# Polink DS-MR-Hu C1 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: 2-8ºC

Catalog No.:
- ☐ DS201C-6 12mL* 120 slides**
- ☐ DS201C-18 36mL* 360 slides**
- ☐ DS201C-60 120mL* 1200 slides**

**Total volume of polymer Conjugates*
*** if use 100µL per slide*

**Intended Use:**

The **Polink DS-MR-Hu C1 Kit** is designed to use with user supplied mouse and rabbit 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 C1 Kit** supplies two polymer enzyme conjugates: HRP-Polymer anti-Mouse IgG and AP-Polymer anti-Rabbit IgG with two chromogens: Emerald (green) and GBI-Permanent Red (red). Simplified steps offer a convenient protocol as the enzyme conjugates are applied to the specimen simultaneously. 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, the antigen indicated by GBI Permanent-Red is expressed at higher concentrations. The **Polink DS-MR-Hu C1 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** | HRP-Polymer anti-Mouse IgG (RTU) | 6mL | 18mL | 60mL |
| **Reagent 2** | AP-Polymer anti-Rabbit IgG (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 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. 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 result.
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)

| Reagent | Staining Procedure | Incubation Time |
|---|---|---|
| 1. Peroxidase and Alkaline Phosphatase Blocking Reagent<br>Not provided<br>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**.<br>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.<br>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 |



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| 3. Preblock<br>(optional) | For paraffin section, Improved formula saves the need for a preblock step.<br>For frozen tissue, preblock may or may not be required depending on fixative. ( Preblock catalogue No.:E07 was Recommended. ) | |
| 4. Primary Antibody Mix: **one Mouse and one Rabbit antibodies**<br><br>Supplied by user | **Note:** Investigator needs to optimize dilution prior to double staining as both GBI Permanent Red and Emerald Chromogen are very strong.<br>a. Apply 2 drops or enough volume of mouse and rabbit primary antibodies mixture to cover the tissue completely. Incubate in moist chamber for 30-60 min. Recommend 30min to shorten total protocol time.<br>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:<br><br>**Reagent 1:** HRP-Polymer anti-Mouse IgG<br>**Reagent 2:** AP-Polymer anti-Rabbit IgG | **Note:** Only make enough mixture for the experiment performed. Mixture is not stable for long term storage.<br>Make sufficient polymer mixture by adding **Reagent 1** HRP-Polymer anti-Mouse IgG and **Reagent 2** AP-Polymer anti-Rabbit IgG at 1:1 ratio, mix well.<br>a. Apply 1 to 2 drops (50-100µL) of the mixture to cover each section.<br>b. Incubate in moist chamber for 30 min.<br>c. Wash with **1X TBS-T only**; 3 times for 2 minutes each. | 30 min |
| 6. **Reagent 3A, 3B, 3C**<br><br>**Reagent 3A:**<br>GBI-Permanent Red Substrate (RTU)<br>**Reagent 3B:**<br>GBI-Permanent Red Activator (5x)<br>**Reagent 3C:**<br>GBI-Permanent Red Chromogen (100x)<br>(**To get maximum sensitivity of AP polymer, Please repeat chromogen step)** | **Note:** Shake GBI-Permanent Red Activator before adding into GBI-Permanent Red Substrate.<br>**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.<br>**b.** [**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.]<br>**c.** 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.**<br>**d.** Rinse well with distilled water. | 10min |
| 7. Counterstain (**Optional**)<br>(**Optional but must be done before Emerald Chromogen step**)<br>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.<br>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.<br>b. Rinse thoroughly with tap water for 1min.<br>c. Put slides in PBS for 5-10 seconds to blue, **DO NOT** over blue.<br>d. Rinse well in distilled or tap water for 1min.<br>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**<br><br>Emerald Chromogen (RTU) | a. Apply 1 to 2 drops (50-100µL) of **Reagent 4** (Emerald Chromogen) to cover the tissue completely.<br>b. Incubate in moist chamber for 5 minutes.<br>c. Wash slides in tap water for 1minute.<br>d. Rinse with distilled water.<br>***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 | **Note: Please wipe off extra water and air dry slides before dehydration and clear.**<br>a. Dehydrate with 85% ethanol 20seconds.<br>b. Dehydrate with 95% ethanol 20seconds.<br>c. Dehydrate with 100% ethanol 20seconds.<br>d. Dehydrate with 100% ethanol 20seconds.<br>e. Dehydrate with 100% ethanol 20seconds.<br>f. Dehydrate with xylene 20seconds.<br>**CAUTION: DO NOT dehydrate with xylene longer than 20 seconds! It will erase GBI-Permanent Red stain!** | 2 min |
| 10. **Reagent 5** | a. Apply 1 drop (50µL) of **Reagent 5** (U-Mount) to cover the tissue | |



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| U-Mount (RTU) | section and apply glass coverslip.<br>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.<br>2. The amount of each protein expressed on tissue may be different.<br>3. Set slides in water too long so that Emerald is washed away.<br>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. | 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 | Missing steps or step reversed. |
| Green Background on the slide | Titer primary antibody. |
| GBI-Permanent Red is leaching | 1. Use fresh 100% ethanol and xylene.<br>2. Slide sat too long in xylene. Do not go over 20seconds! |
| Artifacts on slides | 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 DS201C 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

**DS201C Protocol** is suitable when both mouse and rabbit primary antibodies need or do not need pre-treatment step.

| Protocol Step | DS201C Protocol Reagent / Time | Experiment 1 Date: | Experiment 2 Date: | Experiment 3 Date: | Experiment 4 Date: |
|---|---|---|---|---|---|
| **Step 1** | Peroxidase or Alkaline Phosphatase Block<br>GBI E36xxx is recommended.<br>User supplied | | | | |
| **Step 2** (Optional) | HIER if needed<br>User supplied<br>(up to 60 min) | | | | |
| **Step 3** | Preblock if needed<br>User supplied | | | | |
| **Step 4** | Mouse 1°Ab & Rabbit 1°Ab mixture<br>(30-60 min.) | | | | |
| **Step 5** | **Reagent 1& Reagent 2**<br>HRP-Polymer anti-Mouse IgG and AP-Polymer anti-Rabbit IgG require mixing<br>(30min)<br>Rinse with distilled water. | | | | |
| **Step 6** | **Reagent 3A & Reagent 3B**<br>GBI-Permanent Red requires mixing<br>(10min) | | | | |
| **Step 7** | Counter stain<br>(Do not over counter stain)<br>Hematoxylin User supply<br>Wash with PBS/0.05% Tween20 for 2 min, 3 times. | | | | |
| **Step 8** | **Reagent 4**<br>Emerald Chromogen RTU<br>(5min) | | | | |
| **Step 9** | Dehydrate section<br>20seconds for each step<br>**It is important to follow the protocol.** | | | | |
| **Step 10** | **Reagent 5**<br>U-Mount RTU<br>Mount & coverslip | | | | |
| **Result** | Stain pattern on controls are correct: Fill in Yes or NO | | | | |

The result:


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