Polink TS-MMR-Ms A Kit for Immunohistochemistry Staining

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

Storage: 2-8ºC

Catalog No.:			
	☒	TS308A-6	*24mL (for 120 slides**)
	☐	TS308A-18	*72mL (for 360 slides**)
	☐	TS308A-60	*240mL (for 1200 slides**)

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

Intended Use:

The **Polink TS-MMR-Ms A** 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 mouse/rat tissue or cell samples. Kit has been tested on tissue specimens that are paraffin embedded; however it may be used on frozen or freshly prepared monolayer cell smears. For frozen tissue a lower temperature of 65°C may be used for Antibody Blocker (Reagent 7) to prevent tissue from dissociating from slide. Please read through entire protocol as this protocol requires many step 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-MMR-Ms A** kit from GBI Labs (Golden Bridge International) supplies polymer enzyme conjugates: polymer-HRP anti-mouse IgG, polymer-AP anti-mouse IgG, and polymer-HRP anti-rabbit IgG with three substrates/chromogens; DAB (brown), Emerald (green), and GBI-Permanent Red (Red). **Polink TS-MMR-Ms A** kit is a non-biotin system, avoiding non-specific binding caused by endogenous biotin. A Primer step is used to increase specificity of antibody staining.This kit has been optimized to have no cross detection when detecting two primary antibodies from the same host species using unique blocking system. Optimized protocol allows 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	TS308A-6	TS308A-18	TS308A-60
Reagent 1	Mouse Primer (RTU)	12mL	18mLx2	120mL
Reagent 2	Mouse AP Polymer (RTU)	6mL	18mL	60mL
Reagent 3	Rabbit HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 4A	DAB Substrate (RTU)	15mL	18mLx2	120mL
Reagent 4B	DAB Chromogen (20x)	1.5mL	2mL	6mL
Reagent 5A	GBI-Permanent Red Substrate (RTU)	15mL	18mLx2	120mL
Reagent 5B	GBI-Permanent Red Activator (5x)	3mL	7.2mL	12mLx2
Reagent 5C	GBI-Permanent Red Chromogen (100x)	150µL	360µL	1.2mL
Reagent 6	Antibody Blocker (40x)	15mLx2	50mL	100mL
Reagent 7A	TS-MMR Blocker A (RTU)	12mL	18mLx2	120mL
Reagent 7B	TS-MMR Blocker B (RTU)	12mL	18mLx2	120mL
Reagent 8	Mouse HRP Polymer (RTU)	12mL	18mLx2	120mL
Reagent 9	Emerald Chromogen (RTU)	15mL	18mLx2	120mL
Reagent 10	U-Mount (RTU)	12mL	18mLx2	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 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, 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.
- TS308A 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.
- TS308A Protocol-3 worksheet is suitable when one Mouse & one Rabbit primary antibody are 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 TS308A 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. We recommend **GBI Dual Block E36xx**. b. Rinse the slide using distilled water at least twice.	10min
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. Incubate in moist chamber for 30-60min. 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.	30min
4. **Reagent 1** Mouse Primer (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 1** (Mouse Primer) to cover the tissue completely. Incubate slides in moist chamber for 15 min. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	10min
5. Mix **Reagent 2:** Mouse AP Polymer (RTU) with **Reagent 3:** Rabbit HRP Polymer (RTU)	**Note:** Make sufficient polymer mixture by adding **Reagent 2** (Mouse AP Polymer) and **Reagent 3** (Rabbit HRP 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. a. Apply 1 to 2 drops (50-100µL) of the mixture to cover the tissue completely. b. Incubate in moist chamber for 30 min. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	30min
6. **Reagent 4A&4B** **4A:** DAB Substrate(RTU) **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.	5min

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	d. Wash with **1xTBS-T** only, 3 times for 2 minutes each.	
7. **Reagent 5A, 5B, 5C** **Reagent 5A:** GBI-Permanent Red Substrate (RTU) **Reagent 5B:** GBI-Permanent Red Activator (5x) **Reagent 5C:** GBI-Permanent Red Chromogen (100x) **To get maximum sensitivity of AP polymer, Please repeat chromogen step**	**Note**: Shake GBI-Permanent Red Activator before adding into GBI-Permanent Red Substrate. a. Add 200µL of **Reagent 5B** (Activator) into 1mL of **Reagent 5A** (Substrate) and mix well. Add 10µL 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 well. Add 5µL 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) again of the GBI-Permanent Red working solution to completely cover the tissue for additional 5 to 10min** c. Rinse well with distilled water.	10min
8. **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 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. 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.	10min
9. 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
10. **Reagent 7A** TS-MMR Blocker A (RTU)	a. Apply 2 drops or enough volume of **Reagent 7A** (DS-MMR 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.	30min
11. **Reagent 7B** TS-MMR Blocker B (RTU)	a. Apply 2 drops or enough volume of **Reagent 7B** (DS-MMR 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.	5min
12. 2nd Mouse 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 mouse 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.	30min
13. **Reagent 8** Mouse HRP Polymer (RTU)	c. Apply 1 to 2 drops (50-100µL) of **Reagent 8** (**Mouse HRP Polymer**) to cover the tissue completely. Incubate slides in moist chamber for 15 min. d. Rinse thoroughly with distilled water.	15min
14. Counterstain (**Optional but must be done before Emerald Chromogen step**) Not provided	**Note:** If two antigens are co-localized in the nucleas 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 1min. e. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	5sec
15. **Reagent 9**	a. Apply 1 to 2 drops (50-100µL) of **Reagent 9** (Emerald Chromogen) to cover	5min

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Emerald Chromogen (RTU) **Do hematoxylin first.**	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 to READ:*** 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.	
16.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
17. **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.	

