PolyStain DS Kit - for Mouse and Rat antibody on Mouse tissue -

For co-localization (Emerald/Permanent Red)

NB-23-00124- 3(120 ml)

NB-23-00124- 2(36 ml)

NB-23-00124- 1(12 ml)

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NB-23-00124-1; NB-23-00124-2; NB-23-00124-3

Storage: 2-8ºC

INTENDED USE:

The PolyStain DS Kit is designed to use with user supplied mouse and rat primary antibodies to detect two distinct antigens on mouse tissue or cell samples. This kit has been tested in paraffin–embedded tissues. DS210 kits can be used in frozen specimens or freshly prepared monolayer cell smears. Those kits are designed not to give background on most mouse strains. Double staining is one of most common methods used in immunohistostaining that allows detection of two distinct antigens in a single tissue. PolyStain DS Kit from NeoBiotech Labs supplies two polymer enzyme conjugates: Mouse HRP Polymer and Rat AP Polymer with two distinct substrates/chromogens, Emerald (green color, use with the Mouse HRP Polymer) and Permanent Red (red color, use with the Rat AP Polymer). A Primer step is used to increase specificity of antibody staining. 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	Rat AP Polymer (RTU)	6mL	18mL	60mL
Reagent 2A	Permanent Red Substrate (RTU)	7mL	18mL	60mL
Reagent 2B	Permanent Red Activator (5x)	1.4mL	3.6mL	12mL
Reagent 2C	Permanent Red Chromogen (100x)	70µL	180µL	0.6mL
Reagent 3A	DS-MRt Block A(RTU)	6mL	18mL	60mL
Reagent 3B	DS-MRt Block B(RTU)	6ml	18ml	60mL
Reagent 4	Mouse Primer (RTU)	6mL	18mL	60mL
Reagent 5	Mouse HRP Polymer (RTU)	6mL	18mL	60ml
Reagent 6	Emerald Chromogen (RTU)	7mL	18mL	60mL
Reagent 7	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 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 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.

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 and alkaline phosphatase blocking reagent (NeoPure Dual Enzyme Block NB-23-00193 was Recommended) 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. 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.	
3. Rat primary antibody: Supplied by user	**Note**: Investigator needs to optimize the primary antibodies dilution and incubation time prior to double staining. a. Apply 2 drops or enough volume of rat primary antibody 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

4. Reagent 1: Rat AP Polymer(RTU)	a. Add 2 drops (100µL) or enough volume of **Reagent 1** (Rat AP Polymer) to cover the tissue section and Incubate Room Temperature for 10- 15minutes. b. Wash with 1X TBS-T only; 3 times for 2 minutes each	15 min.
5. Reagent 2A, 2B, 2C **Reagent 2A:** Permanent Red Substrate (RTU) **Reagent 2B:** Permanent Red Activator (5x) **Reagent 2C:** 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 2B** (Activator) into 1mL of **Reagent 2A** (Substrate) and mix well. Add 10µL of **Reagent 2C** (Chromogen) into the mixture and mix well. [**Note**: For fewer slides, Add 100µL of **Reagent 2B** (Activator) into 500µL of **Reagent 2A** (Substrate) and mix well. Add 5µL of **Reagent 2C** (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. Rinse well with distilled water. d. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each	10 min
6. Reagent 3A: DS-MRt Block A (RTU)	a. Add 2 drops (100µL) or enough volume of **Reagent 3A** DS-MRt Block A 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
7. Reagent 3B: DS-MRt Block B (RTU)	a. Add 2 drops (100µL) or enough volume of **Reagent 3B** DS-MRt Block B 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
8. Mouse primary antibody: Supplied by user	**Note**: Investigator needs to optimize the primary antibodies dilution and incubation time prior to double staining. a. Apply 2 drops or enough volume of mouse primary antibody 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
9. Reagent 4: Mouse Primer (RTU)	a. Add 2 drops (100µL) or enough volume of **Reagent 4** (Mouse Primer) to cover the tissue section and Incubate Room Temperature for 15minutes. b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.	15 min.

10. Reagent 5: Mouse HRP Polymer (RTU)	a. Add 2 drops (100µL) or enough volume of **Reagent 5** (Mouse HRP Polymer) to cover the tissue section and incubate at Room Temperature for 15minutes. b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each. c. Rinse well with distilled water.	15 min
11. Counterstain (Optional) Not provided	a. Dip the slide in diluted hematoxylin for 5 seconds. (You may dilute hematoxylin 1:5 in dH_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
12. Reagent 6 Emerald Chromogen (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 6** (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, counter stain first. Do not leave slides sitting in water. Always stain with Emerald chromogen AFTER Permanent Red stain and hematoxylin. Permanent Red removes the Emerald*	5 min

TROUBLE SHOOT:

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	Titer primary antibody.
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

PRECAUTIONS:

Please wear gloves and take other necessary precautions.

FOR RESEARCH USE ONLY

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Work Sheet for NB-23-00124 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

Protocol Step	NB-23-00124 Protocol	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase or Alkaline Phosphatase Block User supplied recommended NB-23-00193				
Step 2	HIER if needed Refer to datasheet				
Step 3	Rat 1°Ab (30-60 min.)				
Step 4	**Reagent 1** Rat AP Polymer (15 min)(Wash with TBS-T only)				
Step 5	**Reagent 2A, 2B&2C** Permanent Red requires mixing! (10min+10min)				
Step 6	**Reagent 3A** DS-MRt Block A(RTU) (30min)				

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Step 7	Reagent 3B DS-MRt Block B(RTU) (5min)				
Step 8	Mouse 1°Ab (30-60 min.)				
Step 9	**Reagent 4** Mouse Primer RTU (15 min)				
Step 10	**Reagent 5** Mouse HRP Polymer (15 min) Wash with PBS/TBS-T and rinse well with distilled water				
Step 11	Counter stain (Do not over counter stain) Hematoxylin User supply Wash with PBS/0.05% Tween20 for 2 min, 3 times				
Step 12	**Reagent 5** Emerald Chromogen RTU (5min)				
Step 13	Dehydrate section 20seconds for each step **It is important to follow the protocol.**				
Step 14	**Reagent 6** U-Mount RTU Mount & coverslip				
Result	Stain pattern on controls are correct: Fill in Yes or NO				

Testing result: