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

(DAB/Permanent Red/Ni-DAB)

**NB-23-00129- 3(240 ml)**

**NB-23-00129- 2(72 ml)**

**NB-23-00129- 1(24 ml)**

**Neo-Biotech**
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NB-23-00129-1; NB-23-00129-2; NB-23-00129-3

**Storage: 2-8ºC**

## 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. Tissue specimens are paraffin embedded; or freshly prepared monolayer cell smears.

Please read through entire protocol as this protocol requires many step that needs 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 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 color); Permanent Red (red color); and DAB-Ni (black color).

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 more than 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 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 | 18mL x 2 | 120mL |

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
5. Beaker
6. Wash buffer: 0.01 M PBS with 0.5% Tween20, pH7.4
7. **1X TBS-T** =50mM Tris HCl, 150mM NaCl, 0.05% Tween-20 pH7.6.
8. Peroxidase and alkaline phosphatase blocking buffer
9. 100% ethanol
10. 100% Xylene
11. 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.
- You may encounter conditions that 1st mouse antibody and one rabbit antibody need HIER and the 3rd protein detected by 2nd mouse 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 mouse antibodies and one rabbit antibody requires HIER.

**Staining protocol NB-23-00129 protocol-1:**

| Reagent | Staining Procedure | Incubation Time (Min.) |
|---|---|---|
| **1.** Peroxidase and phosphatase Blocking Reagent<br><br>Supplied by user | a. Incubate slides in peroxidase and alkaline phosphatase blocking reagent (NeoPure Dual Enzyme Block NB-23-00193 is Recommended) for 10 minutes.<br>b. Rinse the slides using 2 changes of distilled water. | 10 min. |
| **2.** Antigen retrieval **(optional):**<br><br>Refer to primary antibody data sheet | **Note**: Investigator needs to do antigen retrieval only one time during protocol see staining protocol.<br>a. Refer to primary antibody data sheet for antigen retrieval methods.<br>b. Wash with PBS/ 0.05% Tween20 or **1xTBS-T** (See note 9 above) 3 times for 2 minutes each | |
| **3.** Primary Antibody Mix**: Mix one Mouse and one Rabbit primary antibody**<br><br>Supplied by user. | **Note**: Investigator needs to optimize dilution prior to triple staining. **DO NOT** combine the same host species primary antibodies together at this step.<br>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.<br>b. Wash with PBS/ 0.05% Tween20 or **1xTBS-T** 3 times for 2 minutes each. | 30 min |
| **4.** Mix<br><br>**Reagent 1:** Rabbit HRP Polymer (RTU) with<br><br>**Reagent 2:** Mouse AP Polymer (RTU) | **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.<br>a. Apply 1 to 2 drops (50-100µL) of the mixture to cover the tissue completely.<br>b. Incubate in moist chamber for 30 min.<br>**c.** Wash with PBS/ 0.05% Tween20 or **1xTBS-T** 3 times for 2 minutes each | 30 min |
| **5. Reagent 3A&3B**<br><br>**3A:** DAB Substrate(RTU)<br>**3B:** DAB Chromogen (20x) | **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.<br>a. Apply 1 to 2 drops (50-100µL) of your DAB mixture to cover the tissue completely.<br>b. Incubate for 5min.<br>c. Rinse slides in multiple changes of distilled water 3 times for 2min each time or under running tap water for 2minute | 5 min |

| | | |
|---|---|---|
| **6. Reagent 4A, 4B, 4C**<br><br>**Reagent 4A:**<br>Permanent Red Substrate (RTU)<br><br>**Reagent 4B:**<br>Permanent Red Activator (5x)<br><br>**Reagent 4C:**<br>Permanent Red Chromogen (100x) | a. Wash with only 1xTBS-T 3 times for 2 minutes each.<br>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.]<br>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.<br>d. Rinse well with distilled water. | 10 min |
| **7. Reagent 5**<br><br>Antibody Blocker (40x) | **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 65ºC must be used during the Antibody Blocker step to prevent dissociation of the tissue from the slide.<br>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.<br>b. Put slides in heated Antibody Blocker for 10 minutes at 80°C.<br>c. Remove slides from the Antibody blocker; cool slides 5 seconds.<br>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.**<br>e. Wash with PBS/ 0.05% Tween20 or **1xTBS-T** 3 times for 2 minutes each | 10 min |
| **8.** Antigen retrieval:<br><br>**Refer to primary antibody data sheet.** | a. Refer to primary antibody data sheet for antigen retrieval methods.<br>b. Wash with PBS/ 0.05% Tween20 or 1xTBS-T 3 times for 2 minutes each. | UP to 1h |
| **9. Reagent 6A**<br><br>TS-MMR Blocker A (RTU) | 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.<br>b. Wash with PBS/ 0.05% Tween20 or **1xTBS-T** 3 times for 2 minutes each. | 30 min. |

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|---|---|---|
| **10. Reagent 6B**<br><br>TS-MMR Blocker B (RTU) | 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.<br>b. Wash with PBS/ 0.05% Tween20 or **1xTBS-T** 3 times for 2 minutes each. | 5 min. |
| **11. 2nd** Mouse primary antibody<br><br>Supplied by user. | **Note**: Investigator needs to optimize dilution prior to triple staining.<br>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.<br>b. Wash with PBS/ 0.05% Tween20 or **1xTBS-T** 3 times for 2 minutes each. | 30 min. |
| **12. Reagent 7**<br><br>Mouse HRP Polymer (RTU) | 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.<br>b. Wash with PBS/ 0.05% Tween20 or **1xTBS-T** 3 times for 2 minutes each. | 15 min. |
| **13. Reagent 8A, 3B, 8C&8C**<br><br>**8A:** DAB-Ni Substrate (20x)<br>**3B:** DAB Chromogen (20x)<br>**8B:** Hydrogen Peroxide (20x)<br>**8C:** Nickel Solution (7x) | a. Prepare 1mL of distilled water. Add 1 drop of **Reagent 8A** (DAB-Ni Substrate) into 1mL of distilled water. Mix well.<br>b. Add 1 drop of **Reagent 3B** (DAB Chromogen) and 1 drop of concentrated **Reagent 8B** (Hydrogen Peroxide) to the diluted Reagent. Mix well.<br>c. Add 3 drops of **Reagent 8C** (Nickel Solution) to the mixture. Mix well.<br>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.<br>e. Use DAB-Ni working solution within 7 hours and store at 4°C keeping away from light during operation. | 5 min |
| **14. HEMATOXYLIN**<br><br>Not provided | a. Counterstain with 2 drops (100µL) or enough volume of hematoxylin to completely cover tissue. Incubate for 10-15 seconds.<br>b. Rinse thoroughly with tap water for 2-3min.<br>c. Put slides in PBS until show blue color (about ½ - 1min.)<br>d. Rinse well in distilled water | 10 – 15 sec |

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|---|---|---|
| **15. Reagent 9**<br><br>NeoMount Universal (RTU) | a. Apply 1 drop (50µL) of **Reagent 9** (U-Mount) to cover the tissue section and apply glass coverslip.<br>b. 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 SHOOT:**

| PROBLEM | TIPS |
|---|---|
| Uneven stain on 3 primary antibodies | 1. Need to adjust the titer of each antibody.<br>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 coversliped 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. O-Mount, E02-18) 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**

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# Work Sheet for NB-23-00129 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-23-00129 Protocol-1 is suitable when all primary antibodies need pre-treatment or all primary antibodies do not need pre-treatment.

| **Protocol Step** | **NB-23-00129** 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<br><br>User supplied (30-60min) | | | | |
| **Step 4** | **Reagent 1&Reagent 2**<br><br>Rabbit HRP Polymer & Mouse AP Polymer require mixing (30min) | | | | |
| **Step 5** | **Reagent 3A& Reagent 3B**<br><br>DAB requires mixing. (5min) | | | | |
| **Step 6** | **Reagent 4A& Reagent 4B**<br><br>Permanent Red requires mixing. (10min) | | | | |

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|---|---|---|---|---|---|
| **Step 7** | **Reagent 5**<br>Antibody Blocker<br>requires mixing.<br><br>(10min) | | | | |
| **Step 9** | **Reagent 6A**<br><br>DS-MMR Blocker A<br>RTU (30min) | | | | |
| **Step 10** | **Reagent 6B**<br><br>DS-MMR Blocker B<br>RTU (5min) | | | | |
| **Step 11** | Mouse 1°Ab<br><br>User supplied (30-60 min) | | | | |
| **Step 12** | **Reagent 7**<br>Mouse HRP Polymer<br>RTU (15 min) | | | | |
| **Step 13** | **Reagent 8A, 3B, 8B &8C**<br><br>DAB-Ni requires mixing. (5min) | | | | |
| **Step 14** | Counter stain<br>User supplied | | | | |
| **Step 15** | Reagent 9<br>NeoMount Universal<br>RTU | | | | |
| **Result** | **Stain pattern on controls are correct: Fill in Yes or NO** | | | | |

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

**NB-23-00129 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-23-00129** 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)**<br><br>Mouse HRP Polymer RTU (15min) | | | | |
| **Step 5** | **Reagent 3A&3B**<br><br>DAB requires mixing (5 min) | | | | |
| **Step 7** | **Reagent 5**<br><br>Antibody Blocker requires mixing (10min) | | | | |
| **Step 8** | HIER (DAB will not be removed) | | | | |
| **Step 9** | **Reagent 6A (RTU)**<br><br>DS-MMR Blocker A RTU (30min) | | | | |
| **Step 10** | **Reagent 6B (RTU)**<br><br>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**<br><br>Permanent Red requires mixing. (10min) | | | | |
| **Step 13** | Counter stain<br><br>User supplied | | | | |
| **Step 14** | **Reagent 8A, 3B, 8B &8C**<br><br>DAB-Ni requires mixing. (5min) | | | | |
| **Step 15** | Counter stain User supplied | | | | |
| **Step 16** | Reagent 9<br><br>NeoMount Universal **(**RTU) | | | | |
| **Result** | Stain pattern on controls are correct:<br>Fill in Yes or NO | | | | |

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

## Testing result:

**NB-23-00129 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-23-00129** 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**<br><br>Rabbit HRP Polymer & Mouse AP Polymer require mixing. (30min) | | | | |
| **Step 5** | **Reagent 3A&Reagent 3B**<br><br>DAB require mixing. (5min) | | | | |
| **Step 6** | **Reagent 4A&Reagent 4B**<br><br>Permanent Red requires mixing. (10min) | | | | |
| **Step 7** | **Reagent 5**<br>Antibody Blocker required mixing.<br><br>(10min) | | | | |
| **Step 2** | HIER Refer to antibody datasheet. | | | | |
| **Step 7** | **Reagent 6A**<br><br>DS-MMR Blocker A RTU (30min) | | | | |
| **Step 8** | **Reagent 6B**<br><br>DS-MMR Blocker B RTU (5min) | | | | |

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

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

# Testing result: