Polink TS-MMR-Hu B Kit for Immunohistochemistry Staining

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

Storage: 2-8ºC
Important: To obtain optimal staining do not deviate from protocol.

Catalog No.:		
☐	TS301B-6	*24ml (for 120 slides)
☐	TS301B-18	*72ml (for 360 slides)
☐	TS301B-60	*240ml (for 1200 slides)

**Volume of polymer conjugate*

Intended Use:
The **Polink TS-MMR-Hu B 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 human tissue or cell samples. Tissue specimens are paraffin embedded; or freshly prepared monolayer 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-MMR-Hu B Kit** from GBI Labs (Golden Bridge International) supplies polymer enzyme conjugates: Polymer-HRP anti rabbit, Polymer-AP anti mouse and Polymer-HRP anti mouse with three chromogens, DAB (brown color); AP-Red+ (red color); and Ni-DAB (black color). **Polink TS-MMR-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 more than 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 to permanently mount slides with coverslip.

Kit Components:

Component No.	Content	TS301B-6	TS301B-18	TS301B-60
Reagent 1	Rb HRP-Polymer (RTU)	6ml	18ml	60ml
Reagent 2	Ms AP-Polymer (RTU)	6ml	18ml	60ml
Reagent 3A	DAB Substrate (RTU)	12ml	18mlx2	120ml
Reagent 3B	DAB chromogen (20x)	1ml	2ml	6ml
Reagent 4A	AP-Red+ Activator (40x)	1ml	1ml	3ml
Reagent 4B	AP-Red+ Chromogen (40x)	1ml	1ml	3ml
Reagent 4C	AP-Red+ Substrate (20x)	4ml	4ml	12ml
Reagent 5	Antibody Blocker (40x)	2 x 15ml	100ml	3 x 100ml
Reagent 6	Ms HRP-Polymer (RTU)	12ml	36ml	120ml
Reagent 7A	DAB-Ni Substrate Buffer (20x)	1ml	2ml	6ml
Reagent 7B	Hydrogen Peroxide (20X)	1ml	2ml	6ml
Reagent 7C	Nickel Solution (7x)	3ml	6ml	18ml

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.

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.

Equipment or material needed but not provided:
1. Equipment and material for deparaffinization, such as fume absorbing hood, etc.

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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.

Staining protocol A:

Steps / Reagent	**Staining Protocol**	**Incubation Time**
1. Peroxidase and phosphatase Blocking Reagent Supplied by user	a. Incubate slides in peroxidase and phosphatase blocking reagent for 10 minutes. b. Rinse the slide using 2 changes of distilled water.	10 minutes
2. Antigen retrieval if needed: Refer to primary antibody data sheet.	a. Refer to primary antibody data sheet for antigen retrieval methods b. Wash 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 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 30-60 min. Recommend 30min to shorten total protocol time. b. Wash slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	30 minutes
4. Mix **Reagent 1:Rb HRP-Polymer** (RTU) with **Reagent 2:Ms AP-Polymer** (RTU)	**Note:** Make sufficient polymer mixture by adding **Reagent 1** (**Rb HRP-Polymer**) and **Reagent 2** (**Ms 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. 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
5. **Reagent 3A: DAB Substrate** **Reagent 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. Prepare AP-Red Plus at this time (see step 6). 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, 2 each time or under running tap water for 1minute.	5 minutes
6. **Reagents 4A, 4B, 4C** **4A: AP-Red Plus Activator (40x)** **4B: AP-Red Plus Chromogen (40x)** **4C: AP-Red Plus Substrate (20x)**	a. Add 1 drop (50µl) of **Reagent 4A** (**Activator**) and 1 drop of **Reagent 4B** (**Chromogen**) 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 4C** (**Substrate**) to the mixture and mix well. d. Apply 2 drops (100µl) or enough volume of AP-Red Plus mixture to	20 minutes

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	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 running tap water for 1minute.	
7. **Reagent 5** **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 5** 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. Put 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.	10 minutes
8. Antigen retrieval: **Refer to primary antibody data sheet.**	a. Refer to primary antibody data sheet for antigen retrieval methods. Wash slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	
9. 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 slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	30 minutes
10. **Reagent 6** **Ms HRP-Polymer(RTU)**	a. Apply 1 to 2 drops (50-100µl) of **Reagent 6** (**Ms HRP-Polymer**), to cover the tissue completely. Incubate slides in moist chamber for 15 min. b. Wash slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times. c. You need to set up temperature (see step 13) for heat drying process at the end of this step.	15 minutes
11. **Reagent 7A** **DAB-Ni Substrate Buffer (20x)** **Reagent 3B** **DAB Chromogen (20x)** **Reagent 7B** **Hydrogen Peroxide (20x)** **Reagent 7C** **Nickel Solution (7x)**	a. Prepare 1ml of distilled water. Add 1 drop of DAB-Ni Substrate Buffer (**Reagent 7A**) into 1ml of distilled water. Mix well. b. Add 1 drop of DAB Chromogen (**Reagent 3B**) and 1 drop of concentrated Hydrogen Peroxide (**Reagent 7B**) to the diluted Reagent. Mix well. c. Add 3 drops of Nickel Solution (**Reagent 7C**) to the mixture. Mix well. d. Add about 100µl (2 drops) of the pre-diluted DAB-Ni mixture 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. Keep away from light during operation and use the prepared DAB-Ni mixture within 7 hours at 4℃.	10 minutes
12. Counterstain (**Optional**) Not provided	a. Counterstain with 2 drops (100µl) or enough volume of hematoxylin to completely cover tissue. Incubate for 5 to 10 seconds. **DO NOT** over stain with hematoxylin! b. Wash slides thoroughly with tap water for 1 minute. c. Put slides in PBS for 5-10 seconds to blue, **DO NOT** over blue. Wash slides well in distilled or tap water for 1 minute.	5 seconds
13 Use an Aqueous mounting media such as GBI Labs Simpo-Mount **Cat # E03-15 for 15ml** **(Option 1)**	a. Rinse slide with **DISTILLED OR DEIONIZED** WATER, touch the edges of slide on a paper towel to remove excess water and quickly wipe the back of the slide. b. Invert dropper bottle to displace bubbles from the tip-end. c. Squeeze out the first drop onto a paper towel to remove of air bubble on the tip. d. Apply 3 drops of Simpo-Mount to cover the tissue section. **DO NOT** coverslip on top of the Simpo-Mount. e. Rotate the slides to allow Simpo-Mount spread evenly to cover the tissue section. f. Dry for recommend time see data insert.	10min
14.Xylene based mountant Fix slides as follows **(Option 2)**	a. Let the Heat Dry completely. Use method from TS301A on website www.gbilabs.com. b. Place slides into -20°C methanol for 2 minutes. Do not leave longer! Move slides directly to 100% Ethanol, see **step 15**. Do not let dry! **CAUTION: Do not air dry slides after methanol fixation! It will erase AP Red stain!**	2 minutes
15. Cover slip slides with permanent Xylene base mountant. **(Option 2)**	a. 100% Ethanol for **15 seconds**. b. 100% Ethanol for **30-60 seconds**. Longer may wash away AP Red! c. Dip **3 to 5 seconds** in Xylene. Quickly lay slides down.	30 seconds

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	d. 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.	

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
No stain on 1 or 2 antibodies	Missing steps or step reversed
Artifacts on slides	Slides are not completely dried before mount

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 TS301B 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 TS301B	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	Rb HRP-Polymer & Ms AP-Polymer mix (30 min.)				
Step 5	DAB (5 min.)				
Step 6	AP-Red+ (20 min)				
Step 7	Antibody Blocker (10 min.)				
Step 8	HIER if needed				
Step 9	Ms 1°Ab (30-60 min.)				
Step 10	Ms HRP-Polymer (15 min.)				
Step 11	Ni-DAB (5-10 min.)				
Step 12	Counter stain (5 sec.)				
Step 13 (Option 1)	Aqueous mountant				
Step 14 (Option 2)	Heat dry, -20 ºC MeOH Fix (2 min.) **Note: This step is to maintain AP-Red+ staining to dehydrate in EtOH and xylene.**				
Step 15 (Option 2)	Xylene Mount & coverslip				

Testing result:

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