Polink TS-MRR-Hu A Kit

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

Storage: 2-8ºC

Catalog No.:

☐	TS302A-6	*6mL	60 slides**
☐	TS302A-18	*18mL	180 slides**
☐	TS302A-60	*60mL	600 slides**

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

Intended Use:
The **Polink TS-MRR-Hu A Kit** is designed to use with user supplied one mouse primary antibodies and two rabbit primary antibodies to detect three distinct antigens on human tissue or cell samples. This kit has been tested on paraffin embedded tissue specimens. It also can be used for frozen tissue or cell smears. Please read through entire protocol as this protocol requires many steps 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-MRR-Hu A Kit** from GBI Labs (Golden Bridge International) supplies polymer enzyme conjugates: anti-mouse HRP Polymer, anti-rabbit AP Polymer, and anti-rabbit HRP Polymer with three chromogens, DAB (brown); GBI-Permanent Red (red); and Emerald (green). **Polink TS-MRR-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 with the ability to permanently mount slides with coverslip.

Kit Components:

Component No.	Content	TS302A-6	TS302A-18	TS302A-60
Reagent 1	Rabbit AP Polymer (RTU)	6mL	18mL	60mL
Reagent 2	Mouse HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 3A	DAB Substrate (RTU)	12mL	36mL	120mL
Reagent 3B	DAB Chromogen (20x)	1.5mL	2mL	6mL
Reagent 4A	GBI-Permanent Red Substrate (RTU)	15mL	36mL	120mL
Reagent 4B	GBI-Permanent Red Activator (5x)	3mL	7.2mL	24mL
Reagent 4C	GBI-Permanent Red Chromogen (100x)	150µL	360µL	1.2mL
Reagent 5	Antibody Blocker (40x)	30mL	50mL	100mL
Reagent 6A	TS-MRR Blocker A (RTU)	6mL	18mL	60mL
Reagent 6B	TS-MRR Blocker B (RTU)	6mL	18mL	60mL
Reagent 7	Rabbit HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 8	Emerald Chromogen (RTU)	6mL	18mL	60mL
Reagent 9	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 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 pH 7.6. GBI sells 10xTBS-T for your convenience (B11).

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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
5. Beaker
6. Wash buffer: 0.01 M PBS with 0.5% Tween20, pH7.4
7. Peroxidase and alkaline phosphatase blocking buffer
8. 100% ethanol
9. 100% Xylene
10. Hematoxylin
11. 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 2 nd rabbit 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 rabbit antibodies and one mouse antibody requires HIER.

Staining protocol A:

Steps / Reagent	Staining Protocol	Incubation Time
1. Peroxidase and Alkaline Phosphatase Blocking Reagent: Not provided	We recommend using **GBI Dual Block E36.** It is fast, easy and, 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. 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.	30 min
4. **Reagent 1:** Rabbit AP Polymer (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 1** to cover the tissue completely. b. Incubate in moist chamber for 15-30 min. c. Wash slides with PBS/ 0.05% Tween 20 for 2 minutes, 3 times.	15-30 min
5. **Reagent 2:** Mouse HRP Polymer (RTU)	a. Apply 1 to 2 drops of **Reagent 2** to cover the tissue completely. b. Incubate in moist chamber for 15-30 min. c. Wash slides with PBS/ 0.05% Tween 20 for 2 minutes, 3 times.	15-30 min
6. **Reagents 3A, 3B:** **3A:** DAB Substrate (RTU) **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 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. a. Wash with 1xTBS-T only, 3 times for 2 minutes each.	5 min
7. **Reagents 4A, 4B, 4C:** **Reagent 4A:** GBI-Permanent Red Substrate (RTU) **Reagent 4B:** GBI-Permanent Red Activator (5x) **Reagent 4C:** GBI-Permanent Red Chromogen (100x)	**Note**: Shake GBI-Permanent Red Activator before adding into GBI-Permanent Red Substrate. a. Add 200µL of **Reagent 4B** (Activator) into 1mL of **Reagent 4A** (Substrate) and mix well. Add 12µL of **Reagent 4C** (Chromogen) into the mixture and mix well. [**Note: For fewer slides**, add 100µL of **Reagent 4B** (Activator) into 500µL of **Reagent 4A** (Substrate) and mix well. Add 6µL of **Reagent 4C** (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. c. Rinse well with distilled water. **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 10 min.**	10 min

