PolyStain DS Kit - for Rabbit and Rat antibody on Human & Mouse tissue

(Emerald/Permanent Red)

NB-23-00127- 3(120 ml)

NB-23-00127- 2(36 ml)

NB-23-00127- 1(12 ml)

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NB-23-00127-1; NB-23-00127-2; NB-23-00127-3

INTENDED USE:

Storage: 2-8ºC

The PolyStain DS-Rb+Rt/Hu+Ms Kit is designed for use with user supplied rabbit and rat primary antibodies to detect two distinct antigens on human