Polink DS-RR-Hu/Ms A Kit for Immunohistochemistry Staining

Polymer-HRP & AP double staining kit to detect two rabbit primary antibodies on human/mouse tissue with DAB (Brown) and GBI Permanent Red (Red)

Storage: 2-8ºC

Catalog No.:
- ☐ DS204A-6/D80-6A 12mL* 60 slides**
- ☐ DS204A-18 36mL* 180 slides**
- ☐ DS204A-60 120mL* 600slides**

**Total volume of polymer Conjugates*
*** if use 100µl per slide*

Intended Use:
Polink DS-RR-Hu/Ms A Kit is designed to use with user supplied two rabbit antibodies to detect two distinct antigens on human tissue or cell samples. This kit has been tested in paraffin tissue. However, this kit can be used on frozen specimen and freshly prepared monolayer cell smears.
Double staining is one of most common methods used in immunohistostaining that allow revealing two distinct antigens in a single tissue [1, 2]. **Polink DS-RR-Hu/Ms A Kit** from GBI Labs (Golden Bridge International) supplies two polymer enzyme conjugates: HRP polymer anti-Rabbit IgG and AP polymer anti-Rabbit IgG with two distinct substrates/chromogens, DAB (brown color, use with HRP polymer anti-Rabbit IgG) and GBI Permanent Red (red color, use with AP polymer anti-Rabbit IgG). When the AP Polymer anti-Rabbit antigen is present only the GBI Permanent Red will be present or when HRP Polymer anti-rabbit antigen is present only the DAB(brown) will be present. **Polink DS-RR-Hu/Ms A Kit** is non-biotin system that avoids endogenous biotin non-specific binding.

Kit Components:

Component No.	Content	12mL Kit	36mL Kit	120mL Kit
Reagent 1	Rabbit HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 2A	DAB Substrate (RTU)	12mL	15mLx2	70mL
Reagent 2B	DAB Chromogen (20X)	1.5mL	2mL	3.5mL
Reagent 3A	DS-RR-Blocker A (RTU)	6mL	18mL	60mL
Reagent 3B	DS-RR-Blocker B (RTU)	6mL	18mL	60mL
Reagent 4	Rabbit AP Polymer (RTU)	6mL	18mL	60mL
Reagent 5A	GBI-Permanent Red Substrate (RTU)	15mL	18mLx2	70mL
Reagent 5B	GBI-Permanent Red Activator (5x)	3mL	7.2mL	14mL
Reagent 5C	GBI-Permanent Red Chromogen (100x)	150µL	360µL	0.7mL
Reagent 6	Simpo-Mount (RTU)	7mL	18mL	70mL

Recommended Protocol:

1. 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 need to be adhered to the slide tightly to avoid tissue falling off.
3. Paraffin embedded section must be deparffinized with xylene and rehydrated with a graded series of ethanol before staining.
4. Cell smear samples should be made as much monolayer as possible to obtain satisfactory results.
5. Three control slides will aid the interpretation of the result: positive tissue control, reagent control (slides treated with Isotype control reagent), and negative control.
6. Proceed IHC staining: DO NOT let specimen or tissue dry from this point on.
7. 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 pH7.6.

GBI sells 10xTBS-T for your convenience (B11xx)

Reagent	Staining Procedure	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. We recommend **GBI Dual Block E36xx**. b. Rinse the slides using 2 changes of distilled water.	10min
2. HIER Pretreatment: Refer to antibody data sheet.	a. Heat Induced Epitope Retrieval (HIER) may be required for primary antibody. Refer to antibody datasheet. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T(See note 7 above)**; 3 times for 2 minutes each.	
3. Preblock (optional)	For paraffin section, Improved formula saves the need for a preblock step. For frozen tissue, preblock may or may not be required depending on fixative. (Preblock catalogue No.:E07 was Recommended.)	

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4. Rabbit Antibody 1: Supplied by user	***Notes:*** Investigator needs to optimize dilution and incubation times prior to double staining. a. Apply 2 drops or enough volume of rabbit primary antibody 1 to cover the tissue completely. Incubate in moist chamber for 30-60 min. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	30-60min
5.**Reagent 1:** Rabbit HRP polymer (RTU)	a. Apply 1drop (50µL) of **Reagent 1** (Rabbit HRP) polymer to cover each section. b. Incubate in moist chamber for 15 min. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	15min
6. **Reagents 2A, 2B:** 2A: DAB Substrate (RTU) 2B: DAB Chromogen (20x)	a. Add 1 drop or 2 drops (for higher sensitivity and contrast) of **Reagent 2B** (DAB Chromogen) to 1mL **Reagent 2A** (DAB Substrate). Mix well. Protect from light and use within 5 hours. b. Apply 2 drops or enough volume of DAB CHROMOGEN mixture to completely cover tissue. Incubate for 3-10 min. c. Rinse thoroughly with distilled water 4 times, 2 minutes each time. d. Wash with **1X TBS-T only**; 3 times for 2 minutes each.	3-10min
7. **Reagent 3A:** DS-RR-Block A (RTU)	a. Apply 2 drops or enough volume of **Reagent 3A** (DS-RR-Block A) to cover the tissue completely. Mix well on the slide and 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.	30min
8. **Reagent 3B:** DS-RR-Block B (RTU)	a. Apply 2 drops or enough volume of **Reagent 3B** (DS-RR-Block B) to cover the tissue completely. Mix well on the slide and Incubate in moist chamber for 5 min. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	5min
9. Rabbit antibody 2: Supplied by user	***Notes:*** Investigator needs to optimize dilution and incubation times prior to double staining. a. Apply 2 drops or enough volume of rabbit primary antibody 2 to cover the tissue completely. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	30-60min
10. **Reagent 4:** Rabbit AP polymer (RTU)	a. Apply 1drop (50µL) of **Reagent 4** (Rabbit AP polymer) to cover each section. b. Incubate in moist chamber for 15 min. c. Wash with **only 1xTBS-T** 3 times for 2 minutes each.	15min
11. **Reagent 5A, 5B, 5C** **Reagent 5A:** GBI-Permanent Red Substrate (RTU) **Reagent 5B:** GBI-Permanent Red Activator (5x) **Reagent 5C:** 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 5B** (Activator) into 1mL of **Reagent 5A** (Substrate) and mix well. Add 10µL of **Reagent 5C** (Chromogen) into the mixture and mix well. [**Note:** For fewer slides, add 100µL of **Reagent 5B** (Activator) into 500µL of **Reagent 5A** (Substrate) and mix well. Add 5µL of **Reagent 5C** (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.	10min + 5-10mins
12. HEMATOXYLIN Not provided	a. Counterstain with 2 drops (100µL) or enough volume of hematoxylin to completely cover tissue. Incubate for 10-15 seconds. b. Rinse thoroughly with tap water for 2-3 min c. Put slides in PBS until show blue color (about ½ - 1 min.) d. Rinse well in distilled water	
13. **Reagent 6:** Simpo-Mount (RTU)	a. Apply 2 drops (100µL) or enough volume of **Reagent 6** (Simpo-Mount) to cover tissue when tissue is wet. Rotate the slides to allow Simpo-Mount spread evenly. DO NOT coverslip. 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.	30min. in 40-50°C oven Or: overnight at room temperature

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Protocol Notes:

1. The fixation, tissue slide thickness, antigen retrieval and primary antibody dilution and incubation time affect results significantly. Investigator needs to consider all factors and determine optimal conditions when interpreting the result.
2. **GBI-Permanent Red** is insoluble in organic solvent and can be coverslipped as well. however the dehydration steps must be shorter for optimal tissue structure and chromogen signal maintenance.
   Note: Please wipe off extra water and air dry slides before dehydration and clear.
   a. 1x 80% Ethanol 20 seconds;
   b. 1x 95% Ethanol 20 seconds;
   c. 3x 100% Ethanol 20 seconds each;
   d. 1x 100% Xylene 20 seconds;
   e. Add 1 drop of xylene based mountant (Cat. No. O-Mount, E02-18) and coverslip. Press to push the air bubble out.

CAUTION: DO NOT dehydrate in xylene longer than 20 seconds! It will erase GBI-Permanent Red stain!

Precautious:
DAB may be carcinogenic. Please wear gloves and take other necessary precautions.

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 DS204A 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

Protocol Step	DS204A Protocol Reagent/Time	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase & levamisole Block E36 is recommended. User supplied				
Step 2 Optional	HIER if needed User supplied (up to 60 min)				
Step 3 Optional	Preblock User supplied				
Step 4	Rabbit primary antibody 1 User supplied (30-60 min)				
Step 5	**Reagent 1** Rabbit HRP Polymer RTU (15min)				
Step 6	**Reagent 2** DAB requires mixing (5min)				
Step 7	**Reagent3A** DS-RR-Block A RTU (30min)				
Step 8	**Reagent 3B** DS-RR-Block B RTU (5min)				
Step 9	Rabbit primary antibody 2 User supplied (30-60 min)				
Step 10	**Reagent 4** Rabbit AP Polymer RTU (15min) Wash with **1xTBS-T only**.				
Step 11	**Reagent 5A, 5B & 5C** GBI Permanent Red requires mixing (10min)				
Step 12	Counter stain Hematoxylin User supplied				
Step 13	**Reagent 6** Simpo-Mount RTU Do not coverslip!				
Result	Stain pattern on controls are correct: Fill in Yes or NO				

Testing result:

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