Polink TS-GMR-Hu B Kit

Polymer-HRP & AP triple staining kit
Detects goat, mouse, and rabbit primary antibodies on human tissue with DAB(Brown), GBI-Permanent Red (Red), and DAB-Ni (Black)

Storage: 2-8ºC

Catalog No.:
- ☐ TS303B-6 24mL* 120 slides**
- ☐ TS303B-18 72mL* 360 slides**
- ☐ TS303B-60 240mL* 1200 slides**

**Volume of polymer conjugate*
*** if use 100µL per slide*

Intended Use:
The **Polink TS-GMR-Hu B Kit** is designed to use with user supplied goat/mouse/rabbit primary antibodies to detect three distinct antigens on a single human tissue or cell samples. This Kit has been tested on paraffin embedded tissue only; however, it may be used on frozen or freshly prepared monolayer 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-GMR-Hu B Kit from GBI Labs (Golden Bridge International) supplies polymer enzyme conjugates: Polymer-HRP anti-goat, Polymer-AP anti-rabbit and Polymer-HRP anti-mouse with three chromogens, DAB (brown); GBI-Permanent Red (red); and DAB-Ni (black). Polink TS-GMR-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 reaction when detecting three different primary antibodies using our unique blocking system. Simplified steps allow users to complete triple staining within 3 hours (without antigen retrieval) or 4 hours (with antigen retrieval). The well tested protocol provides user to permanently mount slides with coverslip.

Kit Components:

Component No.	Content	TS303B-6	TS303B-18	TS303B-60
Reagent 1	Goat HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 2	Rabbit AP Polymer (RTU)	6mL	18mL	60mL
Reagent 3A	DAB Substrate (RTU)	15mL	18mLx2	120mL
Reagent 3B	DAB Chromogen (20x)	2mL	4mL	6mL
Reagent 4A	GBI-Permanent Red Substrate (RTU)	15mL	18mLx2	120mL
Reagent 4B	GBI-Permanent Red Activator (5x)	3mL	7.2mL	12mLx2
Reagent 4C	GBI-Permanent Red Chromogen (100x)	150µL	360µL	1.2mL
Reagent 5	TS-GMR Blocker (RTU)	12mL	18mLx2	120mL
Reagent 6	Mouse HRP Polymer (RTU)	12mL	18mLx2	120mL
Reagent 7A	DAB-Ni Substrate (20x)	1mL	2mL	6mL
Reagent 7B	Hydrogen Peroxide (20X)	1mL	2mL	6mL
Reagent 7C	Nickel Solution (7x)	3mL	6mL	18mL
Reagent 8	Simpo-Mount (RTU)	15mL	18mLx2	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. 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.
8. 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 (B11xx)

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, Beaker, Timer
4. Wash buffer: 0.01 M PBS with 0.5% Tween20, pH7.4
5. Peroxidase and alkaline phosphatase blocking buffer
6. 100% ethanol, 100% Xylene
7. Hematoxylin, Coverslip

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TS303B Staining Protocol:

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. 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-T containing 0.05% Tween-20 or **1X TBS-T (See note 8 above)**; 3 times for 2 minutes each.	
3. Primary Antibody Mix: **Mix one Goat, 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 goat, 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 minutes
4. Mix **Reagents 1** and **2:** **Reagent 1:** Goat HRP Polymer (RTU) **Reagent 2:** Rabbit AP Polymer (RTU)	**Note:** Make sufficient polymer mixture by mixing **Reagent 1** (Goat HRP Polymer) with **Reagent 2** (Rabbit 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. 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.	30 minutes
5. **Reagents 3A & 3B:** **Reagent 3A:** DAB Substrate (RTU) **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 working solution to cover the tissue completely. b. Incubate for 5min. c. Rinse thoroughly with distilled water. d. Wash with 1xTBS-T only, 3 times for 2 minutes each.	5 minutes
6. **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) **To get maximum sensitivity of AP polymer, repeat chromogen step**	**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 buffer) and mix well. Add 10µL 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 buffer) and mix well. Add 5µL 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. **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.	10 minutes
7. **Reagent 5:** TS-GMR Blocker (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 5** (TS-GMR Blocker) to cover the tissue completely. Incubate for 10min. b. Rinse slides in multiple changes of distilled water 3 times, 2min each time. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	10 minutes
8. **Reagent 6:** Mouse HRP Polymer (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 6** (Mouse HRP Polymer) 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.	15 minutes
9. **Reagents 7A, 3B, 7B, 7C:** **Reagent 7A:** DAB-Ni Substrate(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 DAB-Ni working solution 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°C.	5 minutes
10. Counterstain (**Optional**): Not provided	a. Counterstain with 2 drops (100µL) or enough volume of counterstain solution to completely cover tissue. Incubate for 10-15sec. b. Rinse thoroughly with tap water for 2-3min. c. Rinse well in distilled water.	10-15 seconds
11. **Reagent 8:** Simpo-Mount (RTU)	a. Apply 2 drops (100µL) or enough volume of **Reagent 8** (Simpo-Mount) to cover tissue when tissue is wet. Rotate the slides to allow Simpo-Mount to spread evenly. b. Place slides horizontally in an oven at 40-50°C for at least 30 minutes or leave it at room temperature until slides are thoroughly dried.	

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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 Xylene too long so that GBI-Permanent Red is washed away.
No stain on 1 or 2 antibodies	1. Missing steps or steps reversed.
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 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 TS303B Kit

We designed these work sheets to help you track of each step. When staining fails these sheets help our technical support staff to pinpoint the problem.
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

TS303B Protocol-A is suitable for all primary antibodies need pre-treatment, all primary antibodies do not need pre-treatment or all primary antibodies are not sensitive to pre-treatment.

	Main Protocol Step	**TS303B Protocol-A**	**Experiment 1 Date:**	**Experiment 2 Date:**	**Experiment 3 Date:**	**Experiment 4 Date:**
1	**Step 1**	Peroxidase and phosphatase Block Recommend GBI Labs E36 User supplied				
2	**Step 2**	HIER(Optional)				
3	**Step 3**	Goat 1°Ab, Rabbit 1°Ab& Mouse 1°Ab mix User supplied (30-60 min.)				
4	**Step 4**	**Reagent 1**&**Reagent 2** Goat HRP Polymer & Rabbit AP Polymer require mixing (30 min.)				
5	**Step 5**	**Reagent 3A & Reagent 3B** DAB requires mixing (5 min.)				
6	**Step 6**	**Reagent 4A, Reagent 4B**&**Reagent 4C** GBI-Permanent Red requires mixing (10 min)				
7	**Step 7**	**Reagent 5** TS-GMR Blocker (10min)				
8	**Step 8**	**Reagent 6** Mouse HRP Polymer RTU (15 min)				
9	**Step 9**	**Reagent 7A, Reagent 3B, Reagent 7B & Reagent 7C** DAB-Ni requires mixing RTU (5min)				
10	**Step 10**	Counter stain(10-15sec) User supplied				
11	**Step 11**	**Reagent 8** Simpo-Mount (RTU)				
	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.
Testing result:

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