PolyStain DS Kit - for 2 Mouse antibody on Human tissue

(DAB/Permanent Red)

NB-23-00097- 3(120 ml)

NB-23-00097- 2(36 ml)

NB-23-00097- 1(12 ml)

Neo-Biotech
183, avenue Georges Clémenceau – 92000 Nanterre

Neo-Biotech
183, avenue Georges Clémenceau – 92000 Nanterre

PolyStain DS Kit - for 2 Mouse antibody on Human tissue (DAB/Permanent Red)

NB-23-00097-1; NB-23-00097-2; NB-23-00097-3

Storage: 2-8ºC

INTENDED USE:

The PolyStain DS Kit is designed to use with user supplied two mouse antibodies to detect two 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 one of most common methods used in immunohistostaining that allow revealing two distinct antigens in a single tissue.

PolyStain DS Kit from NeoBiotech Labs supplies two polymer enzyme conjugates: HRP polymer anti-Mouse IgG and AP polymer anti-Mouse IgG with two distinct substrates/chromogens, DAB (brown color, use with HRP polymer anti-Mouse IgG) and Permanent Red (red color, use with AP polymer AntiMouse IgG). PolyStain 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 2A	DAB substrate buffer (RTU)	12ml	18ml	60ml
Reagent 2B	DAB chromogen (20x)	1ml	2ml	3ml
Reagent 3	Antibody Blocker (40x)	15mLx2	50ml	125ml
Reagent 4A	DS-MM Blocker A (RTU)	6ml	18ml	60ml
Reagent 4B	DS-MM Blocker B (RTU)	6mL	18mL	60ml
Reagent 5	AP polymer anti-Mouse IgG (RTU)	6ml	18ml	60ml
Reagent 6A	Permanent Red Substrate (RTU)	7ml	18ml	60ml
Reagent 6B	Permanent Red Activator (5x)	1.4ml	3.6ml	12ml
Reagent 6C	Permanent Red Chromogen (100x)	70 µL	180 µL	0.6ml
Reagent 7	NeoMount Universal (RTU)	15ml	18ml	60ml

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.

Reagent	Staining Procedure	Incubation Time (Min.)
1. Peroxidase Blocking Reagent Not provided	a. Incubate slides in peroxidase and alkaline phosphatase blocking reagent. We recommend **NeoPure Dual Enzyme Block NB-23-00193** for 10 minutes... b. Rinse the slide using distilled water.	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 containing 0.05% Tween-20 for 2 min., 3 times.	
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** was Recommended.)	
4. Mouse Antibody 1: Supplied by user	**Notes**: Investigator needs to optimize dilution and incubation times prior to double staining. a. Apply 2 drops or enough volume of mouse primary antibody 1 to cover the tissue completely. Incubate in moist chamber for 30-60 min. b. Rinse with PBS containing 0.05% Tween-20 for 2 min., 3 times	30 - 60 min.
5.Reagent 1 HRP polymer anti-Mouse IgG (RTU)	a. Apply 2 drops (100µL) of Reagent 1 HRP polymer anti-Mouse IgG to cover each section. b. Incubate in moist chamber for 15 min. c. Rinse with PBS containing 0.05% Tween-20 for 2 min., 3 times	15 min
6. Reagents 2A, 2B **Reagents 2A:**	a. Add 1 drop of Reagent 2B to 1 mL Reagent 2A. Mix well. Protect from light and use within 7 hours.	3-10 min

