Polink TS-MRR-Ms A Kit

(Polymer-HRP&AP triple staining kit)
(Detects one mouse and two rabbit primary antibodies on mouse/rat tissue with DAB (Brown), GBI-Permanent Red (Red), and Emerald (Green))

Storage: 2-8ºC

Catalog No.:
- ☐ TS309A-6 *6mL (60 slides)**
- ☐ TS309A-18 *18mL (180 slides)**
- ☐ TS309A-60 *60mL (600 slides)**

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

Intended Use:
The **Polink TS-MRR-Ms A** Kit is designed to use with user supplied one mouse and two rabbit primary antibodies to detect three distinct antigens on a single mouse/rat tissue or cell samples. Kit has been tested on tissue specimens that are paraffin embedded. For frozen tissue a lower temperature of 65ºC must be used for Antibody Blocker (Reagent 6) to prevent tissue from dissociating from slide. Please read through entire protocol as this protocol requires many steps to be done in the defined order.
Triple staining uses traditional methods in immunohistostaining to reveal three distinct antigens and their co-expression on a single tissue [1, 2]. Polink TS-MRR-Ms A Kit from GBI Labs (Golden Bridge International) supplies polymer enzyme conjugates: polymer-HRP anti-mouse IgG, polymer-HRP anti-rabbit IgG and polymer-AP anti-rabbit IgG with three substrates/chromogens; DAB (brown), Emerald (green), and GBI-Permanent Red (Red). Polink TS-MRR-Ms A 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 two primary antibodies from the same host species using unique blocking system. Simplified steps allow users to complete triple staining within 5 hours (without antigen retrieval) or 6-7 hours (with antigen retrieval). The well tested protocol provides user a method to permanently mount slides with coverslip.

Kit Components:

Component No.	Content	TS309A-6	TS309A-18	TS309A-60
Reagent 1	Mouse Primer (RTU)	6mL	18mL	60mL
Reagent 2	Rabbit AP Polymer (RTU)	6mL	18mL	60mL
Reagent 3	Mouse HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 4A	DAB Substrate (RTU)	12mL	36mL	120mL
Reagent 4B	DAB Chromogen (20x)	1.5mL	2mL	6mL
Reagent 5A	GBI-Permanent Red Substrate (RTU)	15mL	36mL	120mL
Reagent 5B	GBI-Permanent Red Activator (5x)	3mL	7.2mL	24mL
Reagent 5C	GBI-Permanent Red Chromogen (100x)	150µL	360µL	1.2mL
Reagent 6	Antibody Blocker (40x)	30mL	50mL	100mL
Reagent 7A	TS-MRR Blocker A (RTU)	6mL	18mL	60mL
Reagent 7B	TS-MRR Blocker B (RTU)	6mL	18mL	60mL
Reagent 8	Rabbit HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 9	Emerald Chromogen (RTU)	6mL	18mL	60mL
Reagent 10	U-Mount (RTU)	6mL	18mL	NA

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 rabbit primary antibody with mouse 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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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.
- TS309A Protocol-2 worksheet is suitable for one Mouse & one Rabbit primary Abs need pre-treatment, the other Rabbit primary Ab is sensitive to pre-treatment.
- TS309A Protocol-3 worksheet is suitable when one Mouse & one Rabbit primary antibody are sensitive to pre-treatment, but the second Rabbit 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 rabbit antibodies and one mouse antibody requires HIER.

TS309A Staining Protocol 1:

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 min
2. Antigen retrieval (**optional**): Refer to primary antibody data sheet.	**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.	
3. Primary Antibody Mix: **Mix one Mouse and one Rabbit primary antibody:** 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. a. Apply 2 drops or enough volume of mouse and rabbit primary antibody mixture to cover the tissue completely. b. Incubate in moist chamber for 30-60 min. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	30-60 min
4. **Reagent 1:** Mouse Primer (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 1** (**Mouse Primer**) to cover the tissue completely. b. Incubate slides 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 min
5. **Reagents 2:** Rabbit AP Polymer (RTU)	a. Apply 1 to 2 drops of the **Reagent 1** (Rabbit AP Polymer) to cover each section. b. Incubate in moist chamber for 15-30 min. c. Wash with **1X TBS-T only**; 3 times for 2 minutes each. **Note:** longer incubation may increase background.	15-30 min
6. **Reagent 3:** Mouse HRP Polymer (RTU)	a. Apply 1 to 2 drops of **Reagent 2** (Mouse HRP Polymer) to cover each section. b. Incubate in moist chamber for 15-30 min. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	15-30 min
7. **Reagents 4A, 4B:** **Reagents 4A:** DAB Substrate (RTU) **Reagents 4B:** DAB Chromogen (20x)	**Note:** Make enough DAB mix by adding 1 drop of **Reagent 4B** (DAB Chromogen) in 1mL of **Reagent 4A** (DAB Substrate). Mix well. Use within 7 hours store at 4℃. a. Apply 1 to 2 drops (50-100µL) of your DAB mixture to cover the tissue completely. b. Incubate for 5min. c. Rinse thoroughly with distilled water. d. Wash with **1xTBS-T**; 3 times for 2 minutes each.	5 min
8. **Reagents 5A, 5B, 5C:** **Reagent 5A:** GBI-Permanent Red Substrate (RTU) **Reagent 5B:** GBI-Permanent Red Activator (5x) **Reagent 5C:** GBI-Permanent Red Chromogen (100x)	**Note**: First bring **Reagent 5B** and **Reagent 5A** to room temperature. Shake **Reagent 5B** (Activator) before adding into **Reagent 5B** (Substrate). a. Add 200µL of **Reagent 5B** (Activator) into 1mL of **Reagent 5A** (Substrate) and mix until clear. Add 12µL of **Reagent 5C** (Chromogen) into the mixture and mix well. [**Note:** For fewer slides, add 100µL of **Reagent 5B** (Activator) into 500µL of **Reagent 5A** (Substrate) and mix until clear. Add 6µL of **Reagent 5C** (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 drops (100µL) of the GBI-Permanent Red working solution to completely cover the tissue for an additional 5 to 10 min.** c. Rinse well with distilled water.	10 min
9. **Reagent 6:** 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 6** (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 9** if not complete **step 8e** and move on to **step 10.** e. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	10 min

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10. 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
11. **Reagent 7A:** TS-MRR Blocker A (RTU)	a. Apply 2 drops or enough volume of **Reagent 7A** (DS-MRR Blocker A) to cover the tissue completely. Mix well on the slide and incubate in moist chamber for 30 min. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	30 min
12. **Reagent 7B:** TS-MRR Blocker B (RTU)	a. Apply 2 drops or enough volume of **Reagent 7B** (DS-MRR Blocker B) to cover the tissue completely. Mix well on the slide and incubate in moist chamber for 5 min. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	5 min
13. 2nd 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 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. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	30 min
14. **Reagent 8:** Rabbit HRP Polymer (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 8** (Rabbit HRP Polymer) to cover the tissue completely. b. Incubate slides in moist chamber for 15-30 min. c. Rinse thoroughly with distilled water. **Note:** longer incubation may increase background.	15-30 min
15. Counterstain (**Optional but must be done before Emerald Chromogen step**): Not provided	**Note:** If two antigens are co-localized in the nucleus 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. a. Counterstain dip in diluted hematoxylin for 5 seconds for nuclear co- localization or 30 seconds for cytoplasmic or membrane co-localization. **DO NOT** over stain with hematoxylin. b. Rinse thoroughly with tap water for 1min. c. Put slides in PBS for 5-10 seconds to blue, **DO NOT** over blue. d. Rinse well in distilled or tap water for 1 min. e. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	5-30 sec
16. **Reagent 9:** Emerald Chromogen (RTU) **Do hematoxylin first**	a. Apply 1 to 2 drops (50-100µL) of **Reagent 9** (Emerald Chromogen) to cover the tissue completely. b. Incubate slides in humid chamber for 5 minutes. c. Wash slides in tap water for 1 minute. d. Rinse with distilled water. ***Important:*** Emerald Chromogen is water soluble, counter stain first. *Do not leave slides sitting in water*. Always stain Emerald chromogen **AFTER** GBI-Permanent Red stain and hematoxylin. GBI-Permanent Red removes the Emerald.	5 min
17. Dehydrate section: **It is important to follow the protocol**	**Note: Please wipe off extra water and air-dry slides before dehydration and clear.** a. Dehydrate with 85% ethanol 20seconds b. Dehydrate with 95% ethanol 20seconds c. Dehydrate with 100% ethanol 20seconds d. Dehydrate with 100% ethanol 20seconds e. Dehydrate with 100% ethanol 20seconds f. Dehydrate with xylene 20seconds **CAUTION: DO NOT dehydrate in xylene longer than 20 seconds! It will erase GBI-Permanent Red stain!**	2 min
18. **Reagent 10:** U-Mount (RTU)	a. Apply 1 drop (50µL) of **Reagent 10** (U-Mount) to cover the tissue section and apply glass coverslip. b. Apply force to coverslip to **squeeze out any extra mountant** and bubbles for optimal clarity. Removing excess also to prevent leaching of GBI- Permanent Red stain.	

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Troubleshooting:

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. 3. Set slides in water too long so that Emerald is washed away. 4. Set slides in Xylene too long so that GBI-Permanent Red is washed away.
Emerald Chromogen is blue not green when non-co-localized with GBI Permanent Red.	1. Emerald should be green when non colocalized with GBI-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	1. Missing steps or steps reversed.
Green Background on the slide	1. Titer primary antibody.
GBI-Permanent Red is leaching	1. Use fresh 100% ethanol and xylene. 2. Slide sat too long in xylene. Do not go over 20seconds!
Artifacts on slides	1. 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 reagents come into 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 TS309A 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

TS309A Protocol-1 is suitable when all primary antibodies need pre-treatment or all primary antibodies do not need pre- treatment.

