Polink TS-MRRt-Ms B Kit

Polymer-HRP&AP triple staining kit
Detects mouse, rabbit and rat primary antibodies on mouse tissue
With DAB (Brown), GBI-Permanent Red (Red), and DAB-Ni (Black)

Storage: 2-8ºC

Catalog No.:
- ☐ TS312B-6 *6mL (60 slides)**
- ☐ TS312B-18 *18mL (180 slides)**
- ☐ TS312B-60 *60mL (600 slides)**

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

Intended Use:
The **Polink TS-MRRt-Ms B** Kit 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 65ºC 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 [1, 2]. The **Polink TS-MRRt-Ms B** Kit from **GBI Labs** (Golden Bridge International) 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/chromogens; DAB (brown), GBI-Permanent Red (Red) and DAB-Ni (Black). The **Polink TS-MRRt-Ms B** Kit is a non-biotin system, avoiding non-specific binding caused by endogenous biotin. This kit 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	TS312B-6	TS312B-18	TS312B-60
Reagent 1	Mouse HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 2A	DAB Substrate (RTU)	12mL	36mL	120mL
Reagent 2B	DAB Chromogen (20x)	2mL	4mL	12mL
Reagent 3	Antibody Blocker (40x)	30mL	50mL	100mL
Reagent 4	Rat Primer (RTU)	6mL	18mL	60mL
Reagent 5	Rat AP Polymer (RTU)	6mL	18mL	60mL
Reagent 6	Rabbit HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 7A	GBI-Permanent Red Substrate (RTU)	15mL	36mL	120mL
Reagent 7B	GBI-Permanent Red Activator (5x)	3mL	7.2mL	24mL
Reagent 7C	GBI-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	Simpo-Mount (RTU)	6mL	18mL	60mL

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. GBI sells 10xTBS-T for your convenience (B11xx)

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, 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

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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.
- TS312B Protocol-A worksheet is suitable when mouse, rabbit & rat primary antibodies need pre-treatment or mouse is sensitive to pre-treatment.
- TS312B 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.
- TS312B Protocol-C worksheet is suitable for one Rat & one Rabbit primary Abs need pre-treatment, the mouse primary Ab is sensitive to 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 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.

TS312B Protocol A

Steps / Reagent	Staining Protocol	Incubation Time
1. Peroxidase and Alkaline Phosphatase Blocking Reagent: Not provided	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. b. Rinse the slide using distilled water at least twice.	10 minutes
2. Antigen retrieval: **Refer to primary antibody data sheet** Supplied by user	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 8 above)**; 3 times for 2 minutes each.	Up to 1 hour
3. Primary Antibody: **Add Mouse Primary** Supplied by user	**Note:** Investigator needs to optimize dilution prior to triple staining. 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. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	30 minutes
4. **Reagent 1:** Mouse HRP Polymer (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 1** (Mouse HRP Polymer) to cover the tissue completely. b. Incubate in moist chamber for 15 min. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	15 minutes
5. **Reagents 2A, 2B:** **Reagent 2A:** DAB Substrate (RTU) **Reagent 2B:** DAB Chromogen (20x)	**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 4ºC and use within 7 hours. a. Apply 1 to 2 drops (50-100µL) of your DAB working solution to cover the tissue completely. b. Incubate for 5min. c. Rinse slides in multiple changes of distilled water 2min, 3 times or under running tap water for 1 minute.	5 minutes
6. **Reagent 3:** 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 require 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. a. 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. b. Put slides in heated Antibody Blocker for 10 minutes at 80°C. c. Remove slides from the Antibody blocker; cool slides 5 seconds. d. 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.** e. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	10 minutes
7. Antigen retrieval: **Refer to primary antibody data sheet**	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**; 3 times for 2 minutes each.	Up to 1 hour
8. Primary antibody mix: Add Rat and Rabbit primary antibody Supplied by user	**Note**: Investigator needs to optimize dilution prior to triple staining. a. 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. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	30 to 60 minutes
9. **Reagent 4:** Rat Primer (RTU)	**Note:** This step is required for activation of RAT AP Polymer, **DO NOT skip**. a. Apply 1 to 2 drops (50-100µL) of **Reagent 4** (Rat Primer) to cover the tissue completely. b. Incubate in moist chamber for 10 min. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	10 minutes
10. Mix **Reagents 5 and 6:** **Reagent 5:** Rat AP Polymer (RTU) **Reagent 6**: Rabbit HRP Polymer (RTU)	**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. a. Apply 1 to 2 drops (50-100µL) of Polymer mixture to cover the tissue completely. b. Incubate slides in moist chamber for 30 min. c. Wash with **1xTBS-T only;** 3 times for 2 minutes each.	30 minutes

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11. **Reagents 7A, 7B, 7C:** **Reagent 7A:** GBI-Permanent Red Substrate (RTU) **Reagent 7B:** GBI-Permanent Red Activator (5x) **Reagent 7C:** GBI-Permanent Red Chromogen (100x)	**Note**: Shake GBI-Permanent Red Activator before adding into GBI-Permanent Red Substrate. a. 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. [**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.] 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 (100µL) again of the GBI-Permanent Red working solution to completely cover the tissue for additional 5 to 10min.** c. Rinse well with distilled water. **To get maximum sensitivity of AP polymer, repeat chromogen step**	10 minutes
12. **Reagents 9A, 4B, 9B, 9C:** **Reagent 9A:** DAB-Ni Substrate (20x) **Reagent 4B:** DAB Chromogen (20x) **Reagent 9B:** Hydrogen Peroxide (20x) **Reagent 9C:** Nickel Solution (7x)	a. Prepare 1mL of distilled water. Add 1 drop of **Reagent 9A** (DAB-Ni Substrate) into 1mL of distilled water. Mix well. b. Add 1 drop of **Reagent 4B** (DAB Chromogen) and 1 drop of concentrated **Reagent 9B** (Hydrogen Peroxide) to the diluted Reagent. Mix well. c. Add 3 drops of **Reagent 9C** (Nickel Solution) to the mixture. Mix well. 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. e. Use DAB-Ni working solution within 7 hours and store at 4°C keeping away from light during operation.	5 minutes
13. HEMATOXYLIN: Not provided	a. Counterstain with 2 drops (100µL) or enough volume of hematoxylin to completely cover tissue. Incubate for 10-15 seconds. b. Rinse thoroughly with tap water for 2-3min. c. Put slides in PBS until show blue color (about ½ - 1min.) d. Rinse well in distilled water	10-15 seconds
14. **Reagent 10:** Simpo-Mount (RTU)	a. Apply 2 drops (100µL) or enough volume of **Reagent 10** (Simpo-Mount) to cover tissue when tissue is wet. Rotate the slides to allow Simpo-Mount to spread evenly. 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. 2. The amount of each protein expressed on tissue may be different.
No stain on 1 or 2 antibodies	1. Missing steps or steps 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 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. 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 GBI-Permanent Red stain!

Precautions:

Please wear gloves, eye protection, and take other necessary precautions. If any of the reagents 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 TS312B 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 ensure 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 de-paraffinization
- Refer to insert for details of each step

TS312B Protocol A is suitable when mouse, rabbit & rat primary antibodies need pre-treatment or mouse is sensitive to pre-treatment.

	Step/ Protocol	TS312B Protocol A	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	**Step 1**	Peroxidase or Alkaline Phosphatase Block E36 is recommended. User supplied				
2	**Step 2**	HIER(Optional)				
3	**Step 3**	Mouse 1°Ab User supplied (30-60 min)				
4	**Step 4**	**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** GBI-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** Simpo-Mount RTU				
14	**Result**	**Stain pattern on controls is 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:

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TS312B Protocol B is suitable when mouse primary antibody needs pretreatment or rabbit & rat primary antibodies are sensitive to pre-treatment.

	Step/ Protocol	TS312B Protocol B	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	Step 1	Peroxidase or Alkaline Phosphatase Block E36 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** GBI-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** Simpo-Mount RTU				
3	Result	**Stain pattern on controls is 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:

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TS312B Protocol C is suitable when rat and rabbit primary antibodies need pretreatment or mouse primary antibody is sensitive to pretreatment.

	Step/ Protocol	TS312B Protocol C	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	**Step 1**	Peroxidase or Alkaline Phosphatase Block E36 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** GBI-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** Simpo-Mount RTU				
13	**Result**	**Stain pattern on controls is 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:

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