NeoStain Poly DS Kit for Mouse and Rabbit antibody on Human tissue (Emerald/Permanent Red)

NB-23-00088

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Polymer HRP & AP Double Staining Kit Detects Mouse & Rabbit Primary Antibodieson Human Tissue with Permanent Red (Red) and Emerald (Green)

#Cat : NB-23-00088-1 size: 6+6=12ml*, sufficient for 120 slides**
#Cat : NB-23-00088-2 size: 18+18=36ml*, sufficient for 360 slides**
#Cat : NB-23-00088-3 size: 60+60=120ml*, sufficient for 1200 slides**
*Total volume of polymer Conjugates ** if use 100µl per slide

Intended Use:

The NeoStain Poly DS Kit is designed to use with user supplied mouse and rabbit antibodies to detecttwo distinct antigens on human 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 a common method used in immunohistochemistry for the detection of two distinct antigens in a single tissue[1,2]. Neo Biotech NeoStain Poly DS Kit supplies two polymer enzyme conjugates: HRP-Polymer anti-Mouse IgG and AP-Polymer anti-Rabbit IgG with two chromogens: Emerald (green) and Permanent Red (red). Simplified steps offer a convenient protocol as the enzyme conjugates are applied to the specimen simultaneously. A second advantage of Neo Biotech Kit, it allows the researcher to visualize when two proteins are co-localized because of the color change when the chromogens overlap that can be semi-quantitative. For example, if the area of co-localization stains blue, the antigen indicated by Emerald is expressed at higher concentration in the cell and if the color is purple, the antigen indicated by Permanent-Red is expressed at higher concentrations. The NeoStain Poly DS Kit is 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-Mouse IgG (RTU)	6mL	18mL	60mL
Reagent 2	AP-Polymer anti-Rabbit IgG (RTU)	6mL	18mL	60mL
Reagent 3A	Permanent Red Substrate (RTU)	15mL	18mLx2	120mL
Reagent 3B	Permanent Red Activator (5x)	3mL	7.2mL	12mLx2
Reagent 3C	Permanent Red Chromogen (100x)	150µL	360µL	1.2mL
Reagent 4	Emerald Chromogen (RTU)	15mL	18mLx2	120mL
Reagent 5	NeoBio Mount Organic (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 need to be adhered to the slide tightly to avoid tissue falling off.
3. Paraffin embedded section must be deparffinized 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.
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 the result.
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. Neo Biotech sells 10xTBS-T for your convenience (NB-23-00201)

Reagent:

Reagent	**Staining Procedure**	**Incubation Time**
1. Peroxidase and Alkaline Phosphatase Blocking Reagent Not provided We recommend using **NeoPure Dual Enzyme Block NB-23-00193-1/-2.** Fast, easy and it will block endogenous alkaline phosphatase	a. Incubate slides in peroxidase and alkaline phosphatase blocking reagent. We recommend **NeoPure Dual Enzyme Block NB-23-00193-1 /-2**. b. Rinse the slide using distilled water at least twice.	10 min.
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-T containing 0.05% Tween-20 or **1X TBS-T (See note 7 above)**; 3 times for 2 minutes each.	
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 fixative. (Preblock catalogue No.: NB-23-00169-1 /-2 / -3 was Recommended.)	
4. Primary Antibody Mix: **one Mouse and one Rabbit antibodies** Supplied by user	**Note:** Investigator needs to optimize dilution prior to double staining as both Permanent Red and Emerald Chromogen are very strong. a. Apply 2 drops or enough volume of mouse and rabbit 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
5. Polymer mixture: **Reagent 1:** HRP-Polymer anti-Mouse IgG **Reagent 2:** AP-Polymer anti-Rabbit IgG	**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** HRP-Polymer anti- Mouse IgG and **Reagent 2** AP-Polymer anti-Rabbit IgG at 1:1 ratio, mix well. a. Apply 1 to 2 drops (50-100µL) of the mixture to cover each section. b. Incubate in moist chamber for 30 min. c. Wash with **1X TBS-T only**; 3 times for 2 minutes each.	30 min

6. **Reagent 3A, 3B, 3C** **Reagent 3A:** Permanent Red Substrate (RTU) **Reagent 3B:** Permanent Red Activator (5x) **Reagent 3C:** Permanent Red Chromogen (100x)(**To get maximum sensitivity of AP polymer, Please repeat chromogen step)**	**Note:** Shake Permanent Red Activator before adding into 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. b. [**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.] c. Apply 2 drops (100µL) or enough volume of 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 Permanent Red working solution to completely cover the tissue for additional 5 to 10min.** d. Rinse well with distilled water.	5-10 10min

7. 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 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
8. **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 1minute. 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** Permanent Red stain because Permanent Red removes the Emerald and after hematoxylin.	5 min
9.Dehydrate section	**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 Permanent Red stain!**	2 min
10. **Reagent 5:** NeoBio Mount Organic (RTU)	a. Apply 1 drop (50µL) of **Reagent 5** (NeoBio Mount Organic) 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 chromogen.	

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. NeoBio Mount Universeral is an aqueous-based mounting media for immunohistochemistry. It is used as the permanent mounting media for alcohol soluble chromogens such as AP-Red, AEC, and BCIP. NeoBio Mount Universeral does not use a coverslip. However, if you need to coverslip your tissue, after NeoBio Mount Universeral has dried, dip the slide in xylene (1 to 2 seconds), apply an organic mounting solution (such as NeoBio Mount Perm, Cat# NB-23-00156), and place cover glass on the slide. Store slides after they have dried completely.

Precautious:
Please wear gloves and take other necessary precautions.

Remarks:
For research use only.

Storage:
Store at 4°C.

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