Main Protocol Step	TS309A Protocol-1	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase or Alkaline Phosphatase Block E36 is recommended. User supplied				
Step 2	HIER Antigen Retrieval (Optional)				
Step 3	Mouse 1°Ab &Rabbit 1°Ab mix User supplied (30-60 min)				
Step 4	**Reagent 1** Mouse primer RTU (15-30 min)				
Step 5	**Reagent 2** Rabbit AP Polymer (15-30 min)				
Step 6	**Reagent 3:** Mouse HRP Polymer (15-30min)				
Step 7	**Reagent 4A & Reagent 4B** DAB requires mixing. (5 min)				
Step 8	**Reagent 5A, 5B, 5C** GBI-Permanent Red requires mixing (10 min)				
Step 9	**Reagent 6** Antibody Blocker requires mixing. (10min)				
Step 10	Antigen Retrieval				
Step 11	**Reagent 7A** DS-MRR Blocker A RTU (30 min)				
Step 12	**Reagent 7B** DS-MRR Blocker B RTU (5 min)				
Step 13	2nd Rabbit 1°Ab User supplied (30-60 min)				
Step 14	**Reagent 8** Rabbit HRP Polymer RTU (15-30 min)				
Step 15	Counter stain (**Note 2**) User supplied (5-10 sec)				
Step 16	**Reagent 9** Emerald Chromogen RTU (5min)				
Step 17	**It is important to follow the protocol. To maintain stain!** Dehydrate section 20seconds for each step				
Step 18	**Reagent 10** U-Mount RTU Mount & coverslip				
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.

Note: 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:

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TS309A 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	TS309A Protocol-2	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase or Alkaline Phosphatase Block E36 is recommended. User supplied				
Step 13	Rabbit 1°Ab (sensitive to HIER) User supplied (30-60min)				
Step 14	**Reagent 8 (RTU)** Rabbit HRP Polymer RTU (15min)				
Step 7	**Reagent 4A, 4B** DAB requires mixing (5 min)				
Step 9	**Reagent 6** Antibody Blocker requires mixing (10min)				
Step 10	HIER (DAB will not be removed)				
Step 11	**Reagent 7A (RTU)** DS-MRR Blocker A RTU (30min)				
Step 12	**Reagent 7B (RTU)** DS-MRR 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** Mouse primer RTU 15min				
Step 5	**Reagent 2** Rabbit AP Polymer (30min) Wash with 1x TBS-T				
Step 6	**Reagent 3** Mouse HRP Polymer (30min) Wash with 1x TBS-T				
Step 8	**Reagent 5A, Reagent 5B& Reagent 5C** GBI-Permanent Red requires mixing. (10min)				
Step 15	Counter stain (**Note 2**) User supplied (5-10 sec.)				
Step 16	**Reagent 9** Emerald Chromogen RTU (5min.)				
Step 17	**It is important to follow the protocol. To maintain stain!** Dehydrate section 20seconds for each step				
Step 18	**Reagent 10** U-Mount RTU Mount & coverslip				
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.
Note 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:

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TS309A 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	TS309A Protocol-3	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase or Alkaline Phosphatase Block E36 is recommended. User supplied				
Step 3	Mouse 1°Ab & Rabbit 1°Ab mix User supplied (30-60min.)				
Step 4	**Reagent 1** Mouse primer RTU (15min)				
Step 5	**Reagent 2** Rabbit AP Polymer (15-30min)				
Step 6	**Reagent 3** Mouse HRP Polymer (15-30min)				
Step 7	**Reagent 4A&Reagent 4B** DAB require mixing. (5min)				
Step 8	**Reagent 5A, Reagent 5B& Reagent 5C** GBI-Permanent Red requires mixing. (10min)				
Step 9	**Reagent 6** Antibody Blocker required mixing. (10min)				
Step 10	HIER Antigen Retrieval Refer to antibody datasheet.				
Step 11	**Reagent 7A** DS-MRR Blocker A RTU (30min)				
Step 12	**Reagent 7B** DS-MRR Blocker B RTU (5min)				
Step 13	Mouse 1°Ab (Not sensitive to HIER) User supplied (30-60min.)				
Step 14	**Reagent 8** Rabbit HRP Polymer (RTU) (15min.)				
Step 15	Counter stain (**Note2**) User supplied				
Step 16	**Reagent 9** Emerald Chromogen (RTU) (5min)				
Step 17	**It is important to follow the protocol to maintain stain!** Dehydrate section 20seconds for each step				
Step 18	**Reagent 10** U-Mount RTU Mount & coverslip				
Result	**Stain pattern on controls is correct: Fill in Yes or NO**				

Note1: Normal wash steps = Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes 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:

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