Polink TS-GMR-Hu B Kit for Immunohistochemistry Staining

Polymer-HRP & AP triple staining kit to detect 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)	1.5mL	2mL	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	U-Mount (RTU)	3mL	9mL	NA

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. 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.

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

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Staining protocol TS303B protocol:

Steps / Reagent	Staining Protocol	Incubation Time
1. Peroxidase and phosphatase Blocking Reagent Supplied by user	a. Incubate slides in peroxidase and phosphatase blocking reagent (Klear Dual Enzyme Block **E36** is Recommended)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 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 slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	30 minutes
4. Mix **Reagent 1** with **Reagent 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 slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	30 minutes
5. **Reagent 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. Incubate for 5min. b. Rinse slides in multiple changes of distilled water 3 times, 2min each time or under running tap water for 2minute.	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)	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. 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/ 0.05% Tween20 for 2 minutes, 3 times.	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 slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	15 minutes
9. **Reagent 7A,3B,7B,7C&7D** **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.	10-15 seconds

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	c. Rinse well in distilled water.	
11.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 80% ethanol 20seconds. b. Dehydrate with 95% ethanol 20seconds. c. Dehydrate with 100% ethanol 20seconds. d. Dehydrate with 100% ethanol 20seconds. e. Dehydrate with 100% ethanol 20seconds. f. Dehydrate with xylene 20seconds. **CAUTION: DO NOT dehydrate in xylene longer than 20 seconds! It will erase GBI-Permanent Red stain!**	2 min
12. **Reagent 8** U-Mount (RTU)	a. Apply 1 drop (50µL) of **Reagent 8** (U-Mount) to cover the tissue section and apply glass coverslip. b. Apply force to coverslip to squeeze out any extra mountant and bubbles for optimal clarity. Removing excess also to prevent leaching of GBI-Permanent Red stain.	

Trouble shooting

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 step 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.

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.

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 insure 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 after de-paraffinization
- Refer to insert for details of each step

TS303B Protocol-A is suitable for all primary antibodies need pre-treatment, all primary antibodiess 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**	**It is important to follow the protocol. To maintain stain!** Dehydrate section 20seconds for each step				
12	**Step 12**	**Reagent 8** U-Mount RTU Mount & coverslip				
13	**Step 13**	**Stain pattern on controls are correct: Fill in Yes or NO**				
	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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