# Polink TS-MRR-Hu B Kit for Immunohistochemistry Staining

## Polymer-HRP & AP triple staining kit to detect one mouse and two rabbit primary antibodies on human tissue with DAB (Brown), GBI-Permanent Red (Red), and DAB-Ni (Black)

Storage: 2-8ºC

Catalog No.:

| | | | |
|---|---|---|---|
| ☐ | TS302B-6 | *24mL | 120 slides** |
| ☐ | TS302B-18 | *72mL | 360 slides** |
| ☐ | TS302B-60 | *240mL | 1200 slides** |

**Volume of polymer conjugate*
*** If use 100µL per slide*

**Intended Use:**
The **Polink TS-MRR-Hu B 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 embeded 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[1, 2]. **Polink TS-MRR-Hu B Kit** from GBI Labs (Golden Bridge International) supplies polymer enzyme conjugates: anti-mouse HRP Polymer, anti-rabbit AP Polymer, and anti-rabbit HRP Polymer with three chromogens, DAB (brown); GBI-Permanent Red (red); and DAB-Ni (Black). **Polink TS-MRR-Hu B 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 | TS302B-6 | TS302B-18 | TS302B-60 |
|---|---|---|---|---|
| **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** | GBI-Permanent Red Substrate (RTU) | 15mL | 18mLx2 | 120mL |
| **Reagent 4B** | GBI-Permanent Red Activator (5x) | 3mL | 7.2mL | 12mLx2 |
| **Reagent 4C** | GBI-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** | Simpo-Mount (RTU) | 15mL | 18mLx2 | 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 inhibit the activity of the alkaline phosphatase. Note: 1X TBS-T =50mM Tris HCl, 150mM NaCl, 0.05% Tween-20 pH7.6. GBI sells 10xTBS-T for your convenience (B11xx)


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**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.

**Staining main protocol:**
**TS302B Protocol-1**

| **Steps / Reagent** | **Staining Protocol** | **Incubation Time** |
|---|---|---|
| 1. Peroxidase and Alkaline Phosphatase Blocking Reagent<br>Not provided<br>We recommend using **GBI Dual Block E36xx.** Fast, easy and it will block endogenous alkaline phosphatase | a. Incubate slides in peroxidase and alkaline phosphatase blocking reagent. We recommend **GBI Dual Block E36xx**.<br>b. Rinse the slide using distilled water at least twice. | 10min |
| 2. Antigen retrieval (**optional**):<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-T containing 0.05% Tween-20 or **1X TBS-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-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each. | 30min |
| 4. Mix **Reagent 1:** Mouse HRP Polymer (RTU) with<br>**Reagent 2:** Rabbit AP Polymer (RTU) | **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.<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-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each. | 30min |
| 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 thoroughly with distilled water.<br>d. Wash with 1xTBS-T only, 3 times for 2 minutes each. | 5min |
| 6. **Reagent 4A, 4B, 4C**<br><br>**Reagent 4A:**<br>GBI-Permanent Red Substrate (RTU) | **Note**: Shake GBI-Permanent Red Activator before adding into GBI-Permanent Red Substrate.<br>a. 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 | 10min |



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| | | |
|---|---|---|
| **Reagent 4B:**<br>GBI-Permanent Red Activator (5x<br>**Reagent 4C:**<br>GBI-Permanent Red Chromogen (100x)<br>**To get maximum sensitivity of AP polymer, Please repeat chromogen step** | mix well.<br>[**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>b. Apply 2 drops (100µL) or enough volume of GBI-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 GBI-Permanent Red working solution to completely cover the tissue for additional 5 to 10min**<br>c. Rinse well with distilled water. | |
| 7. **Reagent 5**<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) 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.<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.<br>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-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each. | 10min |
| 8. Antigen retrieval:<br>**Refer to primary antibody data sheet.** | a. Refer to primary antibody data sheet for antigen retrieval methods.<br>b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each. | Up to 1 hour |
| 9. **Reagent 6A**<br>TS-MRR Blocker A (RTU) | 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.<br>b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each. | 30min |
| 10. **Reagent 6B**<br>TS-MRR Blocker B (RTU) | 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.<br>b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each. | 5min |
| 11. 2nd rabbit 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 rabbit 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-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each. | 30min |
| 12. **Reagent 7**<br>Rabbit HRP Polymer (RTU) | 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.<br>b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each. | 15min |
| 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. | 5min |
| 14. HEMATOXYLIN<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 | 10-15sec |
| 15. **Reagent 9:** | a. Apply 2 drops (100µL) or enough volume of **Reagent 9** (Simpo-Mount) to | |



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| | | |
|---|---|---|
| Simpo-Mount (RTU) | cover tissue when tissue is wet. Rotate the slides to allow Simpo-Mount spread evenly.<br>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. | |

**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 | 1. 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. **GBI-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. U-Mount, E37-xx) and coverslip. Press to push the air bubble out.

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

**Precautious:**
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.

**Remarks:**
For research use only.

**References:**
1. De Pasquale A, Paterlini P, Quaglino D.*Immunochemical demonstration of different antigens in single cells in paraffin-embedded histological sections.* Clin Lab Haematol. 1982;4(3):267-72.

2. Polak J. M and Van Noorden S. Introduction to Immunocytochemistry Second Edition. Bios Scientific Publishers. P41-54. 1997



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# Work Sheet for TS302B 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

**TS302B Protocol-1** is suitable when all primary antibodies need pre-treatment or all primary antibodiess do not need pre-treatment.

| | Main Protocol Step | TS302B Protocol-1 | Experiment 1 Date: | Experiment 2 Date: | Experiment 3 Date: | Experiment 4 Date: |
|---|---|---|---|---|---|---|
| 1 | Step 1 | Peroxidase and Alkaline Phosphatase Block<br>E36 is recommended.<br>User supplied | | | | |
| 2 | Step 2 | HIER(Optional)<br>Refer to antibody datasheet<br>User supplied | | | | |
| 3 | Step 3 | Mouse 1°Ab &Rabbit 1°Ab mix<br>User supplied (30-60min) | | | | |
| 4 | Step 4 | **Reagent 1&Reagent 2**<br>Mouse HRP Polymer & Rabbit AP Polymer require mixing<br>(30min) | | | | |
| 5 | Step 5 | **Reagent 3A& Reagent 3B**<br>DAB requires mixing.<br>(5min)<br>**Wash with 1x TBS-T after rinse with distilled water.** | | | | |
| 6 | Step 6 | **Reagent 4A& Reagent 4B**<br>GBI-Permanent Red requires mixing.<br>(10min) | | | | |
| 7 | Step 7 | **Reagent 5**<br>Antibody Blocker requires mixing.<br>(10min) | | | | |
| 8 | Step 9 | **Reagent 6A**<br>DS-MRR Blocker A RTU<br>(30min) | | | | |
| 9 | Step 10 | **Reagent 6B**<br>DS-MRR Blocker B RTU (5min) | | | | |
| 10 | Step 11 | Rabbit 1°Ab<br>User supplied (30-60 min) | | | | |
| 11 | Step 12 | **Reagent 7**<br>Rabbit HRP Polymer RTU (15 min) | | | | |
| 12 | Step 13 | **Reagent 8A, 3B, 8B &8C**<br>DAB-Ni requires mixing.<br>(5min) | | | | |
| 13 | Step 14 | Counter stain<br>User supplied | | | | |
| 14 | Step 15 | **Reagent 9**<br>Simpo-Mount 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:



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**TS302B 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 | TS302B Protocol-2 | Experiment 1 Date: | Experiment 2 Date: | Experiment 3 Date: | Experiment 4 Date: |
|---|---|---|---|---|---|---|
| **1** | **Step 1** | Peroxidase or Alkaline Phosphatase Block<br>E36 is recommended.<br>User supplied | | | | |
| **2** | **Step 11** | Rabbit 1°Ab (sensitive to HIER)<br>User supplied (30-60min) | | | | |
| **3** | **Step 12** | **Reagent 7 (RTU)**<br>Rabbit HRP Polymer RTU<br>(15min) | | | | |
| **4** | **Step 5** | **Reagent 3A&3B**<br>DAB requires mixing (5 min) | | | | |
| **5** | **Step 7** | **Reagent 5**<br>Antibody Blocker requires mixing<br>(10min) | | | | |
| **6** | **Step 2** | HIER<br>Refer to antibody datasheet<br>(DAB will not be removed)<br>User supplied | | | | |
| **7** | **Step 9** | **Reagent 6A (RTU)**<br>DS-MRR Blocker A RTU<br>(30min) | | | | |
| **8** | **Step 10** | **Reagent 6B (RTU)**<br>DS-MRR Blocker B RTU (5min) | | | | |
| **9** | **Step 3** | Mouse 1°Ab & Rabbit 1°Ab mix<br>(Abs requires HIER)<br>User supplied (30-60 min) | | | | |
| **10** | **Step 4** | **Reagent 1&Reagent 2**<br>Mouse HRP Polymer & Rabbit AP Polymer require mixing<br>(30min)<br>**Wash with 1x TBS-T only.** | | | | |
| **11** | **Step 6** | **Reagent 4A & Reagent 4B**<br>GBI-Permanent Red requires mixing.<br>(10min) | | | | |
| **12** | **Step 13** | **Reagent 8A, 3B, 8B &8C**<br>DAB-Ni requires mixing.<br>(5min) | | | | |
| **13** | **Step 14** | Counter stain<br>User supplied | | | | |
| **14** | **Step 15** | **Reagent 9**<br>Simpo-Mount 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:



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**TS302B 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 | TS302B Protocol-3 | Experiment 1 Date: | Experiment 2 Date: | Experiment 3 Date: | Experiment 4 Date: |
|---|---|---|---|---|---|---|
| **1** | **Step 1** | Peroxidase or Alkaline Phosphatase Block<br>E36 is recommended.<br>User supplied | | | | |
| **2** | **Step 3** | Mouse 1°Ab & Rabbit 1°Ab mix<br>User supplied<br>(30-60min.) | | | | |
| **3** | **Step 4** | **Reagent 1&Reagent 2**<br>Mouse HRP Polymer & Rabbit AP Polymer require mixing.<br>(30min) | | | | |
| **4** | **Step 5** | **Reagent 3A&Reagent 3B**<br>DAB require mixing.<br>(5min)<br>**Wash with 1x TBS-T after rinse with distilled water.** | | | | |
| **5** | **Step 6** | **Reagent 4A&Reagent 4B**<br>GBI-Permanent Red requires mixing.<br>(10min) | | | | |
| **6** | **Step 7** | **Reagent 5**<br>Antibody Blocker required mixing.<br>(10min) | | | | |
| **7** | **Step 8** | HIER<br>Refer to antibody datasheet.<br>User supplied | | | | |
| **8** | **Step 9** | **Reagent 6A**<br>DS-MRR Blocker A RTU<br>(30min) | | | | |
| **9** | **Step 10** | **Reagent 6B**<br>DS-MRR Blocker B RTU (5min) | | | | |
| **10** | **Step 11** | Rabbit 1°Ab (sensitive to HIER)<br>User supplied (30-6min.) | | | | |
| **11** | **Step 12** | **Reagent 7**<br>Rabbit HRP Polymer (RTU)<br>(15min.) | | | | |
| **12** | **Step 13** | **Reagent 8A, 3B, 8B &8C**<br>DAB-Ni requires mixing.<br>(5min) | | | | |
| **13** | **Step 14** | Counter stain<br>User supplied | | | | |
| **14** | **Step 15** | **Reagent 9**<br>Simpo-Mount 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:



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