PolyStain DS Kit - for 2 Mouse antibody on Rodent tissue

(DAB/Fast Red)

NB-23-00104-1 (12 ml)

NB-23-00104-2 (36 ml)

NB-23-00104-3 (120 ml)

183, avenue Georges Clémenceau – 92000 Nanterre

NeoBiotech

PolyStain DS Kit - for 2 Mouse antibody on Rodent tissue (DAB/Fast Red)

Cat# NB-23-00104-1; NB-23-00104-2; NB-23-00104-3

INTENDED USE:

Storage: 2- 8ºC

The PolyStain DS Kit is designed to use with two user supplied mouse antibodies to detect two distinct antigens on mouse and rat tissue or cell samples. Specimens can be frozen or paraffin embedded, or freshly prepared monolayer cell smears. Double staining is a common method used in immunohistochemistry that allows for detection of two distinct antigens in a single tissue.

This kit uses an HRP or AP polymer based technology combined with a proprietary blocking buffer system that achieves ultra-sensitivity with no background or cross reactivity.

The PolyStain DS Kit from Neo Biotech supplies the user with primer system to enhance the two polymer enzyme conjugates anti-mouse IgG HRP-polymer and anti-mouse IgG

AP-polymer with two distinct substrates/chromogens, Fast Red and DAB. Fast Red reacts with anti-mouse IgG AP-polymer conjugate to produce a red color. DAB chromogen reacts with anti-Mouse IgG HRP-polymer conjugate to produce a brown color. The PolyStain DS Kit is a non-biotin system that avoids the extra steps involved in blocking non-specific binding due to endogenous biotin.

Please read the protocol carefully and use the experimental record sheet to keep track of your progress throughout the protocol.

KIT COMPONENTS:

Component No.	Content	12mL Kit	36mL Kit	120mL Kit
Reagent 1	Mouse Primer (RTU)	6mL	18mL	60mL
Reagent 2	Mouse HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 3A	DAB Substrate (RTU)	12mL	15mLx2	100mL
Reagent 3B	DAB Chromogen (20x)	1mL	2mL	3mL
Reagent 4	Antibody Blocker (40x)	2x15mL	50mL	100mL
Reagent 5A	DS-MM Blocker A (RTU)	6mL	18mL	60mL
Reagent 5B	DS-MM Blocker B (RTU)	6mL	18mL	60mL
Reagent 6	Mouse AP Polymer (RTU)	6mL	18mL	60mL
Reagent 7A	Fast Red Chromogen	6 tablets	18 tablets	60 tablets
Reagent 7B	Fast Red Substrate (RTU)	5mL x 6	5mL x 18	5mL x 60
Reagent 8	NeoMount Universal	6mL	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. Make sure to start preparing AP-Red+ solution near the end of the secondary antibody incubation.
7. Proceed IHC staining: **DO NOT** let specimen or tissue dry from this point on.

Reagent	**Staining Protocol-1 of NB-23-00104**	**Incubation Time (Min.)**
1. Peroxidase Blocking Reagent Not provided	a. Incubate slides in peroxidase blocking reagent (Ready-to-use 3% H_2O_2 solution) 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	60-90 min
	No background issues go to step 5; if background an issue go to step 3.	
3 Optional: Block step 1 **Reagent** NB-23-00081-1 **Rt Blocking Buffer A**	Not provided in this kit must purchase separately (Reagent NB-23-00081) this block has been a staple in many labs screening mouse primary antibodies on mouse tissue. a. Apply 2 drops or enough volume of Rt blocking buffer A (Reagent **NB-23-00081**) to cover the tissue completely. Incubate in moist chamber for 30min. b. Wash with PBS containing 0.05% Tween-20 for 3 times for 2 min each.	30 min
4. Optional: Block step 2 NB-23-00081 **Rt Blocking Buffer B**	Use this block only if used (**Reagent NB-23-00081-1**) block step 3 was done. a. Apply 2 drops or enough volume of rat blocking buffer B (**Reagent** NB-23-00081) to cover the tissue completely. Incubate in moist chamber for 5min. b. Wash with PBS containing 0.05% Tween-20 for 3 times for 2 min each.	5 min

5. Ms Primary Antibody 1: Supplied by user	**Notes**: Investigator needs to optimize dilution and incubation times prior to double staining. Should use as dilute as possible to prevent cross reaction. 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. Wash with PBS containing 0.05% Tween-20 for 3 times for 2 min each	30-60 min
6. Reagent 1: Mouse Primer (RTU)	a. Apply 1-2 drops of **Reagent 1** (Mouse Primer) or enough to cover each section. b. Incubate in moist chamber for 10 min. c. Wash with PBS containing 0.05% Tween-20 for 3 times for 2 min each.	10 min
7.Reagent 2: Mouse HRP Polymer (RTU)	a. Apply 1-2 drops of **Reagent 2** (Mouse HRP Polymer) to cover each section. b. Incubate in moist chamber for 10 min. c. Wash with PBS containing 0.05% Tween-20 for 3 times for 2 min each.	10 min
8. Reagents 3A, 3B: **3A**: DAB Substrate (RTU) **3B**: DAB Chromogen (20x)	**Note**: Although the DAB step can be done at the end of protocol, we find the DAB chromogen acts as additional shielding between the first mouse and second mouse. We recommend you do this at this step. a. Add 1 drop of **Reagent 3B** (DAB Chromogen) to 1mL **Reagent 3A** (DAB Substrate). Mix well. Protect from light and use within 7 hours. b. Apply 2 drops or enough volume of DAB chromogen mix to completely cover tissue. Incubate for 5 min. c. Rinse well with distilled water. d. Wash with PBS containing 0.05% Tween-20 3 times for 2 min each	5 min
9. Reagent 4: 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. a. Use hot plate or water bath to heat diluted **Reagent 4** to 1x solution (1 part of **Antibody Blocker** in 39 parts of distilled water) to 80-95°C. Make enough volume to cover the tissue in beaker. b. For paraffin embedded tissue, put slides in heated Antibody Blocker for 10 minutes at 95°-100°C. For frozen embedded tissue, put slides in heated Antibody Blocker for 10 minutes at 80°C. c. Cool slides to 55°C. d. Rinse slides in multiple changes of distilled water. e. Wash with PBS/ 0.05% Tween 20	10 min
10. Reagent 5A: DS-MM Blocker A (RTU)	a. Apply 2 drops or enough volume of **Reagent 5A** (DS-MM Blocker A) to cover the tissue completely. Mix well on the slide and Incubate in moist chamber for 30 min. b. Wash with PBS/ 0.05% Tween-20 for 2 minutes, 3 times.	30 min

11. Reagent 5B: DS-MM Blocker B (RTU)	a. Apply 2 drops or enough volume of Reagent 5B (DS-MM Blocker B) to cover the tissue completely. Mix well on the slide and Incubate in moist chamber for 5 min. b. Wash with PBS/ 0.05% Tween-20 for 2 minutes, 3 times	5 min
12. Ms Primary 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. Wash with PBS/ 0.05% Tween-20 for 2 minutes, 3 times	30 – 120 min
13. Reagent 6: Mouse AP Polymer (RTU)	a. Apply 1-2 drops of **Reagent 6** (Mouse AP Polymer) or enough to cover each section. b. Incubate in moist chamber for 15 min. c. Wash with PBS/ 0.05% Tween-20 for 2 minutes, 3 times. d. Rinse twice with distilled water. **Note**: To really intensify Fast Red signal rinse 1x 0.1M Tris pH 8.5 to 9.0	15 min
14. Reagent 7A, 7B: **7A:** Fast Red Chromogen (Tablets) **7B:** Fast Red Substrate (RTU)	**Notes**: It takes about 30 minutes to dissolve the tablet in the substrate buffer. Allow enough time to prepare. a. Dissolve 1 tablet of **Reagent 7A** (Fast Red Chromogen) in 5mL **Reagent 7B** (Fast Red Substrate), vortex until the tablet dissolved completely. Use within 1 hour. b. Apply 2 drops (100µl) or enough volume of Fast Red working solution to completely cover the tissue. Incubate for 10-20 min, observe appropriate color development c. Rinse well with distilled water. **(Fast Red is alcohol soluble; do not dehydrate.)**	10-20 min
15. 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 or Tris pH 7.4 to 8.4 until blue color appears d. Rinse well in distilled water	5 min
16. Reagent 8: NeoMount Universal	a. Apply 2 drops or enough volume of **Reagent 8** (NeoMount Universal) to cover tissue when tissue is wet. Rotate the slides to allow NeoMount Universal spread evenly. **Do Not Coverslip**. 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 NeoMount Universal forms an impervious polymer barrier to organic solvent. Do not use oil directly on the top of dried NeoMount Universal. **Note**: **To coverslip see protocol note 2**	30 min. 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. NeoMount Universal 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. NeoMount Universal does not use a coverslip. However, if you need to coverslip your tissue, after NeoMount Universal has dried, dip the slide in xylene (1 to 2 seconds), apply an organic mounting solution (such as NeoMount Perm, Cat# NB-23-00156), and place cover glass on the slide. Store slides after they have dried completely.

PRECAUTIOUS:

Precautious: DAB may be carcinogenic. Please wear gloves and take other necessary precautions.

FOR RESEARCH USE ONLY

Work Sheet for NB-23-00104 Kit

We designed these work sheets to help you track of each step. When staining fails, these sheets help our technical support staff 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

NB-23-00104 **Protocol-1** is suitable when both mouse primary antibodies need or do not need pre-treatment step.

	Protocol Step	NB-23-00104 Protocol-1 Reagent/Time	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	Step 1	Peroxidase Block User supplied				
2	Step 2 Optional	HIER if needed User supplied (up to 60 min)				
3	Step 3 Optional	NB-23-00081 (30 min)				
4	Step 4 Optional	NB-23-00081 (30 min)				
5	Step 5	Ms 1°Ab #1 User supplied (30-60 min)				
6	Step 6	Reagent 1 Ms Primer RTU (10 min)				
7	Step 7	**Reagent 2** Ms HRP Polymer RTU (10 min)				
8	Step 8	**Reagent 3A & 3B** DAB Requires mixing! (5 min)				
9	Step 9	**Reagent 4** Antibody Blocker(40x) (10 min)				

10	Step 10	**Reagent 5A:** DS-MM Blocker A RTU (30 min)				
11	Step 11	**Reagent 5B:** DS-MM Blocker B RTU (5 min)				
12	Step 12	Ms 1°Ab #2 User supplied (30-60 min)				
13	Step 13	**Reagent 6** Ms AP Polymer RTU (15 min)				
14	Step 14	**Reagent 7A & 7B** Fast Red requires mixing (10-20min)				
15	Step 15	Counter stain Hematoxylin User supplied				
16	Step 16	**Reagent 8** NeoMount Universal Do not coverslip!				
17	Result	t Stain pattern on controls are correct: **Fill in Yes or NO**				

NB-23-00104 Protocol-2 is suitable for one mouse primary antibody needs pre-treatment, the other mouse primary antibody is sensitive to pre-treatment.

	Protocol Step	Protocol Step	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	Step 1	Peroxidase Block User supplied				
2	Step 3 Optional	NB-23-00081 (30 min)				
3	Step 4 Optional	NB-23-00081 (5min)				
4	Step 5	Ms 1°Ab #1 User supplied (30-60 min) 1°Ab is sensitive to pre-treatment				
5	Step 6	**Reagent-1** Ms Primer RTU (10 min)				
6	Step 7	**Reagent-2** Ms HRP Polymer RTU (10 min)				
7	Step 8	**Reagent-3A & 3B** DAB Requires mixing! (5 min)				
8	Step 2	HIER (10-15 min) Cool down (45-60 min) User supplied **Skip antibody blocker step 9 if HIER is done since they will achieve same goal.**				

9	Step 10	**Reagent 5A:** DS-MM Blocking A RTU (30 min)				
10	Step 11	**Reagent 5B:** DS-MM Blocking B RTU (5 min)				
11	Step 12	Ms 1°Ab #2 User supplied (30-60 min)				
12	Step 13	**Reagent 6** Ms AP Polymer RTU (15 min)				
13	Step 14	**Reagent 7A & 7B** Fast Red requires mixing (10-20min)				
14	Step 15	Counter stain Hematoxylin User supply				
15	Step 16	**Reagent 8** : NeoMount Universal -Do not coverslip!				
16	Result	**Stain pattern on controls are correct:** **Fill in Yes or No**				

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