DAB Substrate(RTU) **Reagents 2B:** DAB Chromogen(20x)	b. Apply 2 drops or enough volume of DAB CHROMOGEN mixture to completely cover tissue. Incubate for 3-10 min. c. Rinse thoroughly with distilled water 3 times, 2 minutes each time.	
7. Reagent 3: **Antibody Blocker (40x)** **(Optional)** Must test if antibody/antigen interaction is heat sensitive. **Please skip this step if antigen retrieval is used for 2nd Ms Primary Antibody.**	**Note**: This step will block antibodies of previous step so no cross reaction will occur at end of protocol. HIER can be done immediately after **Antibody Blocker** step if only one primary antibody requires antigen retrieval. For frozen tissue a lower temperature of 65ºC must be used for Antibody Blocker (**Reagent 3**) to prevent tissue from dissociating from slide. a. Use hot plate or water bath to heat diluted Reagent 3 to 1x solution (1 part of Antibody Blocker in 39 parts of distilled water) to 80°C. Make enough volume to cover the tissue in beaker. b. Put slides in heated Antibody Blocker for 10 minutes at 80°C. c. Remove slides from the Antibody blocker; cool slides 5 seconds. d. Rinse slides in multiple changes of distilled water. e. Wash with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	10 min
8. Reagent 4A DS-MM Blocker A(RTU)	a. Apply 2 drops or enough volume of Reagent 4A DSMM Blocker A to cover the tissue completely. Mix well on the slide and Incubate in moist chamber for 30 min. b. Rinse with PBS containing 0.05% Tween-20 for 2 min, 3 times.	30 min
9. Reagent 4B DS-MM Blocker B(RTU)	a. Apply 2 drops or enough volume of Reagent 4B DSMM Blocker B to cover the tissue completely. Mix well on the slide and Incubate in moist chamber for 10 min. b. Rinse with PBS containing 0.05% Tween-20 for 2 min, 3 times.	5 min.
10. Mouse antibody 2: Supplied by user	**Notes**: Investigator needs to optimize dilution and incubation times prior to double staining. a. Apply 2 drops or enough volume of mouse primary antibody 2 to cover the tissue completely. b. Rinse with PBS containing 0.05% Tween-20 for 2 min., 3 times	30- 60 min.
11. Reagent 5 AP polymer anti-Mouse IgG (RTU)	a. Apply 2 drops (100µL) of Reagent 5 AP polymer antiMouse IgG to cover each section. b. Incubate in moist chamber for 15 min. c. Rinse with PBS containing 0.05% Tween-20 for 2 min, 3 times. d. Rinse with tap water.	15 min
12. Reagent 6A, 6B, 6C **Reagent 6A:** Permanent Red Substrate (RTU) **Reagent 6B:** Permanent Red Activator	a. Add 200µL of **Reagent 6B** (Activator) into 1mL of **Reagent 6A** (Substrate buffer) 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 buffer) and mix well. Add 5µL of **Reagent 6C** (Chromogen) into the mixture and mix well.)	10 min

(5x) **Reagent 6C:** Permanent Red Chromogen (100x)	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. c. Rinse well with distilled water.	
13. 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 ½ - 1 min.) d. Rinse well in distilled water.	
14. Reagent 7: NeoMount Universal (RTU**)**	Apply 2 drops (100µL) or enough volume of **Reagent 7** NeoMount Universal to cover tissue when tissue is wet. Rotate the slides to allow NeoMount Universal spread evenly.	

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. 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. NeoMount Perm, 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.

FOR RESEARCH USE ONLY

Neo-Biotech
183, avenue Georges Clémenceau – 92000 Nanterre

Work Sheet for NB-23-00097 Kit

We designed this work sheet to help you track of each step. We recommend you use this sheet to record the actual time of each step conducted as it will be helpful for questions with our technical support. 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

NB-23-00097 Protocol is suitable when both mouse and rabbit primary antibodies need or do not need pre-treatment step

Protocol Step	**NB-23-00097 Protocol**	**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 (Optional)	HIER if needed User supplied (up to 60 min)				
Step 3 Optional	Preblock if needed User supplied				
Step 4	Mouse 1°Ab #1 User supplied (30-60 min.)				
Step 5	**Reagent 1** HRP Polymer anti-Mouse IgG RTU(15min) Rinse with distilled water				

Neo-Biotech
183, avenue Georges Clémenceau – 92000 Nanterre

Step 6	**Reagent 2A &Reagent 2B** DAB requires mixing (10min)				
Step 7	**Reagent 3** Antibody Blocker (10 min)				
Step 8	**Reagent 4A** DS-MM Blocker A RTU (30 min)				
Step 9	**Reagent 4B** DS-MM Blocker B RTU (5 min)				
Step 10	Ms 1°Ab #2 User supplied (30-60 min)				
Step 11	**Reagent 5** AP Polymer anti-Mouse IgG RTU(15min) Rinse with tap water				
Step 12	**Reagent 6A, Reagent 6B &Reagent 6C** Permanent Red requires mixing.(10min)				
Step 13	Counter stain				
Step 14	**Reagent 7** Simpo-Mount RTU				
Result	Stain pattern on controls are correct: Fill in Yes or NO				

Testing result: