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

NB-23-00094-1

NB-23-00094-2

NB-23-00094-3

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#Cat: NB-23-00094-1 **Size: 12mL (60 slides)**
#Cat: NB-23-00094-2 **Size: 36mL (180 slides)**
#Cat: NB-23-00094-3 **Size: 120mL (600slides)**

Storage: 2-8ºC

Intended Use:

The **NeoStain Poly DS Kit** is designed to use with user supplied mouse and rabbit primary antibody to detect two distinct antigens on mouse tissue or cell samples. NB-23-00094, kits can be used on frozen specimens, paraffin–embedded tissues, or freshly prepared monolayer cell smears. NB-23-00094 kits is designed not to give background on most mouse strains however there may be some mouse strains especially when using frozen that require additional blocking; we recommend PureStain Mouse-on-Mouse Kit Blocking A & B solutions (NB-23-00076) to improve specificity of the mouse primary antibody on mouse tissue.

Double staining is one of most common methods used in immunohistostaining that allows detection of two distinct antigens in a single tissue1, 2. **NeoStain Poly DS Kit** from Neo Biotech supplies two polymer enzyme conjugates: Mouse HRP Polymer and Rabbit AP Polymer with two distinct substrates/chromogens, DAB (brown color, use with the Mouse HRP Polymer) and NeoStain ABC Kit, AP, Rabbit, with Permanent Red (red color, use with the Rabbit AP Polymer). A Primer step is used to increase specificity of antibody staining. Both enzyme conjugates are applied to the specimen at the same time and mixed on the slide. This kit offers simplified steps that make for a quicker and easier protocol than that used in a sequential procedure. **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	Mouse Primer (RTU)	6mL	18ml	60ml
Reagent 2	Rabbit AP Polymer (RTU)	6mL	18mL	60ml
Reagent 3	Mouse HRP Polymer (RTU)	6ml	18ml	60ml
Reagent 4A	DAB Substrate (RTU)	15mL	36ml	120ml
Reagent 4B	DAB Chromogen (20x)	1.5mL	2ml	6ml
Reagent 5A	Permanent Red Substrate (RTU)	15mL	36ml	120ml
Reagent 5B	Permanent Red Activator (5x)	3ml	7.2ml	24ml
Reagent 5C	Permanent Red Chromogen (100x)	150ul	360ul	1.2ml
Reagent 6	Simpo-Mount (RTU)	12ml	18mLx2	120ml

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 needs to be adhered to the slide tightly to avoid falling off.
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 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. 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 pH 7.6. Neo Biotech sells 10xTBS-T for your convenience (NB-23-00201).

Reagent	Staining Procedure	Staining Procedure
1. Peroxidase and Alkaline Phosphatase Blocking Reagent: Not provided	We recommend using NeoPure Dual Enzyme Block NB-23-00193. Fast, easy and it will block endogenous alkaline phosphatase. a. Incubate slides in peroxidase and alkaline phosphatase blocking reagent. b. Rinse the slide using distilled water at least twice.	10 min.
2.HIER Pretreatment: Refer to Ab 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. **PureStain Mouse-on-Mouse Kit Blocking A solutions** (NB-23-00076) Not provided	(Optional see protocol note 2) a. Add 2 drops (100µL) or enough volume of **NB-23-00076 PureStain Mouse-on-Mouse Kit Blocking A solutions** to cover the tissue section and incubate. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	30 min.
4. **PureStain Mouse-on-Mouse Kit Blocking A solutions** (NB-23-00076): Not provided	(Optional see protocol note 2) a. Add 2 drops (100µL) or enough volume of **NB-23-00076 PureStain Mouse-on-Mouse Kit Blocking B solutions** to cover the tissue section and incubate. Do not exceed 5min. b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.	5 min.
5. Mouse antibody 1 and Rabbit antibody 2: Supplied by user	Note: Investigator needs to optimize dilution and incubation times prior to double staining. a. Apply 2 drops or enough volume of both Ms Primary Antibody 1 and Rb Primary Antibody 2 to cover the tissue completely. Mix well on the slide and incubate in moist chamber for 30-60 min. b. Wash with PBS-T containing 0.05% Tween-20 **or 1X TBS-T**; 3 times for 2 minutes each.	30-60 min.

6. **Reagent 1:** Mouse Primer (RTU)	a. Add 2 drops (100µL) or enough volume of **Reagent 1** (Mouse Primer) to cover the tissue section and incubate at room temperature for 10-15minutes. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	15 min.
7**. Reagent 2:** Rabbit AP Polymer (RTU)	a. Apply 1-2 drops (50-100µl) of **Reagent 2** (Rabbit AP Polymer) to cover each section. b. Incubate in moist chamber for 15-30 min. c. Wash with **1X TBS-T**; 3 times for 2 minutes each. Note: longer incubation may increase background.	15-30 min.
8**. Reagent 3:** Mouse HRP Polymer (RTU)	a. Apply 2 drops (100µL) or enough volume of **Reagent 6** (Simpo-Mount) to cover tissue when tissue is wet. Rotate the slides to allow Simpo-Mount to 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.	15-30 min.
9**. Reagent 4A and 4B** **Reagent 4A**: DAB Substrate (RTU) **Reagent 4B**: DAB Chromogen (20x)	a. Add 1 drop of **Reagent 4B** to 1 mL of **Reagent 4A**. Mix well. Protect from light and use within 7 hours at 4 ºC. b. Apply 2 drops or enough volume of DAB Chromogen working solution to completely cover tissue. Incubate for 3-10 min. c. Rinse thoroughly with distilled water. d. Wash with 1xTBS-T only, 3 times for 2 minutes each.	5 min.
10. **Reagent 5A, 5B, 5C:** **Reagent 5A**: Permanent Red Substrate (RTU) **Reagent 5B**: Permanent Red Activator (5x) **Reagent 5C**: Permanent Red Chromogen (100x)	Note: Shake Permanent Red Activator before adding into Permanent Red Substrate. a. Add 200µL of **Reagent 5B** (Activator) into 1mL of Reagent 5A (Substrate buffer) and mix well. Add 12µL of Reagent 5C (Chromogen) into the mixture and mix well. [Note: For fewer slides, add 100µL of Reagent 5B (Activator) into 500µL of **Reagent 5A** (Substrate buffer) and mix well. Add 6µL of **Reagent 5C** (Chromogen) into the mixture and mix well]. b. 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 Red working solution to completely cover the tissue for additional 5 to 10min.** c. Rinse well with distilled water. **To get maximum sensitivity of AP polymer, repeat chromogen step**	10 min. OR 10 min+ 10min

11. HEMATOXYLIN: Not provided	a. Counterstain with 2 drops (100µL) 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 30 - 60 sec.) d. Rinse well in distilled water.	
12. **Reagent 6**: Simpo-Mount (RTU) **To coverslip see protocol note** 3	a. Apply 2 drops (100µL) or enough volume of **Reagent 6** (Simpo-Mount) to cover tissue when tissue is wet. Rotate the slides to allow Simpo-Mount to 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.	

Protocol Notes:

1. The fixation, tissue slide thickness, 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. **PureStain Mouse-on-Mouse Kit Blocking** the anti-mouse secondary has been absorbed to rat serum resulting in most mouse strains having no background, however some mouse strains may need additional blocking. PureStain Mouse-on-Mouse Kit Blocking A & B solutions (NB-23-00076) works very well on frozen tissue
3. Neo Biotech-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. NeoBio Mount Perm (Permanent mount for DAB, BCIP/NBT, NB-23-00156) and coverslip. Press to push the air bubble out.

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

Precautions:

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

Work Sheet for NB-23-00094 Kits

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 de-paraffinization
- Refer to insert for details of each step

Step/ Protocol	Protocol NB-23-00094	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase & Alkaline Phosphatase Block (NB-23-00193 is recommended) User supplied				
Step 2	HIER if needed				
Step 3 Optional	PureStain Mouse-on-Mouse Kit Blocking A solutions (NB-23-00076) 30min				
Step 4 Optional	PureStain Mouse-on-Mouse Kit Blocking B solutions (NB-23-00076-2) 5min				
Step 5	Ms 1°Ab & Rb 1°Ab mix (30-60 min.)				
Step 6	**Reagent 1** Mouse Primer RTU (15 min.)				
Step 7	**Reagent 2** Rb AP Polymer (15-30 min.)				
Step 8	**Reagent 3** Ms HRP Polymer (15-30 min.)				
Step 9	**Reagent 4A & 4B** DAB Requires mixing! (5 min.)				
Step 10	**Reagent 5A, 5B, 5C** Permanent Red requires mixing (10 min+5-10min)				
Step 11	Counter stain Hematoxylin User supplied				
Step 12	**Reagent 6** Simpo Mount (RTU) Do not coverslip!				

Testing Result: