



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

(DAB/Permanent Red/Emerald)

NB-23-00128- 3(240 ml)

NB-23-00128- 2(72 ml)

NB-23-00128- 1(24 ml)

PolyStain TS Kit - for 2 Mouse and 1 Rabbit antibody on Human tissue (DAB/Permanent Red/Emerald)

NB-23-00128-1; NB-23-00128-2; NB-23-00128-3

Storage: 2-8°C

INTENDED USE:

The PolyStain TS Kit is designed to use with user supplied two mouse primary antibodies and one rabbit primary antibody to detect three distinct antigens on a single human tissue or cell samples. TS-MMR-Hu has been tested on paraffin embedded tissue only; however it may be used on frozen or freshly prepared monolayer cell smears.

Please read through entire protocol as this protocol requires many steps that needs to be done in their defined order.

Choose from protocol NB-23-00128 protocol-1, NB-23-00128 protocol-2 and NB-23-00128 protocol-3 to accommodate your primary antibodies sensitivity to pre-treatment. 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 Kit from NeoBiotech Labs supplies polymer enzyme conjugates: Polymer-HRP anti-rabbit, Polymer-AP anti-mouse and Polymer-HRP anti-mouse with three chromogens, DAB (brown); Permanent Red (red); and Emerald (green).

PolyStain TS 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 Kit	72mL Kit	240mL Kit
Reagent 1	Rabbit HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 2	Mouse AP Polymer (RTU)	6mL	18mL	60mL
Reagent 3A	DAB Substrate (RTU)	15mL	18mL x 2	120mL
Reagent 3B	DAB Chromogen (20x)	1.5mL	2mL	6mL
Reagent 4A	Permanent Red Substrate (RTU)	15mL	18mL x 2	120mL
Reagent 4B	Permanent Red Activator (5x)	3mL	7.2mL	12mL x 2
Reagent 4C	Permanent Red Chromogen (100x)	150µl	360µl	1.2mL
Reagent 5	Antibody Blocker (40x)	15mL x 2	50mL	100mL
Reagent 6A	TS-MMR Blocker A (RTU)	12mL	18mL x 2	120mL
Reagent 6B	TS-MMR Blocker B (RTU)	12mL	18mL x 2	120mL
Reagent 7	Mouse HRP Polymer (RTU)	12mL	18mL x 2	120mL
Reagent 8	Emerald Chromogen (RTU)	15mL	18mL x 2	120mL
Reagent 9	U-Mount (RTU)	3mL	9mL	NA

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

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. 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 inhibitor the activity of the alkaline phosphatase.

Note: 1X TBS-T =50mM Tris HCl, 150mM NaCl, 0.05% Tween-20 pH7.6.

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, Beaker
5. Wash buffer: 0.01 M PBS with 0.5% Tween20, pH7.4
6. Peroxidase and alkaline phosphatase blocking buffer
7. 100% ethanol
8. 100% Xylene
9. Hematoxylin
10. 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.
- NB-23-00128 Protocol-2 worksheet is suitable for one Mouse & one Rabbit primary Abs need pre-treatment, the other Mouse primary Ab is sensitive to pre-treatment.
- NB-23-00128 Protocol-3 worksheet is suitable when one Mouse & one primary antibody is sensitive to pre-treatment but the second Mouse primary antibody needs pre-treatment.
- 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 mouse antibodies and one rabbit antibody requires HIER.

Staining protocol NB-23-00128 protocol-1:

Reagent	Staining Procedure	Incubation Time (Min.)
<p>1. Peroxidase and phosphatase Blocking Reagent</p> <p>Supplied by user</p>	<p>a. Incubate slides in peroxidase and alkaline phosphatase blocking reagent (NeoPure Dual Enzyme Block NB-23-00193 is Recommended) for 10 minutes.</p> <p>b. Rinse the slides using 2 changes of distilled water.</p>	10 min.
<p>2. Antigen retrieval (optional):</p> <p>Refer to primary antibody data sheet</p>	<p>Note: Investigator needs to do antigen retrieval only one time during protocol see staining protocol.</p> <p>a. Refer to primary antibody data sheet for antigen retrieval methods.</p> <p>b. Wash with PBS/ 0.05% Tween20 or 1xTBS-T (See note 9 above) 3 times for 2 minutes each</p>	
<p>3. Primary Antibody Mix: Mix one Mouse and one Rabbit primary antibody</p> <p>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.</p> <p>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.</p> <p>b. Wash with PBS/ 0.05% Tween20 or 1xTBS-T 3 times for 2 minutes each.</p>	30 min
<p>4. Mix Reagent 1: Rabbit HRP Polymer (RTU) with</p> <p>Reagent 2: Mouse AP Polymer (RTU)</p>	<p>Note: Make sufficient polymer mixture by adding Reagent 1 (Rabbit HRP Polymer) and Reagent 2 (Mouse 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 no mixed polymer.</p> <p>a. Apply 1 to 2 drops (50-100µL) of the mixture to cover the tissue completely.</p> <p>b. Incubate in moist chamber for 30 min.</p> <p>c. Wash with PBS/ 0.05% Tween20 or 1xTBS-T 3 times for 2 minutes each</p>	30 min
<p>5. Reagent 3A&3B</p> <p>3A: DAB Substrate(RTU)</p> <p>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> <p>a. Apply 1 to 2 drops (50-100µL) of your DAB mixture to cover the tissue completely.</p> <p>b. Incubate for 5min.</p> <p>c. Rinse slides in multiple changes of distilled water 3 times for 2min each time or under running tap water for 2minute</p>	5 min

<p>6. Reagent 4A, 4B, 4C</p> <p>Reagent 4A: Permanent Red Substrate (RTU)</p> <p>Reagent 4B: Permanent Red Activator (5x)</p> <p>Reagent 4C: Permanent Red Chromogen (100x)</p>	<p>a. Wash with only 1xTBS-T 3 times for 2 minutes each.</p> <p>b. 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.]</p> <p>c. 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.</p> <p>d. Rinse well with distilled water.</p>	<p>10 min</p>
<p>7. Reagent 5</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> <p>a. Use hot plate or water bath to heat diluted Reagent 5 (Antibody Blocker) 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.</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. If antigen retrieval step is required go directly to step 8 if not complete step 7e and move on to step 9.</p> <p>e. Wash with PBS/ 0.05% Tween20 or 1xTBS-T 3 times for 2 minutes each</p>	<p>10 min</p>
<p>8. Antigen retrieval:</p> <p>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/ 0.05% Tween20 or 1xTBS-T 3 times for 2 minutes each.</p>	<p>UP to 1h</p>
<p>9. Reagent 6A</p> <p>TS-MMR Blocker A (RTU)</p>	<p>a. Apply 2 drops or enough volume of Reagent 6A (DS-MMR 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/ 0.05% Tween20 or 1xTBS-T 3 times for 2 minutes each.</p>	<p>30 min.</p>

<p>10. Reagent 6B</p> <p>TS-MMR Blocker B (RTU)</p>	<p>a. Apply 2 drops or enough volume of Reagent 6B (DS-MMR 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/ 0.05% Tween20 or 1xTBS-T 3 times for 2 minutes each.</p>	<p>5 min.</p>
<p>11. 2nd Mouse primary antibody</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 the 2nd mouse 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/ 0.05% Tween20 or 1xTBS-T 3 times for 2 minutes each.</p>	<p>30 min.</p>
<p>12. Reagent 7</p> <p>Mouse HRP Polymer (RTU)</p>	<p>a. Apply 1 to 2 drops (50-100µL) of Reagent 7 (Mouse HRP Polymer) to cover the tissue completely. Incubate slides in moist chamber for 15 min.</p> <p>b. Wash with PBS/ 0.05% Tween20 or 1xTBS-T 3 times for 2 minutes each.</p>	<p>15 min.</p>
<p>13. Counterstain</p> <p>(Optional but must be done before Emerald Chromogen step)</p> <p>Not provided</p>	<p>Note: If two antigens are co-localized in the nucleuse you want less counter stain to optimize the visualization in the nucleus; however you can counter stain using normal protocol time if antigens are co-localized in cytoplasm or membrane or the three antigens are localized in different cells.</p> <p>a. Counterstain dip in diluted hematoxylin for 5 seconds for nuclear colocalization or 30 seconds for cytoplasmic or membrane co-localization. DO NOT over stain with hematoxylin.</p> <p>b. Rinse thoroughly with tap water for 1min.</p> <p>c. Put slides in PBS for 5-10 seconds to blue, DO NOT over blue.</p> <p>d. Rinse well in distilled or tap water for 1min.</p> <p>e. Wash with PBS/ 0.05% Tween20 or 1xTBS-T 3 times for 2 minutes each.</p>	<p>5 sec</p>
<p>14. Reagent 8</p> <p>Emerald Chromogen (RTU)</p> <p>Do hematoxylin first.</p>	<p>a. Apply 1 to 2 drops (50-100µL) of Reagent 8 (Emerald Chromogen) to cover the tissue completely.</p> <p>b. Incubate slides in humid chamber for 5 minutes.</p> <p>c. Wash slides in tap water for 1 minute.</p> <p>d. Rinse with distilled water.</p> <p>Important to READ: Emerald Chromogen is water soluble, counter stain first. Do not leave slides sitting in water. Always stain Emerald chromogen AFTER Permanent Red stain and hematoxylin. Because Permanent Red removes the Emerald.</p>	<p>5 min</p>

