Polink DS-MR-Ms A Kit for Immunohistochemistry Staining

Polymer-HRP & AP double staining kit to detect a rabbit and a mouse primary antibody on mouse tissue with DAB (Brown) and GBI-Permanent Red(Red).

Storage: 2-8ºC

Catalog No.:

☐	DS233A-6	12mL* 120 slides**
☐	DS233A-18	36mL* 360 slides**
☐	DS233A-60	120mL* 1200slides**

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

Intended Use:

The **Polink DS-MR-Ms A Kit** is designed to use with user supplied mouse and rabbit primary antibody to detect two distinct antigens on mouse tissue or cell samples. DS233 kits can be used on frozen specimens, paraffin–embedded tissues, or freshly prepared monolayer cell smears. DS233 kits is designed not to give background on most mouse strains however there may be some mouse strains especially when using frozen that require additional blocking; we recommend GBI's Klear Mouse Block (D54-xx) to improve specificity of the mouse primary antibody on mouse tissue.

Double staining is one of most common methods used in immunohistostaining that allows detection of two distinct antigens in a single tissue[1, 2]. **Polink DS-MR-Ms A Kit** from GBI Labs-Inc supplies two polymer enzyme conjugates: Mouse HRP Polymer and Rabbit AP Polymer with two distinct substrates/chromogens, DAB (brown color, use with the Mouse HRP Polymer) and GBI-Permanent Red(red color, use with the Rabbit AP Polymer). A Primer step is used to increase specificity of antibody staining. Both enzyme conjugates are applied to the specimen at the same time and mixed on the slide. This kit offers simplified steps that make for a quicker and easier protocol than that used in a sequential procedure. **Polink DS-MR-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	Mouse Primer (RTU)	12mL	18mLx2	120mL
Reagent 2	Mouse HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 3	Rabbit AP Polymer (RTU)	6mL	18mL	60mL
Reagent 4A	DAB Substrate (RTU)	15mL	18mLx2	120mL
Reagent 4B	DAB Chromogen (20x)	1.5mL	2mL	6mL
Reagent 5A	GBI-Permanent Red Substrate (RTU)	15mL	18mLx2	120mL
Reagent 5B	GBI-Permanent Red Activator (5x)	3mL	7.2mL	12mLx2
Reagent 5C	GBI-Permanent Red Chromogen (100x)	150µL	360µL	1.2mL
Reagent 6	Simpo-Mount (RTU)	12mL	18mLx2	120mL

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 needs to be adhered to the slide tightly to avoid falling off.
3. Paraffin embedded sections must be deparaffinized with xylene and rehydrated with a graded series of ethanol before staining.
4. Cell smear samples should be made up to as much of a 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 slide using distilled water at least twice.	10 min.
2. HIER Pretreatment: Refer to Ab data sheet.	a. Heat Induced Epitope Retrieval (HIER) may be required for primary antibody suggested by vendor. 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. **Klear Mouse Block A (D54-A)**	a. Add 2 drops (100µL) or enough volume of **D54-A Klear Ms Blocking A** to	30 min.

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Not provided **(optional see protocol note 2)**	cover the tissue section and Incubate. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	
4. **Klear Mouse Block B (D54-B)** Not provided **(optional see protocol note 2)**	a. Add 2 drops (100µL) or enough volume of **D54-B Klear Ms Blocking B** to cover the tissue section and Incubate. **Do not** exceed 5min. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	5 min.
5. Mouse antibody 1 and Rabbit antibody 2: Supplied by user	**Note:** Investigator needs to optimize dilution and incubation times prior to double staining. a. Apply 2 drops or enough volume of both Ms Primary Antibody 1 and Rb Primary Antibody 2 to cover the tissue completely. Mix well on the slide and 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.	30-60 min.
6. **Reagent 1** Mouse Primer(RTU)	a. Add 2 drops (100µL) or enough volume of **Reagent 1** (Mouse Primer) to cover the tissue section and Incubate Room Temperature for 10-15minutes. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	15min
7. **Reagent 2 and 3** **Reagent 2 :** Mouse HRP Polymer(RTU) **Reagent 3:** Rabbit AP Polymer(RTU)	**Note:** Make sufficient polymer mixture by adding **Reagent 2** (Mouse HRP Polymer) and **Reagent 3** (Rabbit AP Polymer) at 1:1 ratio, mix well. Do Not mix more than you need for the experiment because the polymer mixture is not stable for long term storage. a. Apply 1 to 2 drops (50-100µl) of the mixture to cover the tissue completely. b. 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.
8. **Reagent 4A and 4B** **Reagent 4A:** DAB Substrate (RTU) **Reagent 4B:** DAB Chromogen(20x)	a. Add 1 drop of **Reagent 4B to** 1 mL of **Reagent 4A**. Mix well. Protect from light and use within 7 hours at 4 ºC. b. Apply 2 drops or enough volume of DAB Chromogen working solution to completely cover tissue. Incubate for 3-10 min. c. Rinse thoroughly with distilled water. d. Wash with 1xTBS-T only, 3 times for 2 minutes each. **DAB is a predicted carcinogen, wear gloves.**	5 min.
9. **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, Please 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 buffer) 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 buffer) 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.	10 min. + 5-10 min
10. 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 30 - 60 sec.) d. Rinse well in distilled water.	
11. **Reagent 6:** Simpo-Mount (RTU) **To coverslip see protocol note 3**.	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. 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.	

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. **Klear Mouse Block** the anti-mouse secondary has been absorbed to rat serum resulting in most mouse strains having no background, however some mouse strains may need additional blocking. **Klear Mouse Block (D54-xx)** works very well on frozen tissue.
3. **GBI-Permanent Red** is insoluble in organic solvent and can be coversliped as well. however the dehydration steps must be shorter for optimal tissue structure and chromogen signal maintenance.

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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 DS233A 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

Step/ Protocol	Protocol DS233A	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase & Alkaline Phosphatase Block (**E36** is recommended) User supplied				
Step 2	HIER if needed				
Step 3 Optional	Klear Mouse Block A (D54-A) 30min				
Step 4 Optional	Klear Mouse Block B (D54-B) 5min				
Step 5	Ms 1°Ab & Rb 1°Ab mix (30-60 min.)				
Step 6	**Reagent 1** Mouse Primer RTU (15 min.)				
Step 7	**Reagent 2 & 3** Ms HRP Polymer & Rb AP Polymer mix (30 min.)				
Step 8	**Reagent 4A & 4B** DAB Requires mixing! (5 min.)				
Step 9	**Reagent 5A, 5B, 5C** GBI-Permanent Red Requires mixing! (10 min+5-10min)				
Step 10	Counter stain Hematoxylin User supplied				
Step 11	**Reagent 6** Simpo Mount(RTU) Do not coverslip!				

Testing result:

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