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8. **Reagent 5:** 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 5** (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 8** if not complete **step 7e** and move on to **step 9.** e**.** Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	10 min
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 6A:** TS-MRR Blocker A (RTU)	a. Apply 2 drops or enough volume of **Reagent 6A** (DS-MRR Blocker A) to cover the tissue completely. b. Mix well on the slide and 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.	30 min
11. **Reagent 6B:** TS-MRR Blocker B (RTU)	a. Apply 2 drops or enough volume of **Reagent 6B** (DS-MRR Blocker B) to cover the tissue completely. b. Mix well on the slide and incubate in moist chamber for 5 min. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	5 min
12. 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. b. Incubate in moist chamber for 30-60 min. Recommend 30 minutes to shorten total protocol time. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	30 min
13. **Reagent 7:** Rabbit HRP Polymer (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 7** (Rabbit HRP Polymer) to cover the tissue completely. b. Incubate slides in moist chamber for 15 min. c. Rinse thoroughly with distilled water.	15 min
14. 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 1min. e. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	5 sec
15. **Reagent 8:** Emerald Chromogen (RTU) **Do hematoxylin first**	a. Apply 1 to 2 drops (50-100µL) of **Reagent 8** (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. **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 because GBI-Permanent Red removes the Emerald.**	5 min
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 20 seconds a. Dehydrate with 95% ethanol 20 seconds b. Dehydrate with 100% ethanol 20 seconds c. Dehydrate with 100% ethanol 20 seconds d. Dehydrate with 100% ethanol 20 seconds e. Dehydrate with xylene 20 seconds **CAUTION: DO NOT dehydrate in xylene longer than 20 seconds! It will erase GBI-Permanent Red stain!**	2 min
17. **Reagent 9:** U-Mount (RTU)	a. Apply 1 drop (50µL) of **Reagent 9** (U-Mount) to cover the tissue section and apply glass coverslip. Apply force to coverslip to **squeeze out any extra mount** 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.	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	Missing steps or steps reversed.
Green Background on the slide	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	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 TS302A 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

TS302A Protocol-1 is suitable when all primary antibodies need pre-treatment, or all primary antibodies do not need pre-treatment.

	Main Protocol Step	TS302A 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**: Rabbit AP Polymer (15-30min)				
5	Step 5	**Reagent 2:** Mouse HRP Polymer (15-30min)				
6	Step 6	**Reagent 3A& Reagent 3B** DAB requires mixing. (5min)				
7	Step 7	**Reagent 4A& Reagent 4B** GBI-Permanent Red requires mixing. (10min)				
8	Step 8	**Reagent 5** Antibody Blocker requires mixing. (10min)				
9	Step 10	**Reagent 6A** DS-MRR Blocker A RTU (30min)				
10	Step 11	**Reagent 6B** DS-MRR Blocker B RTU (5min)				
11	Step 12	Rabbit 1°Ab User supplied (30-60 min)				
12	Step 13	**Reagent 7** Rabbit HRP Polymer RTU (15 min)				
13	Step 14	Counter stain (**Note 2**) User supplied (5-10 sec)				
14	Step 15	**Reagent 8** 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 9** 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.
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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TS302A 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	TS302A 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**	Rabbit 1°Ab (sensitive to HIER) User supplied (30-60min)				
3	**Step 13**	**Reagent 7 (RTU)** Rabbit HRP Polymer RTU (15min)				
4	**Step 6**	**Reagent 3A&3B** DAB requires mixing (5 min)				
5	**Step 8**	**Reagent 5** Antibody Blocker requires mixing (10min)				
6	**Step 9**	HIER (DAB will not be removed)				
7	**Step 10**	**Reagent 6A (RTU)** DS-MRR Blocker A RTU (30min)				
8	**Step 11**	**Reagent 6B (RTU)** DS-MRR 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** Rabbit AP Polymer (15-30min) Wash with 1x TBS-T				
11	**Step 5**	**Reagent 2** Mouse HRP Polymer (15-30min) Wash with 1x TBS-T				
12	**Step 7**	**Reagent 4A & Reagent 4B** GBI-Permanent Red requires mixing. (10min)				
13	**Step 14**	Counter stain (**Note 2**) User supplied (5-10 sec.)				
14	**Step 15**	**Reagent 8** 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 9** 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 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 results:

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TS302A 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	TS302A 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** Rabbit AP Polymer (15-30min)				
4	**Step 5**	**Reagent 2** Mouse HRP Polymer (15-30min)				
5	**Step 6**	**Reagent 3A&Reagent 3B** DAB require mixing. (5min)				
6	**Step 7**	**Reagent 4A&Reagent 4B** GBI-Permanent Red requires mixing. (10min)				
7	**Step 8**	**Reagent 5** Antibody Blocker required mixing. (10min)				
8	**Step 9**	HIER Refer to antibody datasheet.				
9	**Step 10**	**Reagent 6A** DS-MRR Blocker A RTU (30min)				
10	**Step 11**	**Reagent 6B** DS-MRR Blocker B RTU (5min)				
11	**Step 12**	Rabbit 1°Ab (sensitive to HIER) User supplied (30-6min.)				
12	**Step 13**	**Reagent 7** Rabbit HRP Polymer (RTU) (15min.)				
13	**Step 14**	Counter stain (**Note2**) User supplied				
14	**Step 15**	**Reagent 8** 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 9** 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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