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), AP (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. For frozen tissue a lower temperature of 65°C may be used for Antibody Blocker (Reagent 6) 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 AP-Red+ (Red). **Polink TS-MMR-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 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	**Ms AP-Primer** (RTU)	12ml	36ml	120ml
Reagent 2	**Ms AP-Polymer** (RTU)	6ml	18ml	60ml
Reagent 3	**Rb HRP-Polymer** (RTU)	6ml	18ml	60ml
Reagent 4A	**DAB substrate** (RTU)	12ml	18ml x 2	120ml
Reagent 4B	**DAB chromogen** (20x)	1.5 ml	2ml	6ml
Reagent 5A	**AP-Red+ Enhancer** (40x)	1ml	1ml	2ml
Reagent 5B	**AP-Red+ Solution** (40x)	1ml	1ml	2ml
Reagent 5C	**AP-Red+ Substrate** (20x)	4ml	4ml	8ml
Reagent 6	**Antibody Blocker** (40x)	2 x 15ml	100ml	3 x 100ml
Reagent 7	**Ms HRP-Primer** (RTU)	12ml	36ml	120ml
Reagent 8	**Ms HRP-Polymer** (RTU)	12ml	36ml	120ml
Reagent 9	**Emerald Chromogen** (RTU)	12ml	36ml	120ml

HRP = Horseradish Peroxidase AP = Alkaline Phosphatase Ms = Mouse Rb = Rabbit

Protocol Notes:

1. 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 deparffinized 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:** Incubate 1st primary mouse antibody with rabbit antibody. Never combine two antibodies from the same host species in one incubation step.
8. The fixation, tissue slide 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.

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.

9700 Harbour Place, Suite 129. Mukilteo, WA 98275, USA Tel: 425.493.1801 Fax: 425.493.1803 http://www.gbi-inc.com

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. Slides warmer (or hair dyer, hybridizer, vacuum oven) that can keep temperature to 85°C during slides heat drying process
7. Wash buffer: 0.01 M PBS with 0.5% Tween20, pH7.4
8. Peroxidase and alkaline phosphatase blocking buffer
9. 100% ethanol
10. 100% Xylene
11. Hematoxylin
12. 100% methanol at -20°C
13. Mounting medium
14. 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 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.

You may use the work sheet provided in the kit to remind you steps of each experiment.

Triple Staining Protocol TS308A

Steps / Reagent	**Staining Protocol**	**Incubation Time**
1. Peroxidase and alkaline phosphatase Blocking Reagent Supplied by user	a. Incubate slides in peroxidase and alkaline phosphatase blocking reagent for 10 minutes. b. Rinse slides using 2 changes of distilled water.	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 slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	Up to 1 hour
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. a. Apply 2 drops or enough volume of mouse and rabbit primary antibody mixture to cover the tissue completely. Incubate in moist chamber for 30-60 min. Recommend 30min to shorten total protocol time. b. Wash slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	30 minutes
4. **Reagent 1: Ms AP-Primer**	**Note:** This step is required for specificity and activation of Ms AP-Polymer. **DO NOT** skip. a. Apply 1 to 2 drops (50-100µl) of **Reagent 1 (Ms AP-Primer)** to cover the tissue completely. b. Incubate in moist chamber for 10 min. c. Wash slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	10 minutes
5. Mix **Reagent 2** (**Ms AP-Polymer**) with **Reagent 3** (**Rb HRP-Polymer**)	**Note:** Make sufficient polymer mixture by adding **Reagent 2 (Ms AP-Polymer)** and **Reagent 3 (Rb 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 the 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 slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	30 minutes
6. **Reagent 4A: DAB Substrate** (RTU) **Reagent 4B: DAB Chromogen** (20x)	**Note:** Make enough DAB mix by adding 1 drop of **Reagent 4B** (**DAB chromogen)** to 1ml of **Reagent 4A (DAB substrate).** Mix well. Use within 7 hours. Prepare AP-Red Plus at this time (see step 7).	5 minutes

