Polink DS-GR-Hu/Ms C Kit for Immunohistochemistry Staining

Polymer-HRP and AP Kit to Detect Goat and Rabbit Primary Antibodies on Human or Mouse Tissue with Emerald (Green) and GBI-Permanent Red (Red)

Storage: 2-8ºC

Catalog No.:
- ☐ DS205C-6 12mL* 120 slides**
- ☐ DS205C -18 36mL* 360 slides**
- ☐ DS205C -60 120mL* 1200 slides**

**Total volume of polymer Conjugates*

***If use 100µL per slide*

Intended Use:

Polink DS-GR-Hu/Ms C Kit is designed to use with user supplied goat and rabbit primary antibodies, to detect two distinct antigens on human and mouse 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 most commonly methods used in immunohistostaining for revealing two distinct antigens in a single tissue[1, 2]. **Polink DS-GR-Hu/Ms C Kit** from GBI Labs (Golden Bridge International) supplies two polymer enzyme conjugates: HRP Polymer anti-Goat IgG and AP Polymer anti-Rabbit IgG with two substrates/chromogens, Emerald (Green) and GBI-Permanent Red (Red). Simplified steps offer a convenient protocol as the enzyme conjugates are applied to the specimen simultaneously. GBI-Permanent Red reacts with anti-rabbit AP polymer conjugate to produce the red color. Emerald chromogen reacts with anti-Goat 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. **Polink DS-GR-Hu/Ms C Kit** is a non-biotin system, avoiding blocking steps for endogenous biotin non-specific binding.

Kit Components:

Component No.	Content	6mL Kit	36mL Kit	120mL Kit
Reagent 1	Goat HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 2	Rabbit AP 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 deparffinized with xylene and rehydrated with a graded series of alcohols 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. DO NOT let specimen or tissue dry during protocol.
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 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)

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 distilled water at least twice.	10-20min
2. HIER Pretreatment: Refer to antibody data sheet.	a. Heat Induced Epitope Retrieval (HIER) may be required for primary antibody suggested by vendor.	

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	b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T(See note 8 above)**; 3 times for 2 minutes each.	
3. Primary Antibody Mix: **one Goat and one Rabbit antibody** Supplied by user	**Note:** Investigator needs to optimize dilution prior to double staining. a. Apply 2drops (100µL) or enough volume of goat and rabbit primary antibodies mixture to cover the tissue completely. Incubate in moist chamber for 30-60min. 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.Mix **Reagent 1:** Goat HRP Polymer (RTU) with **Reagent 2** Rabbit AP Polymer (RTU)	**Note:** Only make enough mixture for the experiment performed. Mixture is not stable for long term storage. Make sufficient polymer mixture by adding **Reagent 1** Goat HRP Polymer and **Reagent 2** Rabbit AP Polymer at 1:1 ratio, mix well. a. Apply 2 drops (100µL) or enough volume of the mixture to cover each section. b. Incubate in moist chamber for 30min. c. Wash with **1X TBS-T only**; 3 times for 2 minutes each	30min
5. **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
6. 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 10-30 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
7. **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, 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
8.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.	2 min

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	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!**	
9. **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.	

Protocol Notes:

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, eye protection and take other necessary precautions. If any of the reagent come in contact with skin wash area completely with plenty of water and soap. If irritation develops seek medical attention.

Remarks:
This kit is 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 DS205C Kit

We designed this work sheet to help you keep 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.

- Used for tester to check "√ "each step during the experiment
- Steps follow after de-paraffinization
- Refer to insert for details of each step

DS205C Protocol is suitable when both goat and rabbit primary antibodies need or do not need pre-treatment step.

Step/ Protocol	Protocol of DS205C	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase& alkaline phosphatase Block User supplied				
Step 2	HIER if needed User supplied				
Step 3	Gt 1°Ab & Rb 1°Ab mixture (30-60 min.)				
Step 4	**Reagent 1 & Reagent 2** Goat HRP Polymer (RTU)& Rabbit AP Polymer (RTU) require mixing 30min				
Step 5	**Reagent 3A, Reagent 3B & Reagent 3C** GBI-Permanent Red Requires mixing! 10min				
Step 6	Counter stain (5seconds) (Do not over counter stain) Hematoxylin User supply Wash with PBS/0.05% Tween20 for 2 min, 3 times.				
Step 7	**Reagent 4** Emerald Chromogen (RTU) (5min)				
Step 8	**It is important to follow the protocol to maintain stain!** Dehydrate section 20seconds for each step				
Step 9	**Reagent 5** U-Mount (RTU) Mount & coverslip				
Result	Stain pattern on controls are correct: Fill in Yes or NO				

Testing result:

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