.

For frozen tissues, a lower temperature of 65°C must be used during the Antibody Blocker step (Reagent 3) to prevent dissociation of the tissue from the slide.

Please read through the entire protocol, as all steps must be performed in the defined order to achieve proper staining.

Triple immunohistostaining uses traditional methods to reveal three distinct antigens on a single tissue. PolyStain TS Kit - for Mouse, Rabbit and Rat antibody from Neo BioTech contains the following polymer enzyme conjugates: polymer-HRP anti-mouse IgG, polymer-AP anti-rat IgG and polymer-HRP anti-rabbit IgG with three substrates/chromogen; DAB (brown), Permanent Red (Red) and DAB-Ni (Black).

This kit is a non-biotin system, avoiding non-specific binding caused by endogenous biotin.

It has been optimized to show no cross reaction when detecting more than two primary antibodies from the mouse and rat host species, using our unique blocking system.

Simplified steps allow users to complete triple staining within 5 hours (without antigen retrieval) or 6-7 hours (with antigen retrieval).

This protocol also includes a method to dehydrate, clear and permanently mount slides with coverslip.

KIT COMPONENTS:

Component No.	Content	24 mL Kit	72 mL Kit	240 mL Kit
Reagent 1	Mouse HRP Polymer (RTU)	12 mL	18mLx2	120mL
Reagent 2A	DAB Substrate (RTU)	15mL	18mLx2	120mL
Reagent 2B	DAB Chromogen (20x)	2mL	4 mL	12 mL
Reagent 3	Antibody Blocker (40x)	15mLx2	50mL	100mL
Reagent 4	Rat Primer (RTU)	12mL	18mLx2	120mL
Reagent 5	Rat AP Polymer (RTU)	6mL	18mL	60mL
Reagent 6	Rabbit HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 7A	NeoBioTech-Permanent Red Substrate (RTU)	15mL	18mLx2	120mL
Reagent 7B	NeoBioTech-Permanent Red Activator (5x)	3mL	7.2mL	12mLx2
Reagent 7C	NeoBioTech-Permanent Red Chromogen (100x)	150µL	360µL	1.2mL
Reagent 8A	DAB-Ni Substrate (20x)	1mL	2mL	6mL
Reagent 8B	Hydrogen Peroxide (20X)	1mL	2mL	6mL
Reagent 8C	Nickel Solution (7x)	3mL	6mL	18mL
Reagent 9	NeoMount Universal	15 mL	18mLx2	120 mL

HRP = Horseradish Peroxidase AP = Alkaline Phosphatase Ms = Mouse Rb = Rabbit Rt = Rat

PROTOCOL NOTES:

1. Proper Fixation: To ensure the quality of the staining and obtain reproducible performance, user needs to supply appropriately fixed tissue and well prepared slides.
2. Tissue needs to be adhered to the slide tightly to avoid falling off.
3. Paraffin embedded sections must be deparaffinize with xylene and rehydrated with a graded series of alcohols before staining.
4. Cell smear samples should be prepared as close to a monolayer as possible to obtain satisfactory results.
5. Control slides are recommended for interpretation of results: positive, reagent (slides treated with Isotype control reagent), and negative control.
6. **DO NOT** let specimen or tissue dry during protocol. This will generate false positive and/or false negative signal.

7. The fixation, tissue section thickness, antigen retrieval and primary antibody dilution and incubation time effect results significantly. Investigator needs to consider all factors and determine optimal conditions when interpreting results.
8. We recommend TBS-T to be used as the wash buffer to get the highest sensitivity and clean background. Phosphate in the PBS-T may inhibit the activity of the alkaline phosphatase. Note: 1X TBS-T =50mM Tris HCl, 150mM NaCl, 0.05% Tween-20 pH7.6.