9700 Harbour Place, Suite 129. Mukilteo, WA 98275, USA Tel: 425.493.1801 Fax: 425.493.1803 http://www.gbi-inc.com

	a. Apply 1 to 2 drops (50-100µl) of your DAB mixture to cover the tissue completely. b. Incubate for 5min. c. Rinse slides in multiple changes of distilled water 3 times for 2 minutes each or leave under running tap water for 1minute.	
7. **Reagent for AP-Red+ (5A, 5B, 5C)** **5A: AP-Red+ Enhancer** (40x) **5B: AP-Red+ Solution** (40x) **5C: AP-Red+ Substrate** (20x)	a. Add 1 drop (50µl) of **Reagent 5A** (**Enhancer)** and 1 drop of **Reagent 5B** (**Solution**) to a test tube. Mix well and set at room temperature for 5 minutes. b. Add 2ml of distilled water to the mixture. Mix well. c. Add 4 drops (200µl) of **Reagent 5C** (**Substrate**) to the mixture and mix well. d. Apply 2 drops (100µl) or enough volume of AP-Red Plus mixture to cover the tissue completely. Incubate for 20 minutes observe appropriate color development. e. Rinse slides in multiple changes of distilled water 2 minutes 3 times or leave in running tap water for 1minute.	20 minutes
8. **Reagent 6:** **Antibody Blocker (**40x)	**Note:** This step will block antibodies of previous step so no cross reaction will occur at end of protocol. HIER can be done immediately after **Antibody Blocker** step if only one primary antibody requires antigen retrieval. a. Use hot plate or water bath to heat diluted **Reagent 6** to 1x solution (1 part of **Antibody Blocker** in 39 parts of distilled water) to 80-95°C. Make enough volume to cover the tissue in beaker. b. Place slides in heated Antibody Blocker for 10 minutes at 80-95°C. c. Remove slides from the Antibody blocker; cool slides 5 seconds. d. Rinse slides in multiple changes of distilled water. e. Wash with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	10minutes
9. Antigen retrieval if required. **Refer to primary antibody data sheet**.	a. Refer to primary antibody data sheet for antigen retrieval methods. b. Wash slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	Up to 1 hour
10. 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 **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 slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	30 minutes
11. **Reagent 7: Ms HRP-Primer** (RTU)	**Note:** This step is required for specificity and activation of Ms HRP-Polymer. **DO NOT** skip. a. Apply 1 to 2 drops (50-100µl) of the **Reagent 7 (Ms HRP-Primer)** to cover the tissue completely. b. Incubate in moist chamber for 10 min. c. Wash slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	10 minutes
12. **Reagent 8: (Ms HRP-Polymer)** (RTU)	a. Apply 1 to 2 drops (50-100µl) of **Reagent 8 (Ms HRP-Polymer)** to cover the tissue completely. b. Incubate in moist chamber for 10 min. c. Wash slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times. d. Rinse slides with tap water for 2 minutes.	10 minutes
13. Counterstain (**Optional**) Not provided	a. Counterstain dip in diluted hematoxylin for 5 seconds. (Dilute hematoxylin 1:5 in dH2O). **DO NOT** over stain with hematoxylin. b. Rinse thoroughly with tap water for 15-30 seconds. c. Put slides in PBS for 5 seconds to blue, **DO NOT** over blue. d. Rinse well in distilled or tap water for 15-30 seconds.	5 seconds
14. **Reagent 9:** **Emerald Chromogen** (RTU) **Do counterstain 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 10 seconds! ***Important to READ:*** Emerald Chromogen is water soluble, do counter stain first. *Do not leave slides sitting in water.* Always stain Emerald chromogen **AFTER** AP-Red+ stain because AP-Red+ removes the Emerald and after hematoxylin.	5-10 minutes
15.Heat drying slides: User may choose any drying method to dry slides	First, wipe off excessive water then air dry slides for a few seconds. Choose one of the four heat drying processes to stabilize chromogens. **By hybridizer:** a. Set temperature to 85°C. b. Place slides in the hybridizer and heat for 10 minutes. c. Re-rack slides and allow slides to cool for 1 or 2 minutes before going	10 minutes

