

**Polink DS-MR kit for Double Staining (BCIP/NBT & AEC)**  
(Simultaneous polymer double staining kit for mouse and rabbit antibody  
With BCIP/NBT and AEC)

**Fewer steps and better result than sequential procedure**

Storage: 4-8°C

Catalog No.: D64-6  
Size: 6ml (Polymer Conjugates)  
Good for 50 Slides

**Intended Use:**

The Polink DS-MR kit is designed to use with user supplied mouse antibody and rabbit antibody to detect two distinct antigens on human tissue or cell samples. Specimen can be frozen or paraffin –embedded tissues, and freshly prepared monolayer cell smears.

Double staining is one of most common methods used in immunohistostaining that allow revealing two distinct antigens in a single tissue<sup>1, 2</sup>. Polink DS-MR kit from Golden Bridge International supplies two polymer enzyme conjugates: Goat anti-Rabbit IgG-HRP and Goat anti-Mouse IgG-AP with two distinct substrates/chromogens, AEC (Red color, use with Goat anti-Rabbit IgG-HRP) and BCIP/NBT (Purple/Blue color, use with Goat anti-Mouse IgG-AP). User may apply the two enzyme conjugates onto the specimen at the same time and mix them on the slide. Simplified steps offer user much faster and quicker protocol than a sequential procedure. Polink DS-MR kit is non-biotin system that avoids endogenous biotin non-specific binding.

**Kit Components:**

Component No.	Content	Size
Reagent 1	Ready-to-use Pre-blocking solution	12 ml
Reagent 2	Ready-to-use Goat anti Rabbit IgG-peroxidase (HRP) polymer conjugate	6 ml
Reagent 3	Ready-to-use Goat anti Mouse IgG-alkaline phosphatase(AP) polymer conjugate	6 ml
Reagent 4	BCIP/NBT Solution(Ready to use)	12ml
Reagent 5A	AEC Substrate Buffer (20x)	1ml
Reagent 5B	AEC Chromogen (20x)	2ml
Reagent 5C	Hydrogen Peroxide (20x)	1ml
Reagent 6	Ready-to-use Simpo-Mount solution	12 ml

**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 deparaffinized 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.

Reagent	Staining Procedure	Incubation Time (Min.)
1. Peroxidase Blocking Reagent Not provided	a. Incubate slides in peroxidase blocking reagent (Ready-to-use 3% H <sub>2</sub> O <sub>2</sub> solution) for 10 minutes. b. Rinse the slide using distilled water.	10
2. HIER Pretreatment: Refer to antibody data sheet.	a. Heat Induced Epitope Retrieval (HIER) may be required for primary antibody suggested by vendor. b. Wash with PBS for 2 min., 3 times.	

3. <b>Reagent 1:</b> Pre-Blocking Solution	a. Add 2 drops (100ul) or enough volume of Pre-Blocking Solution to cover the tissue section and Incubate b. Drain or blot off solution. <b>DO NOT RINSE.</b>	10
4. Mouse antibody 1 and Rabbit antibody 2: Supplied by user	<b>Notes:</b> Investigator needs to optimize dilution and incubation times prior to double staining. a. Apply 2 drops or enough volume of both Primary Antibody 1 and Antibody 2 to cover the tissue completely. Mix well on the slide and Incubate in moist chamber for 30-60 min. b. Rinse with PBS containing 0.05% Tween-20 for 2 min., 3 times.	30-60
5. <b>Reagent 2 and 3:</b> Polymer Secondary Antibodies (Ready-to-use)	a. Apply 1 drop (50ul) of Goat anti Rabbit IgG-HRP Polymer Conjugate and 1 drop of Goat anti Mouse IgG-AP Polymer Conjugate to cover each section, mix well on the slide. Or you may prepare secondary antibodies cocktail in advance: 50ul Goat anti Rabbit IgG-HRP Polymer Conjugate plus 50ul Goat anti Mouse IgG-AP Polymer Conjugate per slide b. Incubate in moist chamber for 30 min. c. Rinse with PBS containing 0.05% Tween-20 for 2 min., 3 times.	30
6. <b>Reagents 4:</b> BCIP/NBT Chromogen (Ready-to-use)	a. Apply 2 drops or enough volume of BCIP/NBT CHROMOGEN to completely cover tissue. Incubate for 3-10 min. b. Rinse thoroughly with distilled water.	3-10 min
7. <b>Reagent 5A, 5B, 5C:</b> AEC Chromogen (Concentrated)	a. Add 1 drop (50ul) of reagent <b>5A</b> and 1 drop or <b>2 drops</b> (for higher sensitivity and contrast) of reagent <b>5B</b> and 1 drop of Reagent <b>5C</b> to 1ml distill water. Mix well. Keep away from light and use within 1 hour. b. Apply 2 drops (100 µL) or enough volume of pre-mixed AEC solution to completely cover the tissue. Incubate for 5-15 min, observe appropriate color development c. Rinse well with distilled water. ( <b>AEC is alcohol soluble; do not dehydrate.</b> )	5-10 min
8. HEMATOXYLIN Not provided	a. Counterstain with 2 drops (100 µL) or enough volume of hematoxylin to completely cover tissue. Incubate for 10-15 seconds. b. Rinse thoroughly with tap water for 2-3 min c. Put slides in PBS until show blue color (about ½ - 1 min.) c. Rinse well in distilled water	
9. <b>Reagent 6:</b> Simpo-Mount	a. Apply 2 drops (100 µL) or enough volume to cover tissue when tissue is wet. Rotate the slides to allow Simpo-Mount spread evenly. <b>DO NOT coverslip.</b> 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.	30 min. 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 effect results significantly. Investigator needs to consider all factors and determine optimal conditions when interpret the result.
2. Simpo-Mount is water-based mounting medium for immunohistology. It may be used as the permanent mounting media. It does not need coverslip. However, if you need to coverslip, after Simpo-Mount dried, dip the slide in xylene and take out immediately. Apply O-Mount (organic mounting solution, Catalog No. E02-15) on the tissue and place cover glass on the slide. Store it after dry completely.

**Precautions:**

Please wear gloves and take other necessary precautions.

**Remarks:**

For research use or investigation only. Not for diagnostic or therapeutic use.

**References:**

1. De Pasquale A, Paterlini P, Quaglini 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