EQUIPEMENT OR MATERIAL NEEDED BUT NOT PROVIDED:

1. Equipment and material for deparaffinization, such as fume absorbing hood, etc.
2. Heat source (microwave or hot plate) for HIER and antigen retrieval buffers
3. Thermometer, Timer, Beaker, Coverslip
4. Wash buffer: 0.01 M PBS with 0.5% Tween20, pH7.4 or 50mM Tris HCl, 150mM NaCl, 0.05% Tween-20 pH7.6.
5. Peroxidase and alkaline phosphatase blocking buffer
6. 100% ethanol, 100% Xylene, Hematoxylin

STAINING PROTOCOL SELECTION AND LIMITATION OF THE KIT:

- Most antigens will not be destroyed by heat. However, users need to check if there are proteins on the tissue that are heat sensitive before proceeding with the staining.
- **NB-23-00139** Protocol-A worksheet is suitable when mouse, rabbit & rat primary antibodies need pre-treatment or mouse is sensitive to pretreatment.
- **NB-23-00139** Protocol-B worksheet is suitable when one Rat & one Rabbit primary antibody are sensitive to pre-treatment but the mouse primary antibody needs pre-treatment.
- **NB-23-00139** Protocol-C worksheet is suitable for one Rat & one Rabbit primary Abs need pre-treatment, the mouse primary Ab is sensitive to pretreatment.
- Please read the following table carefully before you start the experiment to ensure the result.
- This kit is not suitable for the following condition: 2 proteins are heat sensitive and detected by rat and mouse antibodies and rabbit antibody requires HIER or 2 proteins are heat sensitive and detected by rabbit and mouse antibodies and rat antibody requires HIER.

NB-23-00139 PROTOCOL-A

Reagent	Staining Protocol	Incubation Time (Min.)
<p>1. Peroxidase and Alkaline Phosphatase Blocking Reagent</p> <p>Not provided</p> <p>Fast, easy and it will block endogenous alkaline phosphatase</p>	<p>a. Incubate slides in peroxidase and alkaline phosphatase blocking reagent. We recommend NeoPure Dual Enzyme Block NB-23-00193.</p> <p>b. Rinse the slide using distilled water at least twice.</p>	10 min.
<p>2. Antigen retrieval:</p> <p>Refer to primary antibody data sheet.</p> <p>Supplied by user.</p>	<p>a. Refer to primary antibody data sheet for antigen retrieval methods.</p> <p>b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T (See note 8 above); 3 times for 2 minutes each.</p>	Up to 1 hour
<p>3. Primary Antibody:</p> <p>Add Mouse Primary</p> <p>Supplied by user.</p>	<p>Note: Investigator needs to optimize dilution prior to triple staining.</p> <p>a. Apply 2 drops or enough volume of mouse primary antibody mixture to cover the tissue completely. Incubate in moist chamber for 30-60 min. Recommend 30min to shorten total protocol time.</p> <p>b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.</p>	30 minutes
<p>4. Reagent 1:</p> <p>Mouse HRP Polymer (RTU)</p>	<p>a. Apply 1 to 2 drops (50-100µL) of Reagent 1 (Mouse HRP Polymer) to cover the tissue completely.</p> <p>b. Incubate in moist chamber for 15 min.</p> <p>c. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.</p>	15 minutes
<p>5. Reagent 2A&2B</p> <p>Reagent 2A: DAB Substrate (RTU)</p> <p>Reagent 2B: DAB Chromogen (20x)</p>	<p>Note: Make enough DAB working solution by adding 1 drop of Reagent 2B (DAB Chromogen) in 1mL of Reagent 2A (DAB Substrate). Mix well. Store at 4°C and use within 7 hours.</p> <p>a. Apply 1 to 2 drops (50-100µL) of your DAB working solution to cover the tissue completely.</p> <p>b. Incubate for 5min.</p> <p>c. Rinse slides in multiple changes of distilled water 2min, 3 times or under running tap water for 1minute</p>	5 minutes
<p>6. Reagent 3:</p> <p>Antibody Blocker (40x)</p>	<p>Note: This step will block antibodies of previous step so no cross reaction will occur in this protocol. HIER can be done immediately after Antibody Blocker step if the primary antibodies requires antigen retrieval. For frozen tissues, a lower temperature of 65°C must be used during the Antibody Blocker step to prevent dissociation of the tissue from the slide.</p>	10 minutes

