Polink DS-MR-Hu A2 Kit for Immunohistochemistry Staining

Polymer-HRP&AP Double Staining Kit to Distinct A Mouse and A Rabbit Primary Antibodies on Human Tissue with DAB (Brown) and GBI-Permanent Red (Red)

Storage: 4-8ºC

Catalog No.:
- ☐ DS202A-6/D32-6A 12mL* 120 slides**
- ☐ DS202A-18 36mL* 360 slides**
- ☐ DS202A-60 120mL* 1200 slides**

**Volume of polymer conjugate*
*** If using 100µL per slide*

Intended Use:

The **Polink DS-MR-Hu A2 Kit** is designed to use with user supplied mouse and rabbit antibody to detect two distinct antigens on human tissue or cell samples. This kit has been tested in paraffin tissue, but may also be used on frozen specimens and freshly prepared monolayer cell smears.
Double staining is one of most common methods used in immunohistostaining for revealing two distinct antigens in a single tissue [1, 2]. **Polink DS-MR-Hu A2 Kit** from Golden Bridge International supplies two polymer enzyme conjugates: AP-Polymer anti-Mouse IgG and HRP-Polymer anti-Rabbit IgG with two distinct chromogens, DAB (brown) and GBI-Permanent Red(red). User may apply the two enzyme conjugates onto the specimen at the same time and mix them on the slide. Simplified steps allow for a much faster protocol than sequential procedures. **Polink DS-MR-Hu A2 Kit** is a 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-Rabbit IgG (RTU)	6mL	18mL	60mL
Reagent 2	AP Polymer anti-Mouse IgG(RTU)	6mL	18mL	60mL
Reagent 3A	DAB Substrate (RTU)	12mL	18mLx2	120mL
Reagent 3B	DAB Chromogen (20x)	1.5mL	2mL	6mL
Reagent 4A	GBI-Permanent Red Substrate (RTU)	15mL	18mLx2	120mL
Reagent 4B	GBI-Permanent Red Activator (5x)	3mL	7.2mL	12mLx2
Reagent 4C	GBI-Permanent Red Chromogen (100x)	150µL	360µL	1.2mL
Reagent 5	Simpo-Mount	12mL	18mLx2	120mL

HRP = Horseradish Peroxidase AP = Alkaline Phosphatase Ms = Mouse Rb = Rabbit

Protocol Notes:

1. Proper 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 deparaffinize 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. 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. This will generate false positive and/or false negative signal.
7. The fixation, tissue section thickness, antigen retrieval and primary antibody dilution and incubation time effect 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.	10min
2. Antigen retrieval if needed: Refer to primary antibody data sheet.	a. Refer to primary antibody data sheet for antigen retrieval methods. 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 1h
3. Preblock (optional)	For paraffin section, Improved formula saves the need for a preblock step. For frozen tissue, preblock may or may not be required depending on	

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	fixative. (Preblock catalogue No.:E07 was Recommended.)	
4. Primary Antibody Mix: **Mix one Mouse and one Rabbit primary antibody** Supplied by user.	**Note:** Investigator needs to optimize dilution prior to triple 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.	30-60min
5. **Reagent 1 and 2** **Reagent 1:** HRP Polymer anti-Rabbit IgG(RTU) **Reagent 2:** AP Polymer anti Mouse IgG(RTU)	**Note:** Make sufficient polymer mixture by adding **Reagent 1** HRP Polymer anti-Rabbit IgG and **Reagent 2** AP Polymer anti-Mouse IgG at 1:1 ratio, mix well. Do Not Mix More than you need for the experiment because the polymer mixture may not be as stable as non-mixed polymer. a. Apply 1 to 2 drops (50-100µL) of the mixture to cover the tissue completely. b. Incubate in moist chamber for 30 min. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	30min
6. **Reagent 3A and 3B** **Reagent 3A:** DAB Substrate(RTU) **Reagent 3B:** DAB Chromogen(20x)	**Note:** Make enough DAB mix by adding 1 drop of **Reagent 3B (DAB Chromogen)** in 1mL of **Reagent 3A (DAB Substrate)**. Mix well. Use within 7 hours. 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 1 minute. d. Wash with **1X TBS-T only**; 3 times for 2 minutes each.	5min
7. **Reagent 4A, 4B, 4C** **Reagent 4A:** GBI-Permanent Red Substrate (RTU) **Reagent 4B:** GBI-Permanent Red Activator (5x) **Reagent 4C:** 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 4B** (Activator) into 1mL of **Reagent 4A** (Substrate buffer) and mix well. Add 10µL of **Reagent 4C** (Chromogen) into the mixture and mix well. [**Note:** For fewer slides, Add 100µL of **Reagent 4B** (Activator) into 500µL of **Reagent 4A** (Substrate buffer) and mix well. Add 5µL of **Reagent 4C** (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
8. 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.	
9. **Reagent 5:** Simpo-Mount(RTU)	a. Apply 2 drops (100µL) or enough volume of **Reagent 5** Simpo-Mount to cover tissue when tissue is wet. Rotate the slides to allow Simpo-Mount spread evenly. 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.	

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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 coversliped 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:

DAB may be carcinogenic. 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 DS202A 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

DS202A Protocol is suitable when both mouse and rabbit primary antibodies need or do not need pre-treatment step.

Protocol Step	DS202A Protocol Reagent / Time	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase or Alkaline Phosphatase Block User supplied				
Step 2 (Optional)	HIER if needed User supplied (up to 60 min)				
Step 3	Mouse 1°Ab & Rabbit 1°Ab mixture (30-60 min.)				
Step 4	**Reagent 1& Reagent 2** HRP Polymer anti-Rabbit IgG and AP Polymer anti-Mouse IgG require mixing (30min)				
Step 5	**Reagent 3A & Reagent 3B** DAB Requires mixing! (5 min.)				
Step 6	**Reagent 4A, Reagent 4B& Reagent 4C** GBI-Permanent Red Requires mixing! (10 min)				
Step 7	Counter stain(10-15sec) User supplied				
Step 8	**Reagent 5** Simpo Mount (RTU)				
Result	Stain pattern on controls are correct: Fill in Yes or NO				

The result:

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