



PolyStain TS Kit - for 2 Rabbit and 1 Mouse antibody on Human tissue

(DAB/Permanent Red/Ni-DAB)

NB-23-00131-1 (24ml)

NB-23-00131-2 (72ml)

NB-23-00131-3 (240ml)



PolyStain TS Kit - for 2 Rabbit and 1 Mouse antibody on Human tissue

NB-23-00131-1; NB-23-00131-2; NB-23-00131-3

Storage: 2-8°C

INTENDED USE:

The PolyStain TS-MRR-Hu Kit is designed to use with user supplied one mouse primary antibody and two rabbit primary antibodies to detect three distinct antigens on human tissue or cell samples. This kit has been tested on paraffin embedded tissue specimens, it also can be used for frozen tissue or cell smears. Please read through entire protocol as this protocol requires many step to be done in their defined order. Triple staining uses traditional and non-traditional methods in immunohistostaining to reveal three distinct antigens and their co-expression on a single tissue. PolyStain TS-MRR-Hu Kit from NeoBiotech Labs supplies polymer enzyme conjugates: anti-mouse HRP Polymer, anti-rabbit AP Polymer, and anti-rabbit HRP Polymer with three chromogen, DAB (brown); Permanent Red (red); and DAB-Ni (Black). PolyStain TS-MRR-Hu Kit is a non-biotin system, avoiding non-specific binding caused by endogenous biotin. This kit has been optimized to have no cross detection when detecting two primary antibodies from the same host species using our unique blocking system. Simplified steps allow users to complete triple staining within 5 hours (without antigen retrieval) or 6 hours (with antigen retrieval). The well tested protocol provides user with the ability to permanently mount slides with coverslip.

KIT COMPONENTS

Component No.	Content	24mL	72mL	240mL
Reagent 1	Mouse HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 2	Rabbit AP Polymer (RTU)	6mL	18mL	60mL
Reagent 3A	DAB Substrate (RTU)	15mL	18mLx2	120mL
Reagent 3B	DAB Chromogen (20x)	2mL	4mL	12mL
Reagent 4A	Permanent Red Substrate (RTU)	15mL	18mLx2	120mL
Reagent 4B	Permanent Red Activator (5x)	3mL	7.2mL	12mLx2
Reagent 4C	Permanent Red Chromogen (100x)	150µL	360µL	1.2mL
Reagent 5	Antibody Blocker (40x)	15mLx2	50mL	100mL
Reagent 6A	TS-MRR Blocker A (RTU)	12mL	18mLx2	120mL
Reagent 6B	TS-MRR Blocker B (RTU)	12mL	18mLx2	120mL
Reagent 7	Rabbit HRP Polymer (RTU)	12mL	18mLx2	120mL
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 (RTU)	15mL	18mLx2	120mL

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

RECOMMENDED PROTOCOL:

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. **Important:** Never combine two antibodies from the same host species in one incubation step. Incubate 1st primary mouse antibody with rabbit antibody.
8. 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.
9. 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. NeoBiotech sells 10xTBS-T for your convenience (**NB-23-00201**)

Equipment 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
4. Timer
5. Beaker
6. Wash buffer: 0.01 M PBS with 0.5% Tween20, pH7.4
7. Peroxidase and alkaline phosphatase blocking buffer
8. 100% ethanol
9. 100% Xylene
10. Hematoxylin
11. Coverslip

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.
- You may encounter conditions that 1st mouse antibody and one rabbit antibody need HIER and the 3rd protein detected by 2nd rabbit antibody is heat sensitive. In this situation you may download our triple color staining protocol from our web site.
- 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 2 rabbit antibodies and one mouse antibody requires HIER.

Reagent	Staining Procedure	Incubation Time (Min.)
<p>1. Peroxidase and Alkaline Phosphatase Blocking Reagent Not provided Fast, easy and it will block endogenous alkaline phosphatase</p>	<p>a. Incubate slides in PEROXIDASE BLOCKING REAGENT we recommend NeoPure Dual Enzyme Block NB-23-00193 b. Rinse the slide using distilled water.</p>	10 min.
<p>2. Antigen retrieval (optional): Refer to primary antibody data sheet.</p>	<p>Note: Investigator needs to do antigen retrieval only one time during protocol see staining protocol. a. Refer to primary antibody data sheet for antigen retrieval methods. b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T (See note 9 above); 3 times for 2 minutes each.</p>	
<p>3. Primary Antibody Mix: one Mouse and one Rabbit primary antibody Supplied by user.</p>	<p>Note: Investigator needs to optimize dilution prior to triple staining. DO NOT combine the same host species primary antibodies together at this step. a. Apply 2 drops or enough volume of mouse and rabbit primary antibody mixture to cover the tissue completely. Incubate in moist chamber for 30-60min. Recommend 30min to shorten total protocol time. b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.</p>	30 min.

<p>4. Mix Reagent 1: Mouse HRP Polymer (RTU) with Reagent 2: Rabbit AP Polymer (RTU)</p>	<p>Note: Make sufficient polymer mixture by adding Reagent 1 (Mouse HRP Polymer) and Reagent 2 (Rabbit AP Polymer) at 1:1 ratio, mix well. Do not mix more than you need for the experiment because the polymer mixture may not be as stable as non-mixed polymer.</p> <ol style="list-style-type: none"> Apply 1 to 2 drops (50-100µL) of the mixture to cover the tissue completely. Incubate in moist chamber for 30 min. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each. 	<p>30 min</p>
<p>5. Reagent 3A&3B 3A: DAB Substrate(RTU) 3B: DAB Chromogen (20x)</p>	<p>Note: Make enough DAB mix by adding 1 drop of Reagent 3B (DAB Chromogen) in 1mL of Reagent 3A (DAB Substrate). Mix well. Use within 7 hours store at 4°C.</p> <ol style="list-style-type: none"> Apply 1 to 2 drops (50-100µL) of your DAB mixture to cover the tissue completely. Incubate for 5min. Rinse thoroughly with distilled water. Wash with 1xTBS-T only, 3 times for 2 minutes each. 	<p>5 min.</p>
<p>6. Reagent 4A, 4B, 4C Reagent 4A: Permanent Red Substrate (RTU) Reagent 4B: Permanent Red Activator (5x) Reagent 4C: Permanent Red Chromogen (100x) To get maximum sensitivity of AP polymer, Please repeat chromogen step</p>	<p>Note: Shake Permanent Red Activator before adding into Permanent Red Substrate.</p> <ol style="list-style-type: none"> Add 200µL of Reagent 4B (Activator) into 1mL of Reagent 4A (Substrate) and mix well. Add 10µL of Reagent 4C (Chromogen) into the mixture and mix well. [Note: For fewer slides, Add 100µL of Reagent 4B (Activator) into 500µL of Reagent 4A (Substrate) and mix well. Add 5µL of Reagent 4C (Chromogen) into the mixture and mix well.] 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 drops (100µL) again of the Permanent Red working solution to completely cover the tissue for additional 5 to 10min Rinse well with distilled water. 	<p>10 min</p>
<p>7. Reagent 5 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> <ol style="list-style-type: none"> Use hot plate or water bath to heat diluted Reagent 5 (Antibody Blocker) to 1x solution (1 part of Antibody Blocker in 39 parts of 	<p>10 min.</p>

	<p>distilled water) to 80°C. Make enough volume to cover the tissue in beaker.</p> <p>b. Put slides in heated Antibody Blocker for 10 minutes at 80°C.</p> <p>c. Remove slides from the Antibody blocker; cool slides 5 seconds.</p> <p>d. Rinse slides in multiple changes of distilled water.</p> <p>e. If antigen retrieval step is required go directly to step 8 if not complete step 7e and move on to step 9.</p> <p>f. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.</p>	
<p>8. Antigen retrieval: Refer to primary antibody data sheet.</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; 3 times for 2 minutes each.</p>	UP to 1 hour
<p>9. Reagent 6A TS-MRR Blocker A (RTU)</p>	<p>a. Apply 2 drops or enough volume of Reagent 6A (DS-MRR Blocker A) to cover the tissue completely. Mix well on the slide and incubate in moist chamber for 30 min.</p> <p>b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.</p>	30 min
<p>10. Reagent 6B TS-MRR Blocker B (RTU)</p>	<p>a. Apply 2 drops or enough volume of Reagent 6B (DS-MRR Blocker B) to cover the tissue completely. Mix well on the slide and Incubate in moist chamber for 5 min.</p> <p>b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.</p>	5 min.
<p>11. 2nd rabbit primary antibody 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 the 2nd rabbit primary antibody to cover the tissue completely. Incubate in moist chamber for 30-60 min. Recommend 30 minutes 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 min
<p>12. Reagent 7 Rabbit HRP Polymer (RTU)</p>	<p>a. Apply 1 to 2 drops (50-100µL) of Reagent 7 (Rabbit HRP Polymer) to cover the tissue completely. Incubate slides in moist chamber for 15 min.</p> <p>b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.</p>	15 min
<p>13. Reagent 8A, 3B, 8C&8C 8A: DAB-Ni Substrate (20x) 3B: DAB Chromogen (20x)</p>	<p>a. Prepare 1mL of distilled water. Add 1 drop of Reagent 8A (DAB-Ni Substrate) into 1mL of distilled water. Mix well.</p> <p>b. Add 1 drop of Reagent 3B (DAB Chromogen) and 1 drop of concentrated Reagent 8B (Hydrogen Peroxide) to the diluted Reagent. Mix well.</p> <p>c. Add 3 drops of Reagent 8C (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</p>	5 min.

<p>8B: Hydrogen Peroxide (20x)</p> <p>8C: Nickel Solution (7x)</p>	<p>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>	
<p>14. 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>	<p>10 – 15 sec</p>
<p>15. Reagent 9: NeoMount Universal (RTU)</p>	<p>a. Apply 2 drops (100µL) or enough volume of Reagent 9 (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>	<p>Refer to insert</p>

PROBLEM	TIPS
Uneven stain on 3 primary antibodies	<ol style="list-style-type: none"> 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 mountant (Cat. No. NeoMount Perm 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!