	<ol style="list-style-type: none"> Use hot plate or water bath to heat diluted Reagent 3 (Antibody Blocker) to 1x solution (1 part of Antibody Blocker in 39 parts of distilled water) to 80°C. Make enough volume to cover the tissue in beaker. Put slides in heated Antibody Blocker for 10 minutes at 80°C. Remove slides from the Antibody blocker; cool slides 5 seconds. Rinse slides in multiple changes of distilled water. If antigen retrieval step is required go directly to step 7 if not complete step 6e and move on to step 8. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each. 	
<p>7. Antigen retrieval:</p> <p>Refer to primary antibody data sheet.</p>	<ol style="list-style-type: none"> Refer to primary antibody data sheet for antigen retrieval methods. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each. 	Up to 1 hour
<p>8. Primary antibody mix:</p> <p>Add Rat and Rabbit primary antibody</p> <p>Supplied by user</p>	<p>Note: Investigator needs to optimize dilution prior to triple staining.</p> <ol style="list-style-type: none"> Apply 2 drops or enough volume of the rat and rabbit primary antibody mixture to cover the tissue completely. Incubate in moist chamber for 30-60 min. Recommend 30 minutes to shorten total protocol time. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each. 	30 to 60 minutes
<p>9. Reagent 4:</p> <p>Rat Primer(RTU)</p>	<p>Note: This step is required for activation of RAT AP Polymer, DO NOT skip.</p> <ol style="list-style-type: none"> Apply 1 to 2 drops (50-100µL) of Reagent 4 (Rat Primer) to cover the tissue completely. Incubate in moist chamber for 10 min. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each. 	10 minutes
<p>10. Mix</p> <p>Reagent 5: Rat AP Polymer(RTU) with</p> <p>Reagent 6: Rabbit HRP Polymer(RTU)</p>	<p>Note: Mix Reagent 5 (Rat AP Polymer) with Reagent 6 (Rabbit HRP Polymer) at 1:1 ratio, do not mix more than you will need for experiment. This mixture is not stable long term for either polymer.</p> <ol style="list-style-type: none"> Apply 1 to 2 drops (50-100µL) of Polymer mixture to cover the tissue completely. Incubate slides in moist chamber for 30 min. Wash with 1xTBS-T only; 3 times for 2 minutes each. 	30 minutes
<p>11. Reagent 7A, 7B, 7C</p> <p>Reagent 7A: Permanent Red Substrate (RTU)</p>	<p>Note: Shake Permanent Red Activator before adding into Neo Permanent Red Substrate.</p> <ol style="list-style-type: none"> Add 200µL of Reagent 7B (Activator) into 1mL of Reagent 7A (Substrate) and mix well. Add 10µL of Reagent 7C (Chromogen) into the mixture and mix well. 	10min

<p>Reagent 7B: Permanent Red Activator (5x)</p> <p>Reagent 7C: Permanent Red Chromogen (100x)</p> <p>get maximum sensitivity of AP polymer, Please repeat chromogen step</p>	<p>[Note: For fewer slides, Add 100µL of Reagent 7B (Activator) into 500µL of Reagent 7A (Substrate) and mix well. Add 5µL of Reagent 7C (Chromogen) into the mixture and mix well.]</p> <p>b. Apply 2 drops (100µL) or enough volume of Permanent Red working solution to completely cover the tissue. Incubate for 10 min, observe appropriate color development. To increase AP signal aspirate or tap off chromogen and apply 2-3 (100µL) again of the Permanent Red working solution to completely cover the tissue for additional 5 to 10min.</p> <p>c. Rinse well with distilled water.</p>	
<p>12. Reagent 9A, 4B, 9C&9C</p> <p>9A: DAB-Ni Substrate (20x)</p> <p>4B: DAB Chromogen (20x)</p> <p>9B: Hydrogen Peroxide (20x)</p> <p>9C: Nickel Solution (7x)</p>	<p>a. Prepare 1mL of distilled water. Add 1 drop of Reagent 9A (DAB-Ni Substrate) into 1mL of distilled water. Mix well.</p> <p>b. Add 1 drop of Reagent 4B (DAB Chromogen) and 1 drop of concentrated Reagent 9B (Hydrogen Peroxide) to the diluted Reagent. Mix well.</p> <p>c. Add 3 drops of Reagent 9C (Nickel Solution) to the mixture. Mix well.</p> <p>d. Add about 100µL (2 drops) of DAB-Ni working solution to each slide and incubate in an enclosed chamber at room temperature for about 5 minutes. When appropriate color is developed, rinse under tap water gently for about 1-2 minutes.</p> <p>e. Use DAB-Ni working solution within 7 hours and store at 4°C keeping away from light during operation.</p>	5min
<p>13. HEMATOXYLIN Not provided</p>	<p>a. Counterstain with 2 drops (100µL) or enough volume of hematoxylin to completely cover tissue. Incubate for 10-15 seconds.</p> <p>b. Rinse thoroughly with tap water for 2-3min.</p> <p>c. Put slides in PBS until show blue color (about ½ - 1min.)</p> <p>d. Rinse well in distilled water</p>	10-15sec
<p>14. Reagent 10: NeoMount Universal (RTU)</p>	<p>a. Apply 2 drops (100µL) or enough volume of Reagent 10 (NeoMount Universal) to cover tissue when tissue is wet. Rotate the slides to allow NeoMount Universal spread evenly.</p> <p>b. Place slides horizontally in an oven at 40-50°C for at least 30 minutes or leave it at room temperature until slides are thoroughly dried.</p>	

TROUBLE SHOOT:

Problem	Tips
Uneven stain on 3 primary antibodies	1. Need to adjust the titer of each antibody. 2. The amount of each protein expressed on tissue may be different.
No stain on 1 or 2 antibodies	Missing steps or step reversed.

PROTOCOL NOTES:

1. The fixation, tissue slide thickness, antigen retrieval and primary antibody dilution and incubation time affect results significantly. Investigator needs to consider all factors and determine optimal conditions when interpreting the result.
2. Permanent Red is insoluble in organic solvent and can be coverslipped as well. however the dehydration steps must be shorter for optimal tissue structure and chromogen signal maintenance.

Note: Please wipe off extra water and air dry slides before dehydration and clear.

- a. 1x 80% Ethanol 20 seconds;
- b. 1x 95% Ethanol 20 seconds;
- c. 3x 100% Ethanol 20 seconds each;
- d. 1x 100% Xylene 20 seconds;
- e. Add 1 drop of xylene based NeoMount Perm (Cat. No. O-Mount, NB-23-00156) and coverslip.
Press to push the air bubble out.

CAUTION: DO NOT dehydrate in xylene longer than 20 seconds! It will erase Permanent Red stain!

PRECAUTIONS:

Please wear gloves, eye protection and take other necessary precautions. If any of the reagent come in contact with skin wash area completely with plenty of water and soap. If irritation develops seek medical attention.

FOR RESEARCH USE ONLY

We designed this work sheet to help you track of each step. We recommend you use this sheet to record the actual time of each step conducted as it will be helpful for questions with our technical support. To insure that all steps are done properly, we recommend that the user fill in the actual time of their experimental step and any variation.

Results will vary if time recommendations are not followed. RTU translates to ready to use.

- Used for tester to check “√” each step during the experiment
- Steps follow after de-paraffinization
- Refer to insert for details of each step

NB-23-00139 Protocol A is suitable when mouse, rabbit & rat primary antibodies need pre-treatment or mouse is sensitive to pre-treatment.