<p>15. Dehydrate section</p> <p>It is important to follow the protocol.</p>	<p>Note: Please wipe off extra water and air dry slides before dehydration and clear.</p> <ol style="list-style-type: none"> Dehydrate with 85% ethanol 20seconds. Dehydrate with 95% ethanol 20seconds. Dehydrate with 100% ethanol 20seconds. Dehydrate with 100% ethanol 20seconds. Dehydrate with 100% ethanol 20seconds. Dehydrate with xylene 20seconds. <p>CAUTION: DO NOT dehydrate in xylene longer than 20 seconds! It will erase Permanent Red stain!</p>	<p>2 min.</p>
<p>16. Reagent 9</p> <p>U-Mount (RTU)</p>	<ol style="list-style-type: none"> Apply 1 drop (50µL) of Reagent 9 (U-Mount) to cover the tissue section and apply glass coverslip. Apply force to coverslip to squeeze out any extra mountant and bubbles for optimal clarity. Removing excess also to prevent leaching of Permanent Red stain. 	

TROUBLE SHOOTING:

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. 3. Set slides in water too long so that Emerald is washed away. 4. Set slides in Xylene too long so that Permanent Red is washed away.
Emerald Chromogen is blue not green when non co-localized with Permanent Red.	Emerald should be green when non colocalized with Permanent Red. If Emerald chromogen is blue the titer on the primary antibody is not dilute enough for the protocol. Re-titer primary antibodies individually first
No stain on 1 or 2 antibodies	Missing steps or step reversed.
Green Background on the slide	Titer primary antibody.
Permanent Red is leaching	<ol style="list-style-type: none"> 1. Use fresh 100% ethanol and xylene. 2. Slide sat too long in xylene. Do not go over 20seconds!
Artifacts on slides	Slides not completely dried before mount. Use fresh 100% Ethanol and xylene.

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

Work Sheet for NB-23-00128 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-23-00128 Protocol-1 is suitable when all primary antibodies need pre-treatment or all primary antibodies do not need pre-treatment.

Protocol Step	NB-23-00128 Protocol-1	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase or Alkaline Phosphatase Block NB-23-00193 is recommended. supplied				
Step 2	HIER(Optional)				
Step 3	Mouse 1°Ab & Rabbit 1°Ab mix User supplied (30-60min)				
Step 4 Optional	Reagent 1&Reagent 2 Rabbit HRP Polymer & Mouse AP Polymer require mixing (30min)				
Step 5	Reagent 3A& Reagent 3B DAB requires mixing. (5min)				
Step 6	Reagent 4A, Reagent 4B& Reagent 4C Permanent Red requires mixing. (10min)				

Step 7	Reagent 5 Antibody Blocker requires mixing. (10min)				
Step 9	Reagent 6A DS-MMR Blocker A RTU (30min)				
Step 10	Reagent 6B DS-MMR Blocker B RTU (5min)				
Step 11	Mouse 1°Ab User supplied (30-60 min)				
Step 12	Reagent 7 Mouse HRP Polymer RTU (15 min)				
Step 13	Counter stain(Note 2) User supplied (5-10 sec)				
Step 14	Reagent 8 Emerald Chromogen RTU (5min)				
Step 15	It is important to follow the protocol. To maintain stain! Dehydrate section 20seconds for each step				
Step 16	Reagent 9 U-Mount RTU Mount & coverslip				
Result	Stain pattern on controls are correct: Fill in Yes or NO				

Note:

1. *Normal wash steps = Wash with PBS containing 0.05% Tween-20 or 1x TBS-T for 3 times for 2 min each.

