Polink DS-MM-Ms A Kit for Immunohistochemistry Staining

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

Storage: 2-8ºC

Cat No.: ☐DS212A-6 12mL* for 60 slides**
☐DS212A-18 36mL* for 180slides**
☐DS212A-60 120mL* for 600slides**

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

Intended Use:

The **Polink DS-MM-Ms A Kit** is designed to use with two user supplied mouse antibodies to detect two distinct antigens on mouse tissue or cell samples. Specimens can be frozen or paraffin embedded, or freshly prepared monolayer cell smears. We recommend you use Klear Rat Blocking Buffer (D102-A& D102-B) when staining mouse tissue or frozen mouse 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 A 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, Permanent Red and DAB. Permanent Red reacts with anti-mouse IgG AP-polymer conjugate to produce a red color. DAB chromogen reacts with anti-mouse IgG HRP-polymer conjugate to produce a brown color. **Polink DS-MM-Ms A 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 Primer (RTU)	6mL	18mL	60mL
Reagent 2	Mouse HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 3A	DAB Substrate (RTU)	12mL	15mLx2	70mL
Reagent 3B	DAB Chromogen (20x)	1.5mL	2mL	3.5mL
Reagent 4	Antibody Blocker(40x)	2x15mL	50mL	100mL
Reagent 5A	DS-MM Blocker A (RTU)	6mL	18mL	60mL
Reagent 5B	DS-MM Blocker B (RTU)	6mL	18mL	60mL
Reagent 6	Mouse AP Polymer (RTU)	6mL	18mL	60mL
Reagent 7A	GBI-Permanent Red Substrate (RTU)	15mL	18mLx2	70mL
Reagent 7B	GBI-Permanent Red Activator (5x)	3mL	7.2mL	14mL
Reagent 7C	GBI-Permanent Red Chromogen (100x)	150µL	360µL	0.7mL
Reagent 8	Simpo Mount (RTU)	6mL	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. 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 (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 slide using distilled water.	10 min

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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.	60-90 min
	No background issues go to step 5; if background an issue go to step 3.	
3 **Optional**: Block step 1 **Reagent D102-A** **Rat Blocking Buffer A**(RTU)	Not provided in this kit must purchase separately Klear Rat blocking buffer (**Reagent D102-xx)** 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 A (**Reagent D102-A)** to cover the tissue completely. Incubate in moist chamber for 30min. b. Wash with PBS containing 0.05% Tween-20 for 3 times for 2 min each.	30 min
4. **Optional**: Block step 2 **Reagent D102-B** **Rat Blocking Buffer B** (RTU)	Use this block only if **Reagent D102-A** was used in step 3. a. Apply 2 drops or enough volume of rat blocking buffer B (**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.	30-60min
6.**Reagent 1:** Mouse Primer (RTU)	a. Apply 1-2 drops of **Reagent 1** (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.	10 min
7.**Reagent 2:** Mouse HRP Polymer (RTU)	a. Apply 1-2 drops of **Reagent 2** (Mouse HRP Polymer) 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.	10 min
8. **Reagents 3A, 3B:** **3A:** DAB Substrate (RTU) **3B:** DAB Chromogen (20x)	***Note:*** Although the DAB step can be done at the end of protocol, we find the DAB chromogen acts as additional shielding between the first mouse and second mouse. We recommend you do this step now. a. Add 1 drop of **Reagent 3B** (DAB chromogen) to 1mL **Reagent 3A** (DAB Substrate)**.** Mix well. Store at 4°C, protect from light and use within 7 hours. b. Apply 2 drops or enough volume of DAB chromogen mixture to completely cover tissue. Incubate for 5 min. c. Rinse well with distilled water. d. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	5 min
9. **Reagent 4:** 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 8.**	**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 4** 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. e. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	10min
10. **Reagent 5A:** DS-MM Blocker A (RTU)	a. Apply 2 drops or enough volume of **Reagent 5A** (DS-MM Blocker A) to cover the tissue completely. Mix well on the slide and Incubate in moist chamber for 30 min. b. Wash with PBS/ 0.05% Tween-20 for 2 minutes, 3 times.	30 min
11. **Reagent 5B:** DS-MM Blocker B (RTU)	a. Apply 2 drops or enough volume of **Reagent 5B** (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.	5min
12. 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.	30-60min
13. **Reagent 6:**	a. Apply 1-2 drops of **Reagent 6** (Mouse AP Polymer) or enough to cover each section.	15 min

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Mouse AP Polymer (RTU)	b. Incubate in moist chamber for 15 min. c. Wash with **1X TBS-T only**; 3 times for 2 minutes each **Note**:To intensify GBI Permanent Red signal rinse with 1x 0.1M Tris pH 8.5 to 9.0.	
14. **Reagent 7A, 7B, 7C** **Reagent 7A:** GBI-Permanent Red Substrate (RTU) **Reagent 7B:** GBI-Permanent Red Activator (5x) **Reagent 7C:** 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 7B** (Activator) into 1mL of **Reagent 7A** (Substrate) and mix well. Add 10µL of **Reagent 7C** (Chromogen) into the mixture and mix well. [Note: For fewer slides, Add 100µL of **Reagent 7B** (Activator) into 500µL of **Reagent 7A** (Substrate) and mix well. Add 5µL of **Reagent 7C** (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.
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 or enough volume of **Reagent 8** (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.	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 effect results significantly. Investigator needs to consider all factors and determine optimal conditions when interpret 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:
This kit is 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 DS212A 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

DS212A 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	DS212A Protocol-1 Reagent/Time	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	**Step 1**	Peroxidase Block 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** Ms Primer RTU (10 min)				
7	**Step 7**	**Reagent 2** Ms HRP Polymer RTU (10 min)				
8	**Step 8**	**Reagent 3A & 3B** DAB Requires mixing! (5 min)				
9	**Step 9**	**Reagent 4** Antibody Blocker(40x) (10 min)				
10	**Step 10**	**Reagent 5A:** DS-MM Blocker A RTU (30 min)				
11	**Step 11**	**Reagent 5B:** DS-MM Blocker B RTU (5 min)				
12	**Step 12**	Ms 1°Ab #2 User supplied (30-60 min)				
13	**Step 13**	**Reagent 6** Ms AP Polymer RTU (15 min)				
14	**Step 14**	**Reagent 7A, 7B, & 7C** GBI-Permanent Red requires mixing (10min)				
15	**Step 15**	Counter stain Hematoxylin User supplied				
16	**Step 16**	**Reagent 8**				

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		Simpo-Mount(RTU) Do not coverslip!				
17	**Result**	**Stain pattern on controls are correct: Fill in Yes or NO**				

DS212A Protocol-2 is suitable for one mouse primary antibody needs pre-treatment, the other mouse primary antibody is sensitive to pre-treatment.

	Protocol Step	DS212A Protocol-2 Reagent/Time	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	**Step 1**	Peroxidase Block 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** Ms Primer RTU (10 min)				
6	**Step 7**	**Reagent 2** Ms HRP Polymer 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 9 if HIER is done since they will achieve same goal.**				
9	**Step 10**	**Reagent 5A:** DS-MM Blocking A RTU (30 min)				
10	**Step 11**	**Reagent 5B:** DS-MM Blocking B RTU (5 min)				
11	**Step 12**	Ms 1°Ab #2 User supplied (30-60 min)				
12	**Step 13**	**Reagent 6** Ms AP Polymer RTU (15 min)				
13	**Step 14**	**Reagent 7A, 7B & 7C** GBI Permanent Red requires mixing (10-20min)				
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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