Polink DS-MM-Hu C Kit for Immunohistochemistry Staining

Polymer-HRP&AP double staining kit to detect two mouse antibodies on Human tissue with GBI-Permanent-Red (Red) and Emerald (Green)

Storage: 2-8ºC

Catalog No.: ☐ DS203C-6 12mL* 60 slides**
☐ DS203C-18 36mL* 180 slides**
☐ DS203C-60 120mL* 600 slides**
**Total volume of polymer Conjugates*
*** if use 100µL per slide*

Intended Use:
The **Polink DS-MM-Hu C Kit** is designed to use with user supplied two mouse primary 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 the most common methods used in immunohistochemistry to evaluate two distinct antigens in a single tissue[1, 2]. **Polink DS-MM-Hu C Kit** from GBI Labs (Golden Bridge International) supplies two polymer enzyme conjugates: HRP polymer anti-Mouse IgG and AP polymer anti-Mouse IgG with two distinct substrates/chromogens, Emerald(Green color, use with HRP polymer anti-Mouse IgG) and GBI Permanent-Red (Red color, use with AP polymer anti-Mouse IgG). Simplified steps offer a much faster protocol and the blocking buffers prevent false positives when using two primary antibodies from the same host species. Another advantage of GBI C-Kit, it allows the researcher to visualize when two proteins are co-localized because of the color change when the chromogens overlap that can be semi-quantitative. For example, if the area of co-localization stains blue, the antigen indicated by Emerald is at higher concentration in the cell and if the color is purple than the antigen indicated by GBI Permanent-Red is expressed at higher concentrations. **Polink DS-MM-Hu 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	AP Polymer anti-Mouse IgG (RTU)	6mL	18mL	60mL
Reagent 2A	GBI-Permanent Red Substrate (RTU)	7mL	18mL	60mL
Reagent 2B	GBI-Permanent Red Activator (5x)	1.4mL	3.6mL	12mL
Reagent 2C	GBI-Permanent Red Chromogen (100x)	70µL	180µL	0.6mL
Reagent 3	Antibody Blocker (40x)	2x15mL	50mL	125mL
Reagent 4A	DS-MM Blocker A (RTU)	6mL	18mL	60mL
Reagent 4B	DS-MM Blocker B (RTU)	6mL	18mL	60mL
Reagent 5	HRP Polymer anti-Mouse IgG (RTU)	6mL	18mL	60mL
Reagent 6	Emerald Chromogen (RTU)	7mL	18mL	70mL
Reagent 7	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 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)

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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.	10 min
2. HIER Pretreatment: Refer to antibody 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. Pre-block (optional)	For paraffin section, Improved polymer formula saves the need for a pre-block step. However some primary antibodies may still require it, this information should be determined by the user prior to using DS kit. For frozen tissue, pre-block may or may not be required depending on fixative. (Pre-block catalogue No.:E07 was Recommended.)	
4. Mouse 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 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
5.**Reagent 1** AP polymer anti-Mouse IgG (RTU)	a. Apply 2 drops (100µL) of **Reagent 1** (AP polymer anti-Mouse IgG) to cover each section. b. Incubate in moist chamber for 15 min. c. Wash with **1X TBS-T only**; 3 times for 2 minutes each.	15 min
6. **Reagent 2A, 2B, 2C** **Reagent 2A:** GBI-Permanent Red Substrate (RTU) **Reagent 2B:** GBI-Permanent Red Activator (5x **Reagent 2C:** 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 2B** (Activator) into 1mL of **Reagent 2A** (Substrate buffer) and mix well. Add 10µL of **Reagent 6C**(Chromogen) into the mixture and mix well. [**Note:** For fewer slides, Add 100µL of **Reagent 2B** (Activator) into 500µL of **Reagent 2A** (Substrate buffer) and mix well. Add 5µL of **Reagent 2C**(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
7. **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.**	**Note:** This step will block antibodies of previous step so no cross reaction will occur at end of protocol. HIER can be done immediately after **Antibody Blocker** step if only one primary antibody requires antigen retrieval. For frozen tissue a lower temperature of 65ºC must be used for Antibody Blocker (**Reagent 3**) to prevent tissue from dissociating from slide. 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°C. Make enough volume to cover the tissue in beaker. b. Put slides in heated Antibody Blocker for 10 minutes at 80°C. c. Remove slides from the Antibody blocker; cool slides 5 seconds. d. Rinse slides in multiple changes of distilled water.	10 min

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	e. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	
8. **Reagent 4A** DS-MM Blocker A	a. 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. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	30 min
9. **Reagent 4B** DS-MM Blocker B	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 10 min. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	5 min
10. Mouse 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
11. **Reagent 5** HRP polymer anti-Mouse IgG (RTU)	a. Apply 2 drops (100µL) of **Reagent 5** (HRP polymer anti-Mouse IgG) 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. d. Rinse with tap water.	15 min
12. Counterstain (**Optional**) Not provided	a. Dip the slide in diluted hematoxylin for 5 seconds. (you may dilute hematoxylin 1:5 in dH2O). **DO NOT** over stain with hematoxylin. b. Rinse thoroughly with tap water for 2min. c. Put slides in PBS for 5 seconds to blue, **DO NOT** over blue. d. Rinse well in distilled or tap water for 2min. e. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	5 seconds
13. **Reagent 6** Emerald Chromogen(RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 6** (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, do counter stain first. *Do not leave slides sitting in water.* Always stain Emerald chromogen **AFTER** GBI-Permanent Red stain because GBI-Permanent Red removes the Emerald and after hematoxylin.	5 min
14.Dehydrate section	**Note: Please wipe off extra water and air dry slides before dehydration and clear.** a. Dehydrate with 85% ethanol 20seconds. b. Dehydrate with 95% ethanol 20seconds. c. Dehydrate with 100% ethanol 20seconds. d. Dehydrate with 100% ethanol 20seconds. e. Dehydrate with 100% ethanol 20seconds. f. Dehydrate with xylene 20seconds. **CAUTION: DO NOT dehydrate with xylene longer than 20 seconds! It will erase GBI-Permanent Red stain!**	2 min
15. **Reagent 7** U-Mount(RTU)	a. Apply 1 drop (50µL) of **Reagent 7** (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.	1. 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	1. Missing steps or step reversed.
Green Background on the slide	1. Titer primary antibody. 2. Use 10% Donkey serum, goat or horse serum as a preblock.
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	1. Slides not completely dried before mount. Use fresh 100% Ethanol and xylene.

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

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Work Sheet for DS203C 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

DS203C Protocol is suitable when both mouse primary antibodies need or do not need pre-treatment step.

Protocol Step	DS203C Protocol Reagent / 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 (Optional)	HIER if needed User supplied (up to 60 min)				
Step 3 (optional)	Preblock if needed User supplied				
Step 4	Mouse 1°Ab #1 User supplied (30-60 min.)				
Step 5	**Reagent 1** AP Polymer anti-Mouse IgG RTU(15min) Rinse with distilled water.				
Step 6	**Reagent 2A,Reagent 2B& Reagent 2C** GBI-Permanent Red requires mixing (10min)				
Step 7	**Reagent 3** Antibody Blocker (10 min)				
Step 8	**Reagent 4A** DS-MM Blocker A RTU (30 min)				
Step 9	**Reagent 4B** DS-MM Blocker B RTU (5 min)				
Step 10	Ms 1°Ab #2 User supplied (30-60 min)				
Step 11	**Reagent 5** HRP Polymer anti-Mouse IgG RTU(15min)				
Step 12	Counter stain (Do not over counter stain) Hematoxylin User supply Wash with PBS/0.05% Tween20 for 2 min, 3 times.				
Step 13	**Reagent 6** Emerald Chromogen RTU (5min)				
Step 14	Dehydrate section 20seconds for each step **It is important to follow the protocol.**				
Step 15	**Reagent 7** U-Mount RTU Mount & coverslip				

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Result	Stain pattern on controls are correct: Fill in Yes or NO				

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