9700 Harbour Place, Suite 129. Mukilteo, WA 98275, USA Tel: 425.493.1801 Fax: 425.493.1803 http://www.gbi-inc.com

	to the next step. **CAUTION: DO NOT** touch hot slides with bare hands! **By slide warmer:** a. Set temperature to 85°C. b. Place slide in the slide warmer and heat for 10 minutes. c. Re-rack the slides and allow slides to cool for 1 or 2 minutes before going to next step. **CAUTION: DO NOT** touch hot slides with bare hands! **By vacuum oven:** a. Preheat Vacuum oven to 85°C. b. Place the slide rack tissue side up in the oven and apply vacuum to >10in of Hg. Heat for 5 minutes. c. Take out slides and allow slides to cool for 1 or 2 minutes before going to next step. **CAUTION: DO NOT** touch hot slides with bare hands! **By hair dryer:** (**CAUTION:** To prevent electrocution move the slide tray to a dry area in the lab. Hair Dryer and Water do not mix!) a. Turn hair dryer on High. b. Hold hair dryer about 2 inches above slides. c. Slowly move the Hair dryer back and forth over the slides! **DO NOT** let the dryer stay over one tissue more than 3 seconds. d. Blow air over sections until Emerald turns from blue to green. It takes 2 minutes of waving the hair dryer back and forth over the slides. e. Check every 15 seconds after the first two minutes for green. f. Re-rack slides and allow slides to cool for 1 or 2 minutes before going to next step. **CAUTION: DO NOT** touch hot slides with bare hands.	
16.Fix slides in -20°C methanol supplied by user.	a. Let the hot slides cool down for 30 seconds in a -20°C freezer. b. Place slides into -20°C methanol for 2 minutes. Move slides directly to 100% Ethanol **step 17**. Do not leave longer in MeOH! Do not let dry! **CAUTION: Do not air dry slides after methanol fixation! It will erase AP Red stain!**	2 minutes
17. Cover slip slides with permanent Xylene base mountant.	a. 100% ethanol for **10 seconds**. b. 100% Ethanol for **10 seconds**. Longer time may wash away AP Red! c. Dip **3 to 5 seconds** in Xylene. Longer time will wash away Emerald! d. Quickly lay slides down. e. Add Xylene based mountant to slides and apply glass coverslip. Apply force to coverslip to squeeze out any extra mountant and bubble for optimal clarity.	30 seconds

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. 3. Set slides in water too long so that AP-Red is washed away. 4. Set slides in Xylene too long so that Emerald is washed away.
No stain on 1 or 2 antibodies	Missing steps or step reversed.
Emerald did not turn green	Drying process is too short.
Artifacts on slides	Slides are not completely dried before mount.

Remarks:
For research use only.

9700 Harbour Place, Suite 129. Mukilteo, WA 98275, USA Tel: 425.493.1801 Fax: 425.493.1803 http://www.gbi-inc.com

Work Sheet for TS308A kit

We designed work sheet to help you track each step since triple color staining takes many steps. You may use this sheet for our technical support staff to review if needed.

- Used for tester to check "√ "each step during the experiment.
- Steps follow after de-paraffinization.
- Refer to insert for details of each step.

Step/ Protocol	Protocol TS308A	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase Block				
Step 2	HIER if needed				
Step 3	Ms 1°Ab & Rb 1°Ab mix (30-60 min.)				
Step 4	Ms AP-Primer (10 min.)				
Step 5	Ms AP-Polymer & Rb HRP-Polymer mix (30 min.)				
Step 6	DAB (5 min.)				
Step 7	AP-Red+ (20 min)				
Step 8	Antibody Blocker (10 min.)				
Step 9	HIER if needed				
Step 10	Ms 1°Ab (30-60 min.)				
Step 11	Ms HRP-Primer (10 min.)				
Step 12	Ms HRP-Polymer (10 min.)				
Step 13	Counter stain (5 sec.)				
Step 14	Emerald (5-10 min.)				
Step 15	Heat drying (10 min.)				
Step 16	-20°C MeOH Fix (2 min.)				
Step 17	Mount & coverslip				

Testing result:

9700 Harbour Place, Suite 129. Mukilteo, WA 98275, USA Tel: 425.493.1801 Fax: 425.493.1803 http://www.gbi-inc.com