PRECAUTION:

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.

Work Sheet for NB-23-00131 Kit

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-23-00131 Protocol-1 is suitable when all primary antibodies need pre-treatment or all primary antibodies do not need pre-treatment.

	Main Protocol Step	NB-23-00131 Protocol-1	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	Step 1	Peroxidase and Alkaline Phosphatase Block NB-23-00193 is recommended. User supplied				
2	Step 2	HIER(Optional) Refer to antibody datasheet User supplied				
3	Step 3	Mouse 1°Ab &Rabbit 1°Ab mix User supplied (30-60min)				
4	Step 4	Reagent 1&Reagent 2 Mouse HRP Polymer & Rabbit AP Polymer require mixing (30min)				
5	Step 5	Reagent 3A& Reagent 3B DAB requires mixing. (5min)				

		Wash with 1x TBS-T after rinse with distilled water.				
6	Step 6	Reagent 4A & Reagent 4B Permanent Red requires mixing.(10min)				
7	Step 7	Reagent 5 Antibody Blocker requires mixing. (10min)				
8	Step 9	Reagent 6A DS-MRR Blocker A RTU (30min)				
9	Step 10	Reagent 6B DS-MRR Blocker B RTU (5min)				
10	Step 11	Rabbit 1°Ab User supplied (30-60 min)				
11	Step 12	Reagent 7 Rabbit HRP Polymer RTU (15 min)				
12	Step 13	Reagent 8A, 3B, 8B & 8C DAB-Ni requires mixing. (5min)				
13	Step 14	Counter stain User supplied				
14	Step 15	Reagent 9 NeoMount Universal RTU				
	Result	Stain pattern on controls are correct: Fill in Yes or NO				

Note: 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-23-00131 Protocol-2 is suitable when one Mouse & one Rabbit primary antibodies need pre-treatment, but the second Rabbit primary antibodies is sensitive to pre-treatment.

	Main Protocol Step	NB-23-00131 Protocol-2	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 11	Rabbit 1°Ab (sensitive to HIER) User supplied (30-60min)				
3	Step 12	Reagent 7 (RTU) Rabbit HRP Polymer RTU (15min)				
4	Step 5	Reagent 3A&3B DAB requires mixing (5 min)				
5	Step 7	Reagent 5 Antibody Blocker requires mixing (10min)				
6	Step 2	HIER Refer to antibody datasheet (DAB will not be removed) User supplied				
7	Step 9	Reagent 6A (RTU) DS-MRR Blocker A RTU (30min)				
8	Step 10	Reagent 6B (RTU) DS-MRR Blocker B RTU (5min)				
9	Step 3	Mouse 1°Ab & Rabbit 1°Ab mix (Abs requires HIER) User supplied (30-60 min)				
10	Step 4	Reagent 1&Reagent 2 Mouse HRP Polymer & Rabbit AP Polymer require mixing (30min) Wash with 1x TBS-T only.				
11	Step 6	Reagent 4A & Reagent 4B Permanent Red requires mixing. (10min)				

12	Step 13	Reagent 8A, 3B, 8B & 8C DAB-Ni requires mixing. (5min)				
13	Step 14	Counter stain User supplied				
14	Step 15	Reagent 9 NeoMount Universal RTU				
	Result	Stain pattern on controls are correct: Fill in Yes or NO				

Note: 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-23-00131 Protocol-3 is suitable when one Mouse & one Rabbit primary antibodies are sensitive to pre-treatment but the second Rabbit primary antibody needs pre-treatment.

	Main Protocol Step	NB-23-00131 Protocol-3	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 & Rabbit 1°Ab mix User supplied (30-60min.)				
3	Step 4	Reagent 1&Reagent 2 Mouse HRP Polymer & Rabbit AP Polymer require mixing. (30min)				
4	Step 5	Reagent 3A&Reagent 3B DAB require mixing. (5min) Wash with 1x TBS-T after rinse with distilled water.				
5	Step 6	Reagent 4A&Reagent 4B Permanent Red requires mixing. (10min)				
6	Step 7	Reagent 5 Antibody Blocker required mixing. (10min)				
7	Step 8	HIER Refer to antibody datasheet. User supplied				
8	Step 9	Reagent 6A DS-MRR Blocker A RTU (30min)				
9	Step 10	Reagent 6B DS-MRR Blocker B RTU (5min)				

10	Step 11	Rabbit 1°Ab (sensitive to HIER) User supplied (30-6min.)				
11	Step 12	Reagent 7 Rabbit HRP Polymer (RTU) (15min.)				
12	Step 13	Reagent 8A, 3B, 8B &8C DAB-Ni requires mixing. (5min)				
13	Step 14	Counter stain User supplied				
14	Step 15	Reagent 9 NeoMount Universal RTU				
	Result	Stain pattern on controls are correct: Fill in Yes or NO				

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

Testing result:

