Polink DS-MM-Ms B Kit for Immunohistochemistry Staining

Polymer-HRP&AP double staining kit to detect two mouse primary antibodies on mouse/rat tissues with BCIP/NBT(Purple) and AEC (Red)

Storage: 2-8ºC

Cat No.: ☐DS212B-6 12mL* for 60 slides**
☐DS212B-18 36mL* for 180slides**
☐DS212B-60 120mL* for 600slides**

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

Intended Use:

The **Polink DS-MM-Ms B Kit** is designed to use with two user supplied mouse antibodies to detect two distinct antigens on mouse and rat tissue or cell samples. This kit has been tested on paraffin embedded tissue, which can be used for frozen or freshly prepared monolayer cell smears. We recommend GBI labs proprietary Klear Rat Blocking Buffer (D102-A& D102-B) when staining rat tissue or frozen mouse or rat tissue.

Double staining is a common method used in immunohistochemistry that allows for detection of two distinct antigens in a single tissue [1, 2]. This kit uses an HRP or AP polymer based technology combined with a proprietary blocking buffer system that achieves ultra sensitivity with no background or cross reactivity. **Polink DS-MM-Ms B Kit** from GBI labs supplies the user with primer system to enhance the two polymer enzyme conjugates anti-mouse IgG HRP polymer and anti-mouse IgG AP polymer with two distinct substrates/chromogens, GBI-AEC and BCIP/NBT. BCIP/NBT reacts with anti-mouse IgG AP polymer conjugate to produce a purple color. AEC chromogen reacts with anti-mouse IgG HRP-polymer conjugate to produce a red color. **Polink DS-MM-Ms B Kit** is a non-biotin system that avoids the extra steps involved in blocking non-specific binding due to endogenous biotin. Please read the protocol carefully and use the experimental record sheet to keep track of your progress throughout the protocol.

Kit Components:

Component No.	Content	12mL Kit	36mL Kit	120mL Kit
Reagent 1	Mouse AP Polymer (RTU)	6mL	18mL	60mL
Reagent 2	BCIP/NBT (RTU)	7mL	18mL	60mL
Reagent 3	Antibody Blocker (40x)	2x15mL	50mL	100mL
Reagent 4A	DS-MM Blocker A (RTU)	6mL	18mL	60mL
Reagent 4B	DS-MM Blocker B (RTU)	6mL	18mL	60mL
Reagent 5	Mouse Primer (RTU)	6mL	18mL	60mL
Reagent 6	Mouse HRP(AEC) Polymer (RTU)	6mL	18mL	60mL
Reagent 7A	AEC Substrate (20x)	1mL	2mL	4mL
Reagent 7B	AEC Chromogen (20x)	2mL	4mL	8mL
Reagent 7C	Hydrogen Peroxide (20x)	1mL	2mL	4mL
Reagent 8	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 with IHC staining: DO NOT let specimen or tissue dry from this point on.
7. **Note:** 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. **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 (Min.)
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	a. Heat Induced Epitope Retrieval (HIER) may be required for primary antibody.	60-90 min

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to antibody data sheet.	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.	
	No background issues go to step 5; if background an issue go to step 3.	
3. **Optional**: Block step 1 **Reagent D102-A Klear Rat Block A** **free 1mL sample**	Provided in this kit is a 1 ml sample of our Klear Rat blocking buffer (**Reagent D102-A)** this block has been a staple in many labs screening mouse primary antibodies on mouse tissue. a. Apply 2 drops or enough volume of rat blocking buffer (**Reagent D102-A)** to cover the tissue completely. Incubate in moist chamber for 30min. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	30 min
4. **Optional**: Block step 2 **Reagent D102-B Klear Rat Block B** **free 1mL sample**	Use this block only if **Reagent D102-A** was used in step 3. a. Apply 2 drops or enough volume of rat blocking buffer (**Reagent D102-B)** to cover the tissue completely. Incubate in moist chamber for 5min. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	5 min
5. Ms Primary Antibody 1: Supplied by user	***Notes:*** Investigator needs to optimize dilution and incubation times prior to double staining. Should use as dilute as possible to prevent cross reaction. a. Apply 2 drops or enough volume of mouse 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.	30-60 min
6. **Reagent 1:** Mouse AP Polymer	a. Apply 1-2 drops **Reagent 1** (Mouse AP Polymer) or enough to cover each section. b. Incubate in moist chamber for 15 min. c. Wash with PBS containing 0.05% Tween-20 for 3 times for 2 min each. d. Wash with **1X TBS-T only;** 3 times for 2 minutes each.	15 min
7. **Reagent 2:** BCIP/NBT (RTU)	a. Apply 2 drops or enough volume of **Reagent 2** (BCIP/NBT) to completely cover tissue. Incubate for 5-10 min. b. Rinse thoroughly with distilled water. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	5-10min
8. **Reagent 3:** Antibody Blocker (40x) **(Optional)** Must test if antibody/antigen interaction is heat sensitive. **Please skip this step if antigen retrieval is used for 2nd Ms Primary Antibody after step 7.**	**Note:** This step will block antibodies of previous step so no cross reaction will occur at end of protocol. a. Use hot plate or water bath to heat diluted **Reagent 3** to 1x solution (1 part of **Antibody Blocker** in 39 parts of distilled water) to 80-95°C. Make enough volume to cover the tissue in beaker. b. For paraffin embedded tissue, put slides in heated Antibody Blocker for 10 minutes at 95°-100°C. For frozen embedded tissue, put slides in heated Antibody Blocker for 10 minutes at 80°C. c. Cool slides to 55°C. d. Rinse slides in multiple changes of distilled water.	10min
9. **Reagent 4A:** DS-MM Blocker A (RTU)	a. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each. b. Apply 2 drops or enough volume of **Reagent 4A** (DS-MM Blocker A) to cover the tissue completely. Mix well on the slide and 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
10. **Reagent 4B:** DS-MM Blocker B (RTU)	a. Apply 2 drops or enough volume of **Reagent 4B** (DS-MM Blocker 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.	5 min
11. Ms Primary 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 mouse 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.	30-60 min
12.**Reagent 5:** Mouse Primer (RTU)	a. Apply 1-2 drops **Reagent 5** (Mouse Primer) or enough to cover each section. b. Incubate in moist chamber for 10 min. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	15 min
13.**Reagent 6:**	a. Apply 1-2 drops **Reagent 6** Mouse HRP(AEC) Polymer to cover each section.	30 min

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Mouse HRP(AEC) Polymer (RTU)	b. Incubate in moist chamber for 10 min. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	
14. **Reagent 7A, 7B, 7C:** **Reagent 7A:** AEC Substrate (20x) **Reagent 7B:** AEC Chromogen (20x) **Reagent 7C:** Hydrogen Peroxide (20x)	a. Add 1 drop (50µL) of **Reagent 7A** to 1mL distilled water. Mix well . Add 2 drops of **Reagent 7B** and 1 drop of **Reagent 7C** to diluted reagent 1. Mix well. Keep away from light and use within 1 hour. b. Apply 2 drops (100µL) or enough volume of pre-mixed AEC solution to completely cover the tissue. Incubate for 5-15min, observe appropriate color development. c.Rinse well with distilled water. (**AEC is alcohol soluble; do not dehydrate.**)	10min
15. 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 or Tris pH 7.4 to 8.4 until blue color appears. d. Rinse well in distilled water.	5 min
16. **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 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.	30 min. 50°C oven or overnight at room temperature

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. Simpo-Mount is an aqueous-based mounting media for immunohistochemistry. It is used as the permanent mounting media for chromogens such as GBI-Permanent Red, AP-Red, AEC, and BCIP. Simpo-Mount does not use a coverslip. However, if you need to coverslip your tissue, after Simpo-Mount has dried, dip the slide in xylene (1 to 2 seconds), apply an organic mounting solution (such as O-Mount, Cat# E02-18), and place cover glass on the slide. Store slides after they have dried completely.

Precautious:
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 Immnocytochemistry Second Edition. Bios Scientific Publishers. P41-54. 1997

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Work Sheet for DS212B Kit

We designed work sheet to help you track each step. You may use this sheet for our technical support staff to review if needed.
To insure that all steps are done properly, we recommend that the user fill in the actual time of their experimental step. 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

DS212B Protocol-1 is suitable for:
1) Both mouse primary antibodies need pre-treatment;
2) One mouse primary antibody needs pre-treatment and the other one is not sensitive to pretreament.

	Protocol Step	DS212B Protocol-1 Reagent/Time	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	**Step 1**	Peroxidase & Alkaline Phosphatase Block E36 is recommend User supplied				
2	**Step 2** Optional	HIER if needed User supplied (up to 60 min)				
3	**Step 3** Optional	**D102-A** (Rt Blocking Buffer A) RTU (30 min)				
4	**Step 4** Optional	**D102-B** (Rt Blocking Buffer B) RTU (5min)				
5	**Step 5**	Ms 1°Ab #1 User supplied (30-60 min)				
6	**Step 6**	**Reagent 1** Mouse AP Polymer RTU (15 min) **Wash with 1xTBS-T only.**				
7	**Step 7**	**Reagent 2** BCIP/NBT RTU (10 min)				
8	**Step 8**	**Reagent 3** Antibody Blocker(40x) (10 min)				
9	**Step 9**	**Reagent 4A:** DS-MM Blocker A RTU (30 min)				
10	**Step 10**	**Reagent 4B:** DS-MM Blocker B RTU (5 min)				
11	**Step 11**	Ms 1°Ab #2 User supplied (30-60 min)				
12	**Step 12**	**Reagent 5:** Mouse Primer RTU (5 min)				
13	**Step 13**	**Reagent 6** Ms HRP Polymer RTU (15 min)				
14	**Step 14**	**Reagent 7A, 7B & 7C** AEC requires mixing (10min)				
15	**Step 15**	Counter stain Hematoxylin User supplied				
16	**Step 16**	**Reagent 8** Simpo-Mount RTU Do not coverslip!				
16	**Result**	**Stain pattern on controls are correct: Fill in Yes or NO**				

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DS212B Protocol-2 is suitable for one mouse primary antibody needs pre-treatment, the other mouse primary antibody is sensitive to pre-treatment.

	Protocol Step	DS212B Protocol-2 Reagent/Time	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	**Step 1**	Peroxidase & Alkaline Phosphatase Block E36 is recommend User supplied				
2	**Step 3** Optional	**D102-A** (Rat Blocking Buffer A) RTU (30 min)				
3	**Step 4** Optional	**D102-B** (Rat Blocking Buffer B) RTU (5min)				
4	**Step 5**	Ms 1°Ab #1 User supplied (30-60 min) 1°Ab is sensitive to pre-treatment				
5	**Step 6**	**Reagent 1** Mouse AP Polymer RTU (15 min) **Wash with 1xTBS-T only.**				
6	**Step 7**	**Reagent 2** BCIP/NBT RTU (10 min)				
7	**Step 8**	**Reagent 3A & 3B** DAB Requires mixing! (5 min)				
8	**Step 2**	HIER (10-15 min) Cool down (45-60 min) User supplied **Skip antibody blocker step 8 if HIER is done since they will achieve same goal.**				
9	**Step 9**	**Reagent 4A:** DS-MM Blocker A RTU (30 min)				
10	**Step 10**	**Reagent 4B:** DS-MM Blocker B RTU (5 min)				
	Step 11	Ms 1°Ab #1 User supplied (30-60 min) 1°Ab is not sensitive to pre-treatment				
11	**Step 12**	**Reagent 5:** Mouse Primer RTU (5 min)				
12	**Step 13**	**Reagent 6** Ms HRP Polymer RTU (15 min)				
13	**Step 14**	**Reagent 7A, 7B & 7C** AEC requires mixing (10min)				
14	**Step 15**	Counter stain Hematoxylin User supplied				
15	**Step 16**	**Reagent 8** Simpo-Mount RTU Do not coverslip!				
16	**Result**	**Stain pattern on controls are correct: Fill in Yes or No**				

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