	Protocol Step	NB-23-00139 Protocol A	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	Step 1	Peroxidase or Alkaline Phosphatase Block NB-23-00193 is recommended. User supplied				
2	Step 2	HIER(Optional)				
3	Step 3 Optional	Mouse 1°Ab User supplied (30-60 min)				
4	Step 4 Optional	Reagent 1 Mouse HRP Polymer RTU (15 min)				
5	Step 5	Reagent 2A & Reagent 2B DAB requires mixing (5 min)				
6	Step 6	Reagent 3 Antibody Blocker requires mixing (10 min)				
7	Step 8	Rabbit 1°Ab & Rat 1°Ab (30-60 min)				
8	Step 9	Reagent 4 Rat Primer RTU (10 min)				
9	Step 10	Reagent 5 & Reagent 6 Rat AP Polymer & Rabbit HRP Polymer require mixing (30 min) Wash with 1xTBS-T only				

10	Step 11	Reagent 7A, Reagent 7B & Reagent 7C Neo-BioTech Permanent Red requires mixing. (10min)				
11	Step 12	Reagent 8A, 8B, 8C & 2B DAB-Ni requires mixing (5min)				
12	Step 13	Counter stain User supplied				
13	Step 14	Reagent 9 NeoMount Universal RTU				
17	Result	Stain pattern on controls are correct: Fill in Yes or NO				

Note: 1. Normal wash steps = Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.
Testing result:

NB-23-00139 Protocol-B is

suitable when mouse primary

antibody needs pretreatment or rabbit & rat primary antibodies are sensitive to pre-treatment.

	Protocol Step	Protocol Step	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	Step 1	Peroxidase or Alkaline Phosphatase Block NB-23-00193 is recommended. User supplied				
2	Step 8	Rabbit 1°Ab & Rat 1°Ab (30-60 min)				
3	Step 9	Reagent 4 Rat Primer RTU (10 min)				
4	Step 10	Reagent 5 & Reagent 6 Rat AP Polymer & Rabbit HRP Polymer require mixing (30 min)				
5	Step 5	Reagent 2A & Reagent 2B DAB requires mixing (5 min) Wash with 1xTBS-T only after rinse with distilled water.				
6	Step 11	Reagent 7A, Reagent 7B & Reagent 7C Neo BioTech-Permanent Red requires mixing. (10min)				
7	Step 7	HIER Supplied by user				
8	Step 3	Mouse 1°Ab (30-60 min)				
9	Step 4	Reagent 1 Mouse HRP Polymer RTU (15 min)				
10	Step 12	Reagent 8A,8B,8C&2B DAB-Ni requires mixing (5min)				
11	Step 13	Counter stain User supplied				
12	Step 14	Reagent 9 NeoMount Universal RTU				
3	Result	Stain pattern on controls are correct: Fill in Yes or NO				

Note: 1.Normal wash steps = Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.
Testing result:

antibodies need pretreatment or mouse primary antibody is sensitive to pretreatment.

	Protocol Step	Protocol C	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	Step 1	Peroxidase or Alkaline Phosphatase Block NB-23-00193 is recommended. User supplied				
2	Step 3	Mouse 1°Ab User supplied (30-60 min)				
3	Step 4	Reagent 1 Mouse HRP Polymer RTU (15 min)				
4	Step 5	Reagent 2A & Reagent 2B DAB requires mixing (5 min)				
5	Step 7	HIER Supplied by user				
6	Step 8	Rabbit 1°Ab & Rat 1°Ab (30-60 min)				
7	Step 9	Reagent 4 Rat Primer RTU (10 min)				
8	Step 10	Reagent 5 & Reagent 6 Rat AP Polymer & Rabbit HRP Polymer require mixing (30 min) Wash with 1xTBS-T only				
9	Step 11	Reagent 7A, Reagent 7B & Reagent 7C NeoBioTech-Permanent Red requires mixing. (10min)				
10	Step 12	Reagent 8A,8B,8C&2B DAB-Ni requires mixing (5min)				
11	Step 13	Counter stain User supplied				
12	Step 14	Reagent 9 NeoMount Universal RTU				
3	Result	Stain pattern on controls are correct: Fill in Yes or NO				

Note: 1.Normal wash steps = Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.

Testing result: