PolyStain DS Kit - for 2 Mouse antibody on Rodent Tissue

For colocalization (Emerald/Permanent Red)

NB-23-00103-1 (12ml)

NB-23-00103-2 (36ml)

NB-23-00103-3 (120ml)

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Storage: 2-8ºC

INTENDED USE:

The PolyStain DS-MM-Ms C Kit is designed to use with two user supplied mouse antibodies to detect two distinct antigens on mouse and rat tissue or cell samples. The advantage of the C kit series is that it will allow you to visualize when two proteins are co localized by producing a third color blue purple. Specimens can be frozen or paraffin embedded, or freshly prepared monolayer cell smears. We recommend you use Normal Rat serum blocking buffer (NB-23-00190) when staining frozen rat or mouse tissue. Double staining is a common method used in immunohistochemistry that allows for detection of two distinct antigens in a single tissue. This C 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. PolyStain DS-MM-Ms C Kit from NeoBiotech labs 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/chromogen, Permanent Red and Emerald. Permanent Red reacts with anti-mouse IgG AP-polymer conjugate to produce a red color. Emerald chromogen reacts with anti-Mouse IgG HRP-polymer conjugate to produce a green color. However when the chromogen are produced in the same place the color appears blue to purple in color. PolyStain DS-MM-Ms C 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	36mL	120mL
Reagent 1	Mouse Primer (RTU)	6mL	18mL	60mL
Reagent 2	Mouse AP Polymer (RTU)	6mL	18mL	60mL
Reagent 3A	Permanent Red Substrate (RTU)	7mL	18mL	60mL
Reagent 3B	Permanent Red Activator (5x)	1.4mL	3.6mL	12mL
Reagent 3C	Permanent Red Chromogen (100x)	70µL	180µL	0.6mL
Reagent 4	Antibody Blocker (40x)	2x15mL	50mL	125mL
Reagent 5A	DS-MM Blocker A (RTU)	6mL	18mL	60mL
Reagent 5B	DS-MM Blocker B (RTU)	6mL	18mL	60mL
Reagent 6	Mouse HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 7	Emerald Chromogen (RTU)	7mL	18mL	60mL
Reagent 8	U-Mount (RTU)	6mL	18mL	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 with 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 pH7.6. NeoBiotech sells 10xTBS-T for your convenience (**NB-23-00201**)

Reagent	Staining Procedure	Incubation Time (Min.)
1. Peroxidase and Alkaline Phosphatase Blocking Reagent **Not provided** Fast, easy and it will block endogenous alkaline phosphatase	a. Incubate slides in PEROXIDASE BLOCKING REAGENT we recommend NeoPure Dual Enzyme Block NB-23-00193 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. Refer to antibody datasheet. b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T (See note **7 above**); 3 times for 2 minutes each. No background issues go to step 5; if background an issue go to step 3.	60 - 90 min
3. Optional: Block step 1 Reagent Normal Rat serum blocking buffer NB-23-00190 **Not provided**	Provided in this kit is a 1 ml sample of Normal Rat serum blocking buffer NB-23-00190 this block has been a staple in many labs screening mouse primary antibodies on mouse tissue. a. Apply 2 drops or enough volume of Normal Rat serum blocking buffer NB-23-00190 to cover the tissue completely. Incubate in moist chamber for 30min. b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.	30 min.

4. **Optional:** **Block step 2** **Reagent** Normal Rat serum blocking buffer NB-23-00190 **Not provided**	Use this block only if Reagent NB-23-00190 was used in step 3. a. Apply 2 drops or enough volume of rat blocking buffer (Reagent NB-23-00190) to cover the tissue completely. Incubate in moist chamber for 5min. b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.	5 min
5. Ms Primary Antibody 1: Supplied by user	**Note**: 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-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. 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-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.	10 min.
7. Reagent 2: Mouse AP Polymer(RTU)	a. Apply 1-2 drops of **Reagent 2** (Mouse AP Polymer) to cover each section. b. Incubate in moist chamber for 10 min. c. Wash only 1X TBS-T; 3 times for 2 minutes each.	10 min.
8.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. [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.] 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 Permanent Red working solution to completely cover the tissue for additional 5 to 10min. c. Wash well with distilled water.	10 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-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.	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-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each. **Note**: Double stain blocker is not the same as D54.	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-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.	30-60 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-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.	30-60min
13. Reagent 6: Mouse HRP Polymer (RTU)	a. Apply 1-2 drops of **Reagent 6** (Mouse HRP Polymer) or enough to cover each section. b. Incubate in moist chamber for 15 min. c. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.	15 min
14. Counterstain (Optional) Not provided	a. Dip the slide in diluted hematoxylin for 5 seconds. (You may dilute hematoxylin 1:5 in d'H_2O). DO NOT over stain with hematoxylin. b. Rinse thoroughly with tap water for 2min. c. Put slides in PBS for 5 seconds to blue, DO NOT over blue. d. Rinse well in distilled or tap water for 2min. e. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.	5 sec

15. Reagent 7 Emerald Chromogen (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 7** (Emerald Chromogen) to cover the tissue completely. b. Incubate in moist chamber for 5 minutes. c. Wash slides in tap water for 1 minute. 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.
16.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
17. Reagent 8 U-Mount(RTU)	a. Apply 1 drop (50µL) of **Reagent 8** (U-Mount) 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 Permanent Red stain.	

