Polink DS-GRt-Hu/Ms C Kit

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

Storage: 2-8ºC

Catalog No.:

☐	DS206C-6	6mL*	60 slides**
☐	DS206C-18	18mL*	180 slides**
☐	DS206C-60	60mL*	600 slides**

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

Intended Use:
Polink DS-GRt-Hu/Ms C Kit is designed to use with user supplied goat and rat primary antibodies to detect two distinct antigens on human/mouse tissue or cell samples. The kit has been tested on paraffin–embedded human and mouse tissues.

Double staining is one of most common methods used in immunohistostaining that allows for the detection of two distinct antigens in a single tissue[1, 2]. The design of **Polink DS-GRt-Hu/Ms C Kit** from OriGene supply two polymer enzyme conjugates: AP polymer anti-Goat IgG and HRP polymer anti-Rat IgG with two distinct substrates/chromogens, HRP/Emerald (Green) and AP/GBI Permanent Red (red). With this protocol the goat and rat primary antibodies will be applied to the tissue at the same time, but the user will apply the polymer enzyme conjugate to the specimen sequentially. When two proteins are present in the same location a blue or purple color will develop depending on quantity of each antigen screened. If only the anti-goat antigen is present only the GBI- Permanent Red chromogen will be present and if the Rat antigen is present only the Emerald chromogen will be present in the cell. The **Polink DS-GRt-Hu/Ms C Kit** is non-biotin system avoiding endogenous biotin non-specific binding.

Kit Components:

Component No.	Content	DS206C-6	DS206C-18	DS206C-60
Reagent 1	Goat AP Polymer (RTU)	6 mL	18ml	60ml
Reagent 2A	GBI-Permanent Red Substrate (RTU)	15mL	36mL	120mL
Reagent 2B	GBI-Permanent Red Activator (5x)	3mL	7.2mL	14mL
Reagent 2C	GBI-Permanent Red Chromogen (100x)	150µL	360µL	700µL
Reagent 3	DS-Gt/Rt Blocker (RTU)	6mL	18mL	60mL
Reagent 4	Rat Primer (RTU)	6mL	18mL	60mL
Reagent 5	Rat HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 6	Emerald Chromogen (RTU)	6mL	18mL	60mL
Reagent 7	U-Mount (RTU)	6mL	18mL	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 must be adhered to the slide properly to ensure maximum quality staining.
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, reagent control (slides treated with Isotype control reagent).
6. Proceed with IHC staining: **DO NOT** let specimens or tissues 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 results.
8. **Note:** 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. **1X TBS-T** =50mM Tris HCl, 150mM NaCl, 0.05% Tween-20 pH 7.6. GBI sells 10xTBS-T for your convenience (B11xx).

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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 slides using 2 changes of distilled water.	10min
2. HIER Pretreatment: Refer to antibody data sheet	a. Heat Induced Epitope Retrieval (HIER) may be required for primary antibody. Refer to antibody datasheet. 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 Goat and one Rat antibody:** Supplied by user	**Note:** Investigator needs to optimize dilution prior to double staining. a. Apply 2 drops or enough volume of **goat and rat primary** antibodies 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:** Goat AP Polymer (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 1** (Goat AP Polymer) to cover each section. b. Incubate in moist chamber for 15 min. c. Wash only with **1X TBS-T**, 3 times for 2 minutes each.	15 min
5. **Reagents 2A, 2B, 2C: Reagent 2A:** GBI-Permanent Red Substrate (RTU) **Reagent 2B:** GBI-Permanent Red Activator (5x) **Reagent 2C:** GBI-Permanent Red Chromogen (100x)	**Note:** Shake GBI-Permanent Red Activator before adding into GBI-Permanent Red Substrate. a. Add 200µL of **Reagent 2B** (Activator) into 1mL of **Reagent 2A** (Substrate buffer) and mix well. Add 10µL of **Reagent 2C** (Chromogen) into the mixture and mix well. **[Note:** For fewer slides, add 100µL of **Reagent 2B** (Activator) into 500µL of **Reagent 2A** (Substrate buffer) and mix well. Add 5µL of **Reagent 2C** (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 10 min.** c. Rinse well with distilled water. d. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each. **To get maximum sensitivity of AP polymer, repeat chromogen step.**	10 min
6. **Reagent 3:** DS-GRt Blocker (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 3** (DS-GRt Blocker) to cover each section. b. Incubate in moist chamber for 10 min. c. Tap off solution. Wipe away excess but **DO NOT** Rinse.	10 min
7. **Reagent 4:** Rat Primer (RTU)	a. Add 2 drops (100µL) or enough volume of **Reagent 4** (Rat Primer) to cover the tissue section and Incubate at Room Temperature for 10-15 minutes. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	15 min
8**. Reagent 5:** Rat HRP Polymer (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 5** (Rat HRP Polymer) to cover each section. b. Incubate in moist chamber for 10-15 min. c. Wash with distilled water.	15 min
9. Counterstain (**Optional, but recommend being 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 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. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each. **Do not leave longer than 30 seconds in counter stain as the HRP enzyme will start to fail.**	10 to 30 sec
10. **Reagent 6:** Emerald Chromogen (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 6** (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 not leave slides sitting in water*. Always stain with Emerald chromogen **AFTER** GBI- Permanent Red stain and hematoxylin. GBI-Permanent Red removes the Emerald.	

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11.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!**	
12. **Reagent 7:** U-Mount (RTU)	a. Apply 1 (50µL) of **Reagent 7** (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.	

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

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Work Sheet for DS206C 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 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 after de-paraffinization
- Refer to insert for details of each step

Step/ Protocol	Protocol DS206C	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase and alkaline phosphatase Block E36 is recommended. Supplied by user.				
Step 2	HIER if needed				
Step 3	Gt 1°Ab & Rat 1°Ab mix (30-60 min.)				
Step 4	**Reagent 1** Goat AP Polymer RTU (15min) **Wash with 1xTBS-T only.**				
Step 5	**Reagent 2A, 2B, 2C** GBI-Permanent Red requires mixing. (10min)				
Step 6	**Reagent 3** DS-GRt Blocker RTU (10min) **Do Not Rinse** Tap off & go directly to step 7				
Step 7	**Reagent 4** Rat Primer RTU (10-15 min.)				
Step 8	**Reagent 5** Rat HRP Polymer RTU (10-15min)				
Step 9	Counter stain with Hematoxylin Supplied by user				
Step 10	**Reagent 6** Emerald Chromogen RTU (5 min)				
Step 11	Dehydrate section 20seconds for each step **It is important to follow the protocol.**				
Step 12	**Reagent 7** U-mount RTU Mount & coverslip				
Result	Stain pattern on controls is correct: Fill in Yes or NO				

Testing Result:

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