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Polink TS-GMR-Hu A 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 Emerald (Green))

Storage: 2-8ºC

Catalog No.:

☐	TS303A-6	*6mL	60 slides**
☐	TS303A-18	*18mL	180 slides**
☐	TS303A-60	*60mL	600 slides**

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

Intended Use:
The **Polink TS-GMR-Hu A Kit** is designed to be used with user supplied goat, mouse, and rabbit primary antibodies to detect three distinct antigens on human tissue or cell samples. TS-GMR-Hu has been tested on paraffin embedded tissue only; however, it may be used on frozen or freshly prepared monolayer cell smears. Please read through the 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. Polink TS-GMR-Hu A Kit from OriGene supplies polymer enzyme conjugates: Goat HRP Polymer, Rabbit AP Polymer, Mouse HRP Polymer, and three chromogens, DAB (brown), GBI-Permanent Red (red), and Emerald (green). Polink TS-GMR-HU A Kit is a non-biotin system, thus 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). This well tested protocol allows the user to permanently mount slides with coverslips.

Kit Components:

Component No.	Content	TS303A-6	TS303A-18	TS303A-60
Reagent 1	Rabbit AP Polymer (RTU)	6mL	18mL	60mL
Reagent 2	Goat 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	TS-GMR Blocker (RTU)	6mL	18mL	60mL
Reagent 6	Mouse HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 7	Emerald Chromogen (RTU)	6mL	18mL	60mL
Reagent 8	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. 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 (B11).

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TS303A Staining Protocol

Steps / Reagent	Staining Protocol	Incubation Time
1. Peroxidase and Alkaline Phosphatase Blocking Reagent: Not provided	We recommend using **GBI Dual Block E36xx.** 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 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- 60 min. 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 min each.	30 min
4. **Reagents 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 with **1X TBS-T**; 3 times for 2 min each. **Note:** longer incubation may increase background.	15-30 min
5. **Reagents 2:** Goat HRP Polymer (RTU)	a. Apply 1 to 2 drops (50-100μL) of **Reagent 2** to cover the tissue completely. b. Incubate in moist chamber for 15-30 min. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 min each. **Note:** longer incubation may increase background.	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 5 min. c. Rinse thoroughly with distilled water. d. Wash with 1xTBS-T only, 3 times for 2 min 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**: First bring **Reagent 4A** and **Reagent 4B** to room temperature. Shake **Reagent 4B** (Activator) before adding into **Reagent 4A** (Substrate). a. Add 200μL of **Reagent 4B** (Activator) into 1mL of **Reagent 4A** (Substrate) and mix until clear. 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 until clear. 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. **To increase AP signal aspirate or tap off chromogen and apply 2-3 drops 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 min
8. **Reagent 5:** TS-GMR Blocker (RTU)	a. Apply 1-2 drops of **Reagent 5** to cover the tissue completely b. Incubate for 10 min c. Rinse slides in multiple changes of distilled water 3 times for 2 min each. d. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 min each.	10 min
9. **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. b. Incubate slides in moist chamber for 15 min. c. Wash in distilled water for 2 min.	15 min
10. Counterstain: **(Optional. Must be done before Emerald Chromogen step)** Not provided	**Note:** If two antigens are co-localized in the nucleus, reduce counterstain protocol time to optimize visualization. If antigens are co-localized in the cytoplasm or membrane or the antigens are localized in different cells, you can use normal counterstaining protocol time. a. Counterstain dip in diluted hematoxylin for 5 sec for nuclear co-localization or 30 sec for cytoplasmic or membrane co-localization. Do not overstain with hematoxylin. b. Rinse thoroughly with tap water for 1 min. c. Put slides in PBS for 5-10 sec to blue. Do not over blue if two proteins are co-localized in the nucleus. d. Rinse well in distilled water for 2 min. e. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 min each	10-15 sec
11. **Reagent 7:** Emerald Chromogen (RTU)	a. Apply 1-2 drops of **Reagent 7** (Emerald Chromogen) to cover the tissue completely. b. Incubate slides in a humid chamber for 5 min. c. Wash slides in distilled water; 3 times for 30 sec. **Note:** Emerald Chromogen is water soluble. Do counterstain first. Do not leave slides sitting in water. Always stain Emerald Chromogen **AFTER** GBI-Permanent Red stain and **AFTER** hematoxylin because GBI-Permanent Red removes the Emerald.	

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12. Dehydrate section: It is important to follow this protocol	**Note: Wipe off extra water and air dry the slides before dehydration** a. Dehydrate with 80% ethanol 20 seconds b. Dehydrate with 95% ethanol 20 seconds c. Dehydrate with 100% ethanol 20 seconds d. Dehydrate with 100% ethanol 2 seconds e. Dehydrate with 100% ethanol 20 seconds f. Dehydrate with xylene 20 seconds **Caution:** Do not dehydrate in xylene for longer than 20 seconds. It will erase the GBI-Permanent Red stain.	
13. **Reagent 8:** U-Mount (RTU)	a. Apply 1 drop of **Reagent 8** (U-Mount) to cover the tissue section and apply the glass coverslip. b. Apply force to the coverslip to squeeze out any extra mountant and bubbles for optimal clarity. Removing excess also help leaching of GBI-Permanent Red stain.	

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. Slides set in water for too long and the Emerald washed away. 4. Slides set in Xylene for too long and GBI-Permanent Red washed away.
Emerald Chromogen is blue not green when non-co-localized with GBI-Permanent Red	1. Emerald should be green when non-co-localized with GBI-Permanent Red. If Emerald chromogen is blue, then the titer on the primary antibody is not diluted enough for this protocol. Re-titer the primary antibody individually first.
No stain on 1 or 2 antibodies	1. Missing steps or steps reversed.
Green background on the slide	1. Titer primary antibody.
GBI-Permanent Red is leaching	1. Use fresh 100% ethanol and xylene 2. Slides set in Xylene too long. Do not go over 20 seconds
Artifacts on slides	1. Slides not completely dried before mounting. 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 TS303A 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

TS302B Protocol-1 is suitable when 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	TS303A Protocol-1	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase and Alkaline Phosphatase Block E36 is recommended. User supplied				
Step 2	HIER (Optional) Refer to antibody datasheet User supplied				
Step 3	Goat 1°Ab, Rabbit 1°Ab, Mouse 1°Ab mix User supplied (30-60min)				
Step 4	**Reagent 1** Rabbit AP Polymer (15-30min)				
Step 5	**Reagent 2** Goat HRP Polymer (15-30min)				
Step 6	**Reagent 3A, 3B** DAB requires mixing. (5min) **Wash with 1x TBS-T after rinse with distilled water.**				
Step 7	**Reagent 4A, 4B, 4C** GBI-Permanent Red requires mixing. (10min)				
Step 8	**Reagent 5** TS-GMR Blocker (10min)				
Step 9	**Reagent 6** Mouse HRP Polymer (15min)				
Step 10	**Counterstain** User supplied (5-10sec)				
Step 11	**Reagent 7** Emerald Chromogen (5min)				
Step 12	**Dehydrate second** It is important to follow the protocol. (20 sec each step)				
Step 13	**Reagent 8** U-Mount and cover slip				
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.
Note 2: If using as a co-localization staining kit: If antigens are co-localized in nucleus, counterstain and blue should take 5 seconds. If antigens are co-localized in cytoplasm, membrane, or in different cells, counterstain with normal protocol time.
Testing result:

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