Polink DS-RRt-Hu/Ms C Kit

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

Storage: 2-8ºC

Catalog No.:
- ☐ DS211C-6 12mL* 120 slides**
- ☐ DS211C-18 36mL* 360 slides**
- ☐ DS211C-60 120mL* 1200 slides**

**Total volume of polymer Conjugates*
***If using 100µl per slide*

Intended Use:
The **Polink DS-RRt-Hu/Ms C Kit** is designed for use with user supplied rabbit and rat primary antibodies to detect two distinct antigens on human and mouse tissue or cell samples. 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, allowing for the detection of two distinct antigens in a single tissue. Polink DS-RRt-Hu/Ms C Kit from GBI labs supplies the user with two polymer enzyme conjugates: HRP polymer anti-Rat IgG (minimal cross reaction to mouse) and AP polymer anti-Rabbit IgG with two distinct substrates/chromogens, Emerald and GBI-Permanent Red. Emerald chromogen reacts with the anti-Rat HRP polymer conjugate to produce a green color. GBI-Permanent Red reacts with anti-Rabbit AP polymer to produce the subsequent red color. When two proteins are co-localized, a blue/ purple color will develop depending which on antigen is stronger. If only the rat antigen is present only the Emerald chromogen will be present and if the rabbit antigen is present only the GBI-Permanent Red chromogen will be present. Polink DS-RRt-Hu/Ms C Kit is a non-biotin system avoiding 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	Rabbit AP Polymer (RTU)	6mL	18mL	60mL
Reagent 2	Rat-NM HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 3A	GBI-Permanent Red Substrate (RTU)	15mL	18mLx2	120mL
Reagent 3B	GBI-Permanent Red Activator (5x)	3mL	7.2mL	12mLx2
Reagent 3C	GBI-Permanent Red Chromogen (100x)	150µL	360µL	1.2mL
Reagent 4	Emerald Chromogen (RTU)	15mL	18mLx2	120mL
Reagent 5	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 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. 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 interpreting results.
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 pH 7.6. GBI sells 10xTBS-T for your convenience (B11).

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Steps / Reagents	Staining Procedure	Incubation Time
1. Peroxidase and Alkaline Phosphatase Blocking Reagent: Not provided	We recommend using **GBI Dual Block E36xx.** It is fast, easy, and will block endogenous alkaline phosphatase. a. Incubate slides in peroxidase and alkaline phosphatase blocking reagent. b. Rinse the slide using distilled water at least twice.	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 8 above)**; 3 times for 2 minutes each.	Up to 1 hour
3. Primary Antibody Mix: **one Rat and one Rabbit antibody** Supplied by user	**Note:** Investigator needs to optimize primary antibody titer prior to double staining as both GBI-Permanent Red and Emerald Chromogen are very strong. a. Apply 2 drops or enough volume of rat and rabbit 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.	30-60 min
4. **Reagent 1:** Rabbit AP Polymer (RTU)	a. Apply 1 to 2 drops (50-100µl) of **Reagent 1** (Rabbit AP Polymer) to cover each section. b. Incubate in moist chamber for 15-30 min. c. Wash with **1X TBS-T only**; 3 times for 2 minutes each. **Note:** Longer incubation may increase background.	15-30 min
5. **Reagent 2:** Rat-NM HRP Polymer (RTU)	a. Apply 1 to 2 drops of **Reagent 2** (Rat-NM HRP Polymer) to cover each section. b. Incubate in moist chamber for 15-30 min. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	15-30 min
6. **Reagent 3A, 3B, 3C:** **Reagent 3A:** GBI-Permanent Red Substrate (RTU) **Reagent 3B:** GBI-Permanent Red Activator (5x) **Reagent 3C:** GBI-Permanent Red Chromogen (100x)	**Note:** Shake GBI-Permanent Red Activator before adding into GBI- Permanent Red Substrate. a. Add 200µL of **Reagent 3B** (Activator) into 1mL of **Reagent 3A** (Substrate buffer) and mix well. Add 12µL of **Reagent 3C** (Chromogen) into the mixture and mix well. (**Note:** For fewer slides, add 100µL of **Reagent 3B** (Activator) into 500µL of **Reagent 3A** (Substrate buffer) and mix well. Add 6µL of **Reagent 3C** (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. (**To get maximum sensitivity of AP polymer, repeat chromogen step)**	10 min
7. Counterstain: (**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.	5-30 seconds
8. **Reagent 4:** Emerald Chromogen (RTU)	a. Apply 1 to 2 drops (50-100µl) of **Reagent 4** Emerald Chromogen to cover the tissue completely. b. Incubate in moist chamber for 5 minutes. c. Wash slides in tap water for 1minute. 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
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 5:** U-Mount (RTU)	a. Apply 1 drop (50µL) of **Reagent 5** 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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Troubleshooting:

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 steps 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.

Precautions:
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 Immunocytochemistry Second Edition. Bios Scientific Publishers. P41-54. 1997

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Work Sheet for DS211C 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 ensure 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 de-paraffinization
- Refer to insert for details of each step

DS211C Protocol is suitable when both rabbit and rat primary antibodies need or do not need pre-treatment step.

Step/ Protocol	Protocol DS211C	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase or Alkaline Phosphatase Block E36 is Recommended User supplied User supplied				
Step 2	HIER if needed User supplied (up to 60 min)				
Step 3	Rabbit 1°Ab & Rat 1°Ab mix (30-60 min.)				
Step 4	**Reagent 1** Rabbit AP Polymer (15-30 min.)				
Step 5	**Reagent 2** Rat (No Ms) HRP Polymer (15-30 min.)				
Step 6	**Reagent 3A, Reagent 3B & Reagent 3C** GBI-Permanent Red requires mixing (10min)				
Step 7	Counter stain (5-30seconds) (Do not over counter stain) Hematoxylin User supply Wash with PBS/0.05% Tween20 for 2 min, 3 times.				
Step 8	**Reagent 4** Emerald Chromogen RTU (5min)				
Step 9	**It is important to follow the protocol to maintain stain!** Dehydrate section 20 seconds for each step.				
Step 10	**Reagent 5** U-Mount RTU Mount & coverslip				
Result	Stain pattern on controls is correct: Fill in Yes or NO				

Testing result:

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