TROUBLE SHOOTING

Problem	Tips
Uneven stain on 2 primary antibodies	1. Need to adjust the titer of each antibody. 2. The amount of each protein expressed on tissue may be different. 3. Set slides in water too long so that Emerald is washed away. 4. Set slides in Xylene too long so that Permanent Red is washed away.
Emerald Chromogen is blue not green when non co-localized with Permanent Red.	Emerald should be green when not co-localized with Permanent Red. If Emerald chromogen is blue the titer on the primary antibody is not dilute enough for the protocol. Re-titer primary antibodies individually first.
No stain on 1 or 2 antibodies	Missing steps or step reversed.
Green Background on the slide	1. Titer primary antibody. 2. Use 10% Donkey serum, goat or horse serum as a preblock
Permanent Red is leaching	1. Use fresh 100% ethanol and xylene. 2. Slide sat too long in xylene. Do not go over 20seconds!
Artifacts on slides	Slides not completely dried before mount. Use fresh 100% Ethanol and xylene.

PRECAUTION:

Please wear gloves and take other necessary precautions.

For research use only

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Work Sheet for NB-23-00103 Kit

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 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-23-00103 Protocol-1 is suitable when both mouse primary antibodies need or do not need pre-treatment step

	Main Protocol Step	**NB-23-00103 Protocol-1**	**Experiment 1 Date:**	**Experiment 2 Date:**	**Experiment 3 Date:**	**Experiment 4 Date:**
1	**Step 1**	Peroxidase & Alkaline Phosphatase Block User supplied				
2	**Step 2 Optional**	HIER if needed User supplied (up to 60 min)				
3	**Step 3 Optional**	**NB-23-00190** Normal Rat serum blocking buffer (30min)				
4	**Step 4 Optional**	**NB-23-00190** Normal Rat serum blocking buffer (5 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 AP Polymer RTU (10 min) Wash only with TBS-T.				
8	**Step 8**	**Reagent 3A, 3B & 3C** Permanent Red requires mixing (10min)				
9	**Step 9**	**Reagent 4** Antibody Blocker(40x) (10 min)				

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10	**Step 10**	**Reagent 5A** DS-MM Blocker A RTU (30 min) (5 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 HRP Polymer RTU (15 min)				
14	**Step 14**	Counter stain (Do not over counter stain) Hematoxylin User supply Wash with PBS/0.05% Tween20 for 2 min, 3 times				
15	**Step 15**	**Reagent 7** Emerald Chromogen RTU (5min)				
16	**Step 16**	Dehydrate section 20seconds for each step It is important to follow the protocol.				
17	**Step 17**	**Reagent 8** U-Mount RTU Mount & coverslip				
	Result	Stain pattern on controls are correct: Fill in Yes or NO				

NB-23-00103 Protocol-2 is suitable for one mouse primary antibody needs pre-treatment, the other mouse primary antibody is sensitive to pretreatment.

	Main Protocol Step	NB-23-00103 Protocol-2	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	**Step 1**	Peroxidase & Alkaline Phosphatase Block User supplied				
2	**Step 3 Optional**	**NB-23-00190** Normal Rat serum blocking buffer (30min)				
3	**Step 4 Optional**	**NB-23-00190** Normal Rat serum blocking buffer (5 min)				
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 AP Polymer RTU (10 min) Wash only with 1xTBS-T				
7	**Step 8**	**Reagent 3A, 3B & 3C** Permanent Red requires mixing (10min)				
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 Blocker A RTU (30 min)				
10	**Step 11**	**Reagent 5B** DS-MM Blocker B RTU (5 min)				
11	**Step 12**	Ms 1°Ab #2 User supplied (30-60 min)				
12	**Step 13**	**Reagent 6** Ms HRP Polymer RTU (15 min)				

13	**Step 14**	Counter stain (Do not over counter stain) Hematoxylin User supply Wash with PBS/0.05% Tween20 for 2 min, 3 times.				
14	**Step 15**	**Reagent 7** Emerald Chromogen RTU (5min)				
15	**Step 16**	Dehydrate section 20seconds for each step It is important to follow the protocol.				
16	**Step 17**	**Reagent 8** U-Mount RTU Mount & coverslip				
	Result	Stain pattern on controls are correct: Fill in Yes or NO				