Trouble shooting

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 step 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.

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 TS308A 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

TS308A Protocol-1 is suitable when all primary antibodies need pre-treatment or all primary antibodiess do not need pre-treatment.

	Main Protocol Step	TS308A Protocol-1	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 &Rabbit 1°Ab mix User supplied (30-60min)				
4	Step 4	**Reagent 1** Mouse primer RTU 15min				
5	Step 5	**Reagent 2&Reagent 3** Mouse AP Polymer & Rabbit HRP Polymer require mixing (30min)				
6	Step 6	**Reagent 4A& Reagent 4B** DAB requires mixing. (5min)				
7	Step 7	**Reagent 5A, Reagent 5B Reagent 5C** GBI-Permanent Red requires mixing. (10min)				
8	Step 8	**Reagent 6** Antibody Blocker requires mixing. (10min)				
9	Step 10	**Reagent 7A** DS-MMR Blocker A RTU (30min)				
10	Step 11	**Reagent 7B** DS-MMR Blocker B RTU (5min)				
11	Step 12	Mouse 1°Ab User supplied (30-60 min)				
12	Step 13	**Reagent 8** Mouse HRP Polymer RTU (15 min)				
13	Step 14	Counter stain(**Note 2**) User supplied (5-10 sec)				
14	Step 15	**Reagent 9** Emerald Chromogen RTU (5min)				
15	Step 16	**It is important to follow the protocol. To maintain stain!** Dehydrate section 20seconds for each step				
16	Step 17	**Reagent 10** 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-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.
2.*Using as a co-localization staining kit,

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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:

TS308A 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.*

	Main Protocol Step	**TS308A Protocol-2**	**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 12**	Mouse 1°Ab (sensitive to HIER) User supplied (30-60min)				
3	**Step 13**	**Reagent 8 (RTU)** Mouse HRP Polymer RTU (15min)				
4	**Step 6**	**Reagent 4A&4B** DAB requires mixing (5 min)				
5	**Step 8**	**Reagent 6** Antibody Blocker requires mixing (10min)				
6	**Step 9**	HIER (DAB will not be removed)				
7	**Step 10**	**Reagent 7A (RTU)** DS-MMR Blocker A RTU (30min)				
8	**Step 11**	**Reagent 7B (RTU)** DS-MMR Blocker B RTU (5min)				
9	**Step 3**	Mouse 1°Ab & Rabbit 1°Ab mix (Abs requires HIER) User supplied (30-60 min)				
10	**Step 4**	**Reagent 1** Mouse primer RTU 15min				
11	**Step 5**	**Reagent 2&Reagent 3** Mouse AP Polymer & Rabbit HRP Polymer require mixing (30min) Wash with 1x TBS-T				
12	**Step 7**	**Reagent 5A, Reagent 5B& Reagent 5C** GBI-Permanent Red requires mixing. (10min)				
13	**Step 14**	Counter stain(**Note 2**) User supplied (5-10 sec.)				
14	**Step 15**	**Reagent 9** Emerald Chromogen RTU (5min.)				
15	**Step 16**	**It is important to follow the protocol. To maintain stain!** Dehydrate section 20seconds for each step				
16	**Step 17**	**Reagent 10** U-Mount RTU Mount & coverslip				
	Result	**Stain pattern on controls are 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,

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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:

TS308A 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.

	Main Protocol Step	TS308A Protocol-3	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 & Rabbit 1°Ab mix User supplied (30-60min.)				
3	**Step 4**	**Reagent 1** Mouse primer RTU 15min				
4	**Step 5**	**Reagent 2&Reagent 3** Mouse AP Polymer & Rabbit HRP Polymer require mixing. (30min)				
5	**Step 6**	**Reagent 3A&Reagent 3B** DAB require mixing. (5min)				
6	**Step 7**	**Reagent 5A, Reagent 5B& Reagent 5C** GBI-Permanent Red requires mixing. (10min)				
7	**Step 8**	**Reagent 6** Antibody Blocker required mixing. (10min)				
8	**Step 9**	HIER Refer to antibody datasheet.				
9	**Step 10**	**Reagent 7A** DS-MMR Blocker A RTU (30min)				
10	**Step 11**	**Reagent 7B** DS-MMR Blocker B RTU (5min)				
11	**Step 12**	Mouse 1°Ab (Not sensitive to HIER) User supplied (30-60min.)				
12	**Step 13**	**Reagent 8** Mouse HRP Polymer (RTU) (15min.)				
13	**Step 14**	Counter stain(**Note2**) User supplied				
14	**Step 15**	**Reagent 9** Emerald Chromogen (RTU) (5min)				
15	**Step 16**	**It is important to follow the protocol to maintain stain!** Dehydrate section 20seconds for each step				
16	**Step 17**	**Reagent 10** U-Mount RTU Mount & coverslip				
	Result	**Stain pattern on controls are 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.

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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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