2. *Using as a co-localization staining kit,

If antigens are co-localized in nucleus counter stain and blue should be for 5 seconds to blue.

If antigens are co-localized in cytoplasm and membrane or in different cells counter stain using normal protocol time.

Testing result:

NB-23-00128 Protocol-2 is suitable when one Mouse & one Rabbit primary antibodies need pre-treatment, but the second Mouse primary antibodies is sensitive to pre-treatment.

Protocol Step	NB-23-00128 Protocol-2	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase or Alkaline Phosphatase Block NB-23-00193 is recommended. supplied				
Step 11	Mouse 1°Ab (sensitive to HIER) User supplied (30-60min)				
Step 12	Reagent 7 (RTU) Mouse HRP Polymer RTU (15min)				
Step 5	Reagent 3A&3B DAB requires mixing (5 min)				
Step 7	Reagent 5 Antibody Blocker requires mixing (10min)				
Step 2	HIER (DAB will not be removed)				
Step 9	Reagent 6A (RTU) DS-MMR Blocker A RTU (30min)				
Step 10	Reagent 6B (RTU) DS-MMR Blocker B RTU (5min)				
Step 3	Mouse 1°Ab & Rabbit 1°Ab mix (Abs requires HIER) User supplied (30-60 min)				

Step 4	Reagent 1&Reagent 2 Rabbit HRP Polymer & Mouse AP Polymer require mixing (30min) Wash with 1x TBS-T				
Step 6	Reagent 4A, Reagent 4B& Reagent 4C Permanent Red requires mixing. (10min)				
Step 13	Counter stain(Note 2) User supplied (5-10 sec)				
Step 14	Reagent 8 Emerald Chromogen RTU (5min)				
Step 15	It is important to follow the protocol. To maintain stain! Dehydrate section 20seconds for each step				
Step 16	Reagent 9 U-Mount RTU Mount & coverslip				
Result	Stain pattern on controls are correct: Fill in Yes or NO				

Note1: Normal wash steps = Wash with PBS containing 0.05% Tween-20 for 3 times for 2 min each.

Note2: *Using as a co-localization staining kit,

If antigens are co-localized in nucleus counter stain and blue should be for 5 seconds to blue.

If antigens are co-localized in cytoplasm and membrane or in different cells counter stain using normal protocol time.

Testing result:

NB-23-00128 Protocol-3 is suitable when one Mouse & one Rabbit primary antibodies are sensitive to pre-treatment but the second Mouse primary antibody needs pre-treatment.

Protocol Step	NB-23-00128 Protocol-3	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase or Alkaline Phosphatase Block NB-23-00193 is recommended. supplied				
Step 3	Mouse 1°Ab & Rabbit 1°Ab mix User supplied (30-60min.)				
Step 4	Reagent 1&Reagent 2 Rabbit HRP Polymer & Mouse AP Polymer require mixing. (30min)				
Step 5	Reagent 3A&Reagent 3B DAB require mixing. (5min)				
Step 6	Reagent 4A, Reagent 4B& Reagent 4C Permanent Red requires mixing. (10min)				
Step 7	Reagent 5 Antibody Blocker required mixing. (10min)				
Step 8	HIER Refer to antibody datasheet				
Step 9	Reagent 6A DS-MMR Blocker A RTU (30min)				
Step 10	Reagent 6B DS-MMR Blocker B RTU (5min)				

Step 11	Mouse 1°Ab (Not sensitive to HIER) User supplied (30-60min.)				
Step 12	Reagent 7 Mouse HRP Polymer (RTU) (15min.)				
Step 13	Counter stain(Note 2) User supplied (5-10 sec)				
Step 14	Reagent 8 Emerald Chromogen RTU (5min)				
Step 15	It is important to follow the protocol. To maintain stain! Dehydrate section 20seconds for each step				
Step 16	Reagent 9 U-Mount RTU Mount & coverslip				
Result	Stain pattern on controls are correct: Fill in Yes or NO				

Note1: Normal wash steps = Wash with PBS containing 0.05% Tween-20 for 3 times for 2 min each.

Note2: *Using as a co-localization staining kit,

If antigens are co-localized in nuclear counter stain and blue should be for 5 seconds to blue.

If antigens are co-localized in cytoplasm and membrane or in different cells counter stain using normal protocol time.

Testing result: