Polink DS-MR-Ms C Kit for Immunohistochemistry Staining

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

Storage: 2-8ºC

Catalog No.:
- ☐ DS233C-6 12mL* 120 slides**
- ☐ DS233C-18 36mL* 360 slides**
- ☐ DS233C-60 120mL* 1200slides**

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

Intended Use:
The **Polink DS-MR-Ms C 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 for detection of two distinct antigens in a single tissue[1, 2]. **Polink DS-MR-Ms C Kit** from GBI Labs-Inc supplies two polymer enzyme conjugates: Mouse HRP Polymer and Rabbit AP Polymer with two distinct substrates/chromogens, Emerald (green 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. This kit offers simplified steps that make for a quicker and easier protocol than that used in a sequential procedure. **Polink DS-MR-Ms C 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	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	Emerald Chromogen (RTU)	15mL	18mLx2	120mL
Reagent 6	U-Mount (RTU)	12mL	18mLx2	NA

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.	10min
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 Block A** to cover the tissue section and Incubate for 30min.	30min

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Not provided **(optional see protocol note 2)**	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 Block 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.	5min
5. Mouse antibody and Rabbit antibody : Supplied by user	**Note:** Investigator needs to optimize dilution and incubation times prior to double staining, as both GBI-Permanent Red and Emerald Chromogen are very strong. a. Apply 2 drops or enough volume of both Mouse Primary Antibody and Rabbit Primary Antibody 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..	30-60min
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 at 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.	10-15min
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 **1X TBS-T only**; 3 times for 2 minutes each.	30min
8. **Reagent 4A, 4B, 4C** **Reagent 4A:** GBI-Permanent Red Substrate (RTU) **Reagent 4B:** GBI-Permanent Red Activator (5x **Reagent 4C:** 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 4B** (Activator) into 1mL of **Reagent 4A** (Substrate) 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) 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. **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
9. Counterstain (**Optional**) (**Optional but must be done before Emerald Chromogen step**) Not provided	**Note:** If two antigens are co-localized in nuclear you want less counter stain to optimize the visualization in the nucleus; however you can counter stain using normal protocol time if antigens are co-localized in cytoplasm or membrane or those two antigens are localized in different cells. a. Counterstain dip in diluted hematoxylin for 5 seconds for nuclear co-localization or 30 seconds for cytoplasmic or membrane co-localization. **DO NOT** over stain with hematoxylin. b. Rinse thoroughly with tap water for 1min. c. Put slides in PBS for 5-10 seconds to blue, DO NOT over blue. d. Rinse well in distilled or tap water for 1min. e. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each..	5 seconds
10. **Reagent 5** Emerald Chromogen (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 5** (Emerald Chromogen) to cover the tissue completely. b. Incubate in moist chamber for 5 minutes. c. Wash slides in tap water for 1 minute. d. Rinse with distilled water. ***Important to READ:*** Emerald Chromogen is water soluble, counter stain first. *Do not leave slides sitting in water.* Always stain with Emerald chromogen **AFTER** GBI-Permanent Red stain and hematoxylin steps because GBI-Permanent Red removes the Emerald Chromogen.	5min
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 85% ethanol for 20seconds. b. Dehydrate with 95% ethanol for 20seconds. c. Dehydrate with 100% ethanol for 20seconds. d. Dehydrate with 100% ethanol for 20seconds. e. Dehydrate with 100% ethanol for 20seconds. f. Dehydrate with xylene for 20seconds. **CAUTION: DO NOT dehydrate with xylene longer than 20 seconds! It will erase GBI-Permanent Red stain!**	2min
12. **Reagent 6** U-Mount (RTU)	a. Apply 1 drop (50µL) of **Reagent 6** (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.	

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Trouble shoot:

Problem	Tips
Uneven stain on 2 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 water too long so that Emerald is washed away. 4. Set slides in Xylene too long so that GBI-Permanent Red is washed away.
Emerald Chromogen is blue not green when non co-localized with GBI Permanent Red.	Emerald should be green when not co-localized with GBI-Permanent Red. If Emerald chromogen is blue the titer on the primary antibody is not dilute enough for the protocol. Re-titer primary antibodies individually first.
No stain on 1 or 2 antibodies	Missing steps or step reversed.
Green Background on the slide	Titer primary antibody.
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.

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** (sample provided) 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.

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 DS233C kit

We designed this work sheet to help you track of each step. We recommend you use this sheet to record the actual time of each step conducted as it will be helpful for questions with our technical support.
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

DS233C Protocol is suitable when both mouse and rabbit primary antibodies need or do not need pre-treatment step.

Step/ Protocol	**Protocol DS233C/ Reaction time**	**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	Mouse & Rabbit 1°Antibody mix (30-60 min)				
Step 6	**Reagent 1** Mouse Primer RTU (15 min)				
Step 7	**Reagent 2& Reagent 3** Mouse HRP Polymer & Rabbit AP Polymer require mixing (30 min) **Wash only with 1xTBS-T.**				
Step 8	**Reagent 4A, Reagent 4B& Reagent 4C** GBI-Permanent Red requires mixing (10min)				
Step 9	Counter stain (Do not over counter stain) Hematoxylin User supply Wash with PBS/ 0.05% Tween20 for 2 min, 3 times.				
Step 10	**Reagent 5** Emerald Chromogen RTU (5min)				
Step 11	Dehydrate section 20seconds for each step **It is important to follow the protocol.**				
Step 12	**Reagent 6** U-Mount RTU Mount & coverslip				
Result	Stain pattern on controls are correct: Fill in Yes or NO				

Testing result:

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