Polink DS-MRt-Hu C Kit for Immunohistochemistry Double Staining

Polymer HRP and AP Double Staining Kit for Mouse & Rat Primary Antibodies on Human Tissue with Emerald (Green) and GBI-Permanent Red (Red)

Storage: 2-8ºC

Catalog No.: ☐DS209C-6 12mL* for 120 slides**
☐DS209C-18 36mL* for 360 slides**
☐DS209C-60 120mL* for 1200 slides**
*Total volume of polymer Conjugates
** if use 100µL per slide

Intended Use:
The **Polink DS-MRt-Hu C Kit** is designed for use with user supplied mouse and rat primary antibodies to detect two distinct antigens on human tissue or cell samples. The advantage of the C kit series is that it will allow you to visualize when two proteins are co localized by producing a third color blue purple. This kit has been tested on paraffin embedded tissue. However, this kit can be used to stain frozen specimen and/or freshly prepared monolayer cell smears.

Double staining is a common method used in immunohistostaining that allows detection of two distinct antigens in a single tissue[1, 2]. **Polink DS-MRt-Hu C Kit** from GBI labs supplies the user with two polymer enzyme conjugates: anti-Mouse IgG (minimal cross reaction to rat) HRP polymer and anti-rat IgG(minimal cross reaction to mouse) AP polymer with two distinct substrates/chromogens, GBI-Permanent Red and Emerald. GBI-Permanent Red reacts with anti-Rat AP polymer conjugate to produce the red color. Emerald chromogen reacts with anti-Mouse HRP polymer conjugate to produce the green color. When two proteins are co-expressed in the same location, the area of co-localization shows blue color if more Emerald is present and purple blue if more GBI-Permanent Red is present. A Primer step is used to increase specificity of antibody staining. **Polink DS-MRt-Hu C Kit** is a non-biotin system that avoids the extra steps involved in blocking non-specific binding due to endogenous biotin.

Kit Components:

Component No.	Content	12mL Kit	36mL Kit	120mL Kit
Reagent 1	Rat Primer (RTU)	12mL	18mLx2	120mL
Reagent 2	Rat AP Polymer (RTU)	6mL	18mL	60mL
Reagent 3	Mouse HRP 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 tissue falling off.
3. Paraffin embedded section must be deparaffinized with xylene and rehydrated with a graded series of ethanol before staining.
4. Cell smear samples should be prepared as close to a monolayer as possible to obtain satisfactory results.
5. Three control slides are recommended for interpretation of results: positive, reagent (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. GBI-Permanent Red reaction removes the Emerald chromogen. Always do the GBI-Permanent Red reaction first then Emerald.
8. 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)

Step/Reagent	Staining Procedure	Incubation Time
1. Peroxidase and Alkaline Phosphatase Blocking Reagent Not provided	a. Incubate slides in peroxidase and alkaline phosphatase blocking reagent. We recommend **GBI Dual Block E36xx**. b. Rinse the slide using distilled water at least twice.	10-20min

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We recommend using **GBI Dual Block E36xx.** Fast, easy and it will block endogenous alkaline phosphatase		
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 8 above)**; 3 times for 2 minutes each..	1h
3. Primary Antibody Mix: **one Mouse and one Rat primary antibody** Supplied by user	***Notes:*** Investigator needs to optimize primary antibody titer and incubation time prior to double staining as both GBI-Permanent Red and Emerald Chromogen are very strong. a. Apply 2 drops or enough volume of Mouse and Rat primary antibody mixture to cover the tissue completely. Incubate in moist chamber for 30-60 min. Recommend 30min to shorten total protocol time. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each..	30-60min
4. **Reagents 1:** Rat Primer (RTU)	a. Apply 2 drops or enough volume of **Reagent 1** Rat Primer to cover the tissue completely. Incubate in moist chamber for 10min. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	10min
5. **Reagents 2 & 3:** **2:** Rat AP Polymer(RTU) **3:** Mouse HRP Polymer(RTU)	**Note:** Make sufficient polymer mixture by adding **Reagent 2** (Rat AP Polymer) and **Reagent 3** (Mouse HRP Polymer) at 1:1 ratio, mix well. a. Apply 1 to 2 drops (50-100µL) of the mixture to cover each section. b. Incubate in moist chamber for 30 min. c. Wash with **1X TBS-T only**; 3 times for 2 minutes each. **Make enough mixture for the experiment. Do not make extra volume as mixture is not stable for long term storage .**	30min
6. **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 buffer) 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 buffer) 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.	10 min
7. 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 the three 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
8. **Reagent 5**	a. Apply 1 to 2 drops (50-100µL) of **Reagent 5** (Emerald Chromogen) to cover the tissue completely.	5 min

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Emerald Chromogen (RTU)	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.	
9.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 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
10. **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.	

Trouble shooting:

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.
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:
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 DS209C 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

DS209C Protocol is suitable when both mouse and rat primary antibodies need or do not need pre-treatment step.

Protocol Step	DS209C Protocol Reagent / Time	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase or Alkaline Phosphatase Block User supplied				
Step 2 (Optional)	HIER if needed User supplied (up to 60 min)				
Step 3	Mouse 1°Ab & Rat 1°Ab mix (30-60 min.)				
Step 4	**Reagent 1** Rat Primer RTU (10min)				
Step 5	**Reagent 2&Reagent 3** Rat AP Polymer & Mouse HRP Polymer require mixing (30min) Rinse with distilled water.				
Step 6	**Reagent 4A,Reagent 4B&Reagent 4C** GBI-Permanent Red requires mixing (10min)				
Step 7	Counter stain (5seconds) (Do not over counter stain) Hematoxylin User supply Wash with PBS/0.05% Tween20 for 2 min, 3 times.				
Step 8	**Reagent 5** Emerald Chromogen RTU (5min)				
Step 9	**It is important to follow the protocol to maintain stain!** Dehydrate section 20seconds for each step				
Step 10	**Reagent 6** U-Mount RTU Mount & coverslip				
Result	Stain pattern on controls are correct: Fill in Yes or NO				

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