



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: 2-8°C

Catalog No.: [DS203C-6	12mL*	60 slides**
	DS203C-18	36mL*	180 slides**
	DS203C-60	120mL*	600 slides**
	*Total volum	ne of polyn	ner Conjugates
** if	use 100µL per sli	ide	

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.
- 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
Reagent	Stanning Froctaure	Time
 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 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 65°C 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

3 Reagent 4A a. A poly 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. 30 min 0. Reagent 4B 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. 30 min 0. Reagent 4B 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. 5 min 0. Mouse antibody 2: Notes: Investigator needs to optimize dilution and incubation times cach. 30-60 min 10. Mouse antibody 2: Notes: Investigator needs to optimize dilution and incubation times cach. 30-60 min 11. Reagent 5 a. Apply 2 drops or enough volume of mouse primary antibody 2 to cover the tissue completely. 30-60 min 11. Reagent 5 a. Apply 2 drops or enough volume of mouse primary antibody 2 to cover the tissue completely. 30-60 min 12. Counterstain (Optional) a. Apply 2 drops or enough volume of mouse primary antibody 50 to cover and the section. 15 min 12. Counterstain (Optional) a. Apply 2 drops (100µL) of Reagent 5 (IRP polymer anti-Mouse IgG (RTU) b. Incubate in moist chamber for 15 min. 15 min 12. Counterstain (Optional) a. Bigs the polymer antines each. 15 min <t< th=""><th></th><th>Well it DDG T containing 0.050/ T con 1V</th><th></th></t<>		Well it DDG T containing 0.050/ T con 1V	
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30 min 30 min b Wash with PBS-T containing 0.05% Tween-20 or IX TBS-T; 3 times for 2 minutes each. 30 min c) Regent 48 DS-MM Blocker B a Apply 2 drops or enough volume of Regent 48 (DS-MM Blocker B) to cover the tissue completely. Mix well on the side and Incubate in most chamber for 10 min. b Wash with PBS-T containing 0.05% Tween-20 or IX TBS-T; 3 times for 2 minutes each. 10. Mouse antibody 2: Notes: Investignation reads to polumize 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. 10. Mouse antibody 2: Notes: Investignation reads to polumize dilution and incubation times prior to double staining. a Apply 2 drops (oroupt) of Regent 5 (IRP polymer anti- Mouse IgG) to cover each section. 11. Reagent 5 a Apply 2 drops (oroupt) of Reagent 5 (IRP polymer anti- Mouse IgG) to cover each section. 12. Counterstain (Optional) a Dip the slide in diluted hematoxylin for 5 seconds. (you may dilute hematoxylin. 13. Reagent 6 a Apply 1 to 2 drops (oroupt) of Reagent 6 (Emerald Chromogen) to cover the tissue completely. 13. Reagent 6 a Apply 1 to 2 drops (oroupt) of Reagent 6 (Emerald Chromogen AFTER GBI-Permanent Rel stain because GBI-Permanent Rel removes the Emerald and after hematoxylin. 14. Dehydrate section Note: Places with 6 95% ethanol 20seconds. 5 min 15. Wash with PBS-T containing 0.05% Tween-20 or IX TBS-T; 3 times for 2 minutes each. 5 min <td></td> <td></td> <td></td>			
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		prevent leaching of GBI-Permanent Red stain.	

Trouble shoot:

Problem	Tips	
	1.	Need to adjust the titer of each antibody.
Uneven stain on 2 primary antibodies	2.	The amount of each protein expressed on tissue may be different.
Oneven stant on 2 primary antibodies	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	1.	Emerald should be green when not co-localized with GBI-Permanent Red.
non co-localized with GBI Permanent Red.		If Emerald chromogen is blue the titer on the primary antibody is not dilute
lion co-localized with OBI Fermalient Red.		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.
Green Background on the side	2.	Use 10% Donkey serum, goat or horse serum as a preblock.
CDI Dermonant Rad is leaching	1.	Use fresh 100% ethanol and xylene.
GBI-Permanent Red is leaching	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
Artifacts on shues		xylene.

Precautious:

DAB may be carcinogenic. Please wear gloves and take other necessary precautions.

Remarks:

For research use only.

References:

1. <u>De Pasquale A, Paterlini P, Quaglino D</u>. *Immunochemical demonstration of different antigens in single cells in paraffin-embedded histological sections*. <u>Clin Lab Haematol</u>, 1982;4(3):267-72.

2. Polak J. M and Van Noorden S. Introduction to Immnocytochemistry Second Edition. Bios Scientific Publishers. P41-54. 1997

-4

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 " $\sqrt{}$ "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	HIER if needed				
(Optional)	User supplied (up to 60 min)				
Step 3	Preblock if needed				
(optional)	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				

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