Polink DS-GRt-Hu/Ms A Kit for Immunohistochemistry Staining

Polymer-HRP and AP Kit to Detect Goat and Rat Primary Antibodies for Human and Mouse Tissue with DAB (Brown) and GBI-Permanent Red (Red)

Storage: 2-8ºC

Catalog No.:

☐	DS206A-6	12mL*	60 slides**
☐	DS206A-18	36mL*	180 slides**
☐	DS206A-60	120mL*	600 slides**

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

Intended Use:

The **Polink DS-GRt-Hu/Ms A 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. However, this kit can be used on frozen specimen and freshly prepared monolayer cell smears.

Double staining is one of most common methods used in immunohistostaining that allows for revealing two distinct antigens in a single tissue[1, 2]. The **Polink DS-GRt-Hu/Ms A Kit** from GBI Labs (Golden Bridge International) supplies two polymer enzyme conjugates: HRP polymer anti-Goat IgG and AP polymer anti-Rat IgG with two distinct substrates/chromogens, DAB (brown) and GBI Permanent Red (red). User will apply the two enzyme conjugates onto the specimen sequentially. When two proteins are present a brown/red color will develop depending presence and location of the antigen the two colors should be distinct. If only the anti goat antigen is present only the DAB brown chromogen will be present and if the Rat antigen is present only the GBI Permanent Red chromogen will be present. The **Polink DS-GRt-Hu/Ms A Kit** is non-biotin system avoiding endogenous biotin non-specific binding.

Kit Components:

Component No.	Content	12mL Kit	36mL Kit	120mL Kit
Reagent 1	Goat HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 2A	DAB Substrate (RTU)	12mL	15mLx2	70mL
Reagent 2B	DAB Chromogen (20x)	1.5mL	2mL	3.5mL
Reagent 3	DS-GRt Blocker (RTU)	6mL	18mL	60mL
Reagent 4	Rat Primer (RTU)	6mL	18mL	60mL
Reagent 5	Rat AP Polymer (RTU)	6mL	18mL	60mL
Reagent 6A	GBI-Permanent Red Substrate (RTU)	15mL	18mLx2	70mL
Reagent 6B	GBI-Permanent Red Activator (5x)	3mL	7.2mL	14mL
Reagent 6C	GBI-Permanent Red Chromogen (100x)	150µL	360µL	0.7mL
Reagent 7	Simpo-Mount (RTU)	7mL	18mL	70mL

Gt=Goat Rt=Rat

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. **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; Beaker; Timer
4. Wash buffer: 0.01 M PBS with 0.5% Tween20, pH7.4; **1X TBS-T** =50mM Tris HCl, 150mM NaCl, 0.05% Tween-20 pH7.6.

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5. Peroxidase and alkaline phosphatase blocking buffer
6. 100% ethanol; 100% Xylene; Hematoxylin

Steps / Reagent	**Staining Procedure**	**Incubation Time**
1. 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** 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** Goat HRP Polymer (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 1** (Goat HRP Polymer) to cover each section. b. Incubate in moist chamber for 15 min. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	15min
5. **Reagent 2A and 2B** **Reagent 2A:** DAB Substrate (RTU) **Reagent 2B:** DAB Chromogen (20x)	**Note:** Make enough DAB mixture by adding 1 drop of **Reagent 2B** (DAB Chromogen) in 1mL of **Reagent 2A** (DAB Substrate). Mix well. Use within 7 hours at 4°C. a. Apply 1 to 2 drops (50-100µL) of your DAB mixture to cover the tissue completely. b. Incubate for 5min. c. Rinse slides in multiple changes of distilled water 3 times, 2 each time or under running tap water for 1minute. d. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	5min
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. Blot off solution. **DO NOT** Rinse.	10min
7. **Reagent 4** Rat Primer (RTU)	a. Add 2 drops (100µL) or enough volume of **Reagent 4** (Rat Primer) to cover the tissue section b. Incubate at Room Temperature for 10-15minutes. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	10-15min
8**. Reagent 5:** Rat AP Polymer (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 5** (Rat AP Polymer) to cover each section. b. Incubate in moist chamber for 10-15 min. c. Wash with **1X TBS-T only**; 3 times for 2 minutes each.	10-15min
9. **Reagent 6A, 6B, 6C** **Reagent 6A:** GBI-Permanent Red Substrate (RTU) **Reagent 6B:** GBI-Permanent Red Activator (5x **Reagent 6C:** GBI-Permanent Red Chromogen (100x) (**To get maximum sensitivity of AP**	**Note:** Shake GBI-Permanent Red Activator before adding into GBI-Permanent Red Substrate. a. Add 200µL of **Reagent 6B** (Activator) into 1mL of **Reagent 6A** (Substrate) and mix well. Add 10µL of **Reagent 6C** (Chromogen) into the mixture and mix well. [**Note:** For fewer slides, add 100µL of **Reagent 6B** (Activator) into 500µL of **Reagent 6A** (Substrate) and mix well. Add 5µL of **Reagent 6C** (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.**	10min

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polymer, Please repeat chromogen step)	c. Rinse well with distilled water.	
10. HEMATOXYLIN Not provided	a. Counterstain with 2 drops (100µL) or enough volume of hematoxylin to completely cover tissue. Incubate for 5 seconds. **DO NOT** over stain with hematoxylin. b. Rinse thoroughly with tap water for 1 minute. c. Put slides in PBS for 5 seconds to blue, **DO NOT** over blue. d. Rinse well in distilled or tap water for 1 minute.	
11. **Reagent 7:** Simpo-Mount (RTU)	a. Apply 2 drops (100µL) or enough **Reagent 7** (Simpo-Mount) to cover tissue when tissue is wet. Rotate the slides to allow Simpo-Mount spread evenly. DO NOT coverslip. b. Place slides horizontally in an oven at 40-50°C for at least 30 minutes or leave it at room temperature until slides are thoroughly dried. Hardened Simpo-Mount forms an impervious polymer barrier to organic solvent. Do not use oil directly on the top of dried Simpo-Mount. **To coverslip see protocol note 2**.	30min in 40-50°C oven Or: overnight at room temperature

Protocol Notes:

1. 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.
2. **GBI-Permanent Red** is insoluble in organic solvent and can be coverslipped as well. however the dehydration steps must be shorter for optimal tissue structure and chromogen signal maintenance.
   Note: Please wipe off extra water and air dry slides before dehydration and clear.
   a. 1x 80% Ethanol 20 seconds;
   b. 1x 95% Ethanol 20 seconds;
   c. 3x 100% Ethanol 20 seconds each;
   d. 1x 100% Xylene 20 seconds;
   e. Add 1 drop of xylene based mountant (Cat. No. O-Mount, E02-18) and coverslip. Press to push the air bubble out.

CAUTION: DO NOT dehydrate in xylene longer than 20 seconds! It will erase GBI-Permanent Red stain!

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 DS206A 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

Step/ Protocol	Protocol DS206A	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase & levamisole Block E36 is recommended. User supplied				
Step 2	HIER if needed				
Step 3	Gt 1°Ab & Rat 1°Ab mix (30-60 min.)				
Step 4	**Reagent 1** Goat HRP Polymer RTU (15min)				
Step 5	**Reagent 2A & 2B** DAB Chromogen requires mixing. (5min)				
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 AP Polymer RTU (10-15min) Wash with **1xTBS-T only**.				
Step 9	**Reagent 6A, 6B, & 6C** GBI Permanent Red requires mixing. (20 min)				
Step 10	Counter stain Supplied by user				
Step 11	**Reagent 7** Simpo-Mount RTU				

Testing result:

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