Polink DS-MR-Hu C2 Kit for Immunohistochemistry Staining

Polymer-HRP and AP kit to detect mouse and rabbit primary antibodies for human tissue with GBI Permanent-Red (Red) and Emerald (Green)

Storage: 2-8°C

Catalog No.:

☐	DS202C-6	12mL*	120 slides**
☐	DS202C-18	36mL*	360 slides**
☐	DS202C-60	120mL*	1200 slides**

**Total volume of polymer Conjugates*
*** if use 100µLper slide*

Intended Use:
The **Polink DS-MR-Hu C2 Kit** is designed to use with user supplied mouse and rabbit 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 a common method used in immunohistochemistry for the detection of two distinct antigens in a single tissue [1, 2]. GBI Labs **Polink DS-MR-Hu C2 Kit** contains two polymer enzyme conjugates: AP-polymer anti-Mouse IgG and HRP-polymer anti-Rabbit IgG with two distinct chromogens. The GBI Permanent-Red (Red color) is used with AP-polymer anti-Mouse IgG) and Emerald chromogen (Green color) is used with HRP polymer anti-Rabbit IgG). Simplified steps offer a much faster protocol as the enzyme conjugates are applied to the specimen as a mixture. A second 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 expressed 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. The **Polink DS-MR-Hu C2 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 AP Polymer (RTU)	6mL	18mL	60mL
Reagent 2	Rabbit 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. Tissues need to be adhered to the slides 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 and negative tissue controls, and reagent control (slides treated with Isotype control reagent).
6. Proceed to 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 affect results significantly. Investigator needs to consider all factors and determine optimal conditions when interpreting the 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 pH7.6. GBI sells 10xTBS-T for your convenience (B11xx)

Equipment or material needed but not provided:
1. Equipment and material for deparaffinization, such as fume absorbing hood, etc.
2. Heat source (microwave or hot plate) for HIER and antigen retrieval buffers.
3. Thermometer
4. Beaker
5. Timer
9. Wash buffer: 0.01 M PBS with 0.5% Tween20, pH7.4 or 50mM Tris HCl, 150mM NaCl, 0.05% Tween-20 pH7.6.
6. Peroxidase and alkaline phosphatase blocking buffer
7. 100% ethanol
8. 100% Xylene
9. Hematoxylin
10. Coverslip

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Steps / 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 2 changes of 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. After slides have cooled, rinse with dH2O. c. 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. Preblock (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 be required depending on fixative. (Pre-block catalogue No.: E07 was Recommended.)	
4. Primary Antibody Mix: **one Mouse and one Rabbit antibody** Supplied by user	**Note:** Investigator needs to optimize primary antibody dilution prior to double staining. a. Apply 2 drops or enough volume of mouse 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.
5. Polymer mixture: **Reagent 1:** Mouse AP Polymer (RTU) **Reagent 2:** Rabbit HRP Polymer (RTU)	**Note:** Make sufficient polymer mixture by adding **Reagent 1** (Mouse AP Polymer) and **Reagent 2** (Rabbit HRP) at 1:1 ratio, mix well. Use within 30 minutes 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. Extra volume is not necessary and may not be as stable as non-mixture.**	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) (**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 3B** (Activator) into 1mL of **Reagent 3A** (Substrate buffer) and mix well. Add 10µL 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 5µL 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.	10min
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 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
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 1 minute. d. Rinse with distilled water. ***Important to READ:*** Emerald Chromogen is water soluble, counter stain first. *Do*	5 min

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	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.	
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 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!**	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 chromogen.	

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.
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:
Standard laboratory personal protective equipment should be worn: i.e. gloves, eye protection and appropriate lab coat.

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 DS202C 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
- Follow steps after de-paraffinization
- Refer to insert for details of each step

DS202C Protocol is suitable when both mouse and rabbit primary antibodies need or do not need pre-treatment step.

Protocol Step	**DS202C Protocol Reagent / Time**	**Experiment 1 Date:**	**Experiment 2 Date:**	**Experiment 3 Date:**	**Experiment 4 Date:**
Step 1	Peroxidase or Alkaline Phosphatase Block (Recommend E36) 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 & Rabbit 1°Ab mix (30-60 min.)				
Step 5	**Reagent 1**&**Reagent 2** Mouse AP Polymer and Rabbit HRP Polymer require mixing (30min) Rinse with distilled water.				
Step 6	**Reagent 3A,Reagent 3B & Reagent 3C** GBI-Permanent Red requires mixing (10min)				
Step 7	Counter stain (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	Dehydrate section 20seconds for each step **It is important to follow the protocol.**				
Step 10	**Reagent 5** U-Mount RTU Mount & coverslip				
Result	Stain pattern on controls are correct: Fill in Yes or NO				

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