Polink TS-MMR-Hu A 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 Emerald(Green)

Storage: 2-8ºC

Catalog No.:
- ☐ TS301A-6 *24ml (for 120 slides)
- ☐ TS301A-18 *72ml (for 360 slides)
- ☐ TS301A-60 *240ml (for 1200 slides)

Volume of polymer conjugate

Intended Use:

The **Polink TS-MMR-Hu 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 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 steps to be done in their defined order. Choose from protocol TS301A protocol-1, TS301A protocol-2 and TS301A protocol-3 to accommodate your primary antibodies sensitivity to pre-treatment.

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 A 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); AP-Red+(red); and Emerald(green). **Polink TS-MMR-Hu 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 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	TS301A-6	TS301A-18	TS301A-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+ Enhancer (40x)	1ml	1ml	3ml
Reagent 4B	AP-Red+ Solution (40x)	1ml	1ml	3ml
Reagent 4C	AP-Red+ Substrate (20x)	4ml	4ml	12ml
Reagent 5A	TS-MMR Blocker A(RTU)	12ml	18mlx2	120ml
Reagent 5B	TS-MMR Blocker B(RTU)	12ml	18mlx2	120ml
Reagent 6	Ms HRP-Polymer (RTU)	12ml	18mlx2	120ml
Reagent 7	Emerald Chromogen (RTU)	12ml	18mlx2	120ml

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.
2. Heat source (microwave or hot plate) for HIER and antigen retrieval buffers
3. Thermometer

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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.
- TS301A Protocol-2 is suitable for one Ms & one Rb primary Abs need pre-treatment, the other Ms primary Ab is sensitive to pre-treatment.
- TS301A Protocol-3 is suitable when one Ms & one Rb primary antibody is sensitive to pre-treatment but the second Ms 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 TS301A protocol-1:

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 (**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/ 0.05% Tween20 for 2 minutes, 3 times.	
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 Enhancer (40x)** **4B: AP-Red Plus Solution (40x)** **4C: AP-Red Plus Substrate (20x)**	a. Add 1 drop (50µl) of **Reagent 4A** (**Enhancer**) and 1 drop of **Reagent 4B** (**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 4C** (**Substrate**) to the mixture and mix well.	20 minutes

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	d. Rinse slides with tap water. 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 running tap water for 1minute.	
7. **Reagent 5A** **TS-MMR Blocker A(RTU)**	a. Apply 2 drops or enough volume of **Reagent 5A (DS-MMR Blocker A**) to cover the tissue completely. Mix well on the slide and incubate in moist chamber for 30 min. b. Rinse with PBS containing 0.05% Tween-20 for 2 min., 3 times.	30 min
8. **Reagent 5B** **TS-MMR Blocker B(RTU)**	a. Apply 2 drops or enough volume of **Reagent 5B (DS-MMR Blocker B**) to cover the tissue completely. Mix well on the slide and Incubate in moist chamber for 10 min. b. Rinse with PBS containing 0.05% Tween-20 for 2 min., 3 times.	5 min
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 14) for heat drying process at the end of this step.	15 minutes
11. Counterstain (**Optional but must be done before Emerald Chromogen step**) Not provided	**Note:** If two antigens are co-localized in nuclear 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 15-30 seconds. c. Put slides in PBS for 5-10 seconds to blue, **DO NOT** over blue. d. Rinse well in distilled or tap water for 15-30 seconds.	5 seconds
12. **Reagent 7** **Emerald Chromogen (RTU)** **Do hematoxylin first.**	a. Apply 1 to 2 drops (50-100µl) of **Reagent 7** (**Emerald Chromogen**) to cover the tissue completely. b. Incubate slides in humid chamber for 5-10 minutes. c. Wash slides in tap water for 3 times for 15 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.	10 minutes
13.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 to the step 14. **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 step 14. **CAUTION: DO NOT** touch hot slides with bare hands! **By vacuum oven:** a. Preheat Vacuum oven to 85°C. b. Place the slides in 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	10 minutes

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	going to step 14. **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 them cool for 1 or 2 minutes before going to step 14. **CAUTION: DO NOT** touch hot slides with bare hands.	
14.Fix slides	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. Do not leave longer! Do not let dry! Move slides directly to 100% Ethanol, see **step 15**. **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.	a. 100% Ethanol for **10 seconds**. b. 100% Ethanol for **10 seconds**. Longer may wash away AP Red! c. Dip **3 to 5 seconds** in Xylene. Longer may 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

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 AP-Red is washed away
No stain on 1 or 2 antibodies	Missing steps or step reversed
Emerald does not turn green	Drying process is too short.
AP-Red is leaching	Recommend to use 100% fresh methanol and ethanol.
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 TS301A 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

TS301A Protocol-1 is suitable for all primary Abs need pre-treatment or all primary Abs do not need pre-treatment.

	Main Protocol Step	TS301A Protocol-1	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	**Step 1**	Peroxidase Block User supplied				
2	**Step 2**	HIER(Optional)				
3	**Step 3**	Ms 1°Ab & Rb 1°Ab mix User supplied (30-60 min.)				
4	**Step 4**	**Reagent 1** Rb HRP-Polymer & **Reagent 2** Ms AP- Polymer mixing required (30 min.)				
5	**Step 5**	**Reagent 3A&3B** DAB (5 min.) mixing required				
6	**Step 6**	**Reagent 4A,4B&4C** AP-Red+ (20 min) mixing required				
7	**Step 7**	**Reagent 5A (RTU)** DS-MMR Blocker A(30min)				
8	**Step 8**	**Reagent 5B (RTU)** DS-MMR Blocker B(5min)				
9	**Step 9**	Ms 1°Ab User supplied (30-60 min.)				
10	**Step 10**	**Reagent 6 (RTU)** Ms HRP-Polymer (15 min.)				
11	**Step 11**	Counter stain(**Note 2**) User supplied (5-10 sec.)				
12	**Step 12**	**Reagent 7 (RTU)** Emerald (5-10 min.)				
13	**Step 13**	Heat drying (10 min.)				
14	**Step 14**	-20 ºC MeOH Fix User supplied (2 min.)				
15	**Step 15**	Mount & coverslip User supplied				
	Result	**Stain pattern on controls are correct: Fill in Yes or NO**				

Note1: Normal wash steps = Wash with PBS containing 0.05% Tween-20 for 3 times for 2 min 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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TS301A Protocol-2 is suitable for one Ms & one Rb primary Abs need pre-treatment, the other Ms primary Ab is sensitive to pre-treatment.

	Main Protocol Step	TS301A Protocol-2	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	**Step 1**	Peroxidase Block User supplied				
2	**Step 9**	Ms 1°Ab (sensitive to HIER) User supplied (30-60 min.)				
3	**Step 10**	**Reagent 6 (RTU)** Ms HRP-Polymer (15 min.)				
4	**Step 5**	**Reagent 3A&3B** DAB (5 min.) mixing required				
5	**Step 2**	HIER (DAB will not be removed)				
6	**Step 7**	**Reagent 5A (RTU)** DS-MMR Blocker A(30min)				
7	**Step 8**	**Reagent 5B (RTU)** DS-MMR Blocker B(5min)				
8	**Step 3**	Ms 1°Ab & Rb 1°Ab mix (Abs requires HIER) User supplied (30-60 min.)				
9	**Step 4**	**Reagent 1** Rb HRP-Polymer & **Reagent 2** Ms AP- Polymer mixing required (30 min.)				
10	**Step 6**	**Reagent 4A,4B&4C** AP-Red+ (20 min) mixing required				
11	**Step 11**	Counter stain(**Note 2**) User supplied (5-10 sec.)				
12	**Step 12**	**Reagent 7 (RTU)** Emerald (5-10 min.)				
13	**Step 13**	Heat drying (10 min.)				
14	**Step 14**	-20 ºC MeOH Fix User supplied (2 min.)				
15	**Step 15**	Mount & coverslip User supplied				
	Result	**Stain pattern on controls are correct: Fill in Yes or NO**				

Note1: Normal wash steps = Wash with PBS containing 0.05% Tween-20 for 3 times for 2 min 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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TS301A Protocol-3 is suitable when one Ms & one Rb primary antibody is sensitive to pre-treatment but the second Ms primary antibody needs pre-treatment.

	Main Protocol Step	TS301A Protocol-3	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	Step 1	Peroxidase Block User supplied				
2	Step 3	Ms 1°Ab & Rb 1°Ab mix User supplied (30-60 min.)				
3	Step 4	**Reagent 1** Rb HRP-Polymer & **Reagent 2** Ms AP- Polymer mixing required (30 min.)				
4	Step 5	**Reagent 3A&3B** DAB (5 min.) mixing required				
5	Step 6	**Reagent 4A,4B&4C** AP-Red+ (20 min) mixing required				
6	Step 2	HIER				
7	Step 7	**Reagent 5A (RTU)** DS-MMR Blocker A(30min)				
8	Step 8	**Reagent 5B (RTU)** DS-MMR Blocker B(5min)				
9	Step 9	Ms 1°Ab (sensitive to HIER) User supplied (30-60 min.)				
10	Step 10	**Reagent 6 (RTU)** Ms HRP-Polymer (15 min.)				
11	Step 11	Counter stain(**Note2**) User supplied				
12	Step 12	**Reagent 7 (RTU)** Emerald (5-10 min.)				
13	Step 13	Heat drying (10 min.)				
14	Step 14	-20 ºC MeOH Fix User supplied (2 min.)				
15	Step 15	Mount & coverslip User supplied				
	Result	**Stain pattern on controls are correct: Fill in Yes or NO**				

Note1: Normal wash steps = Wash with PBS containing 0.05% Tween-20 for 3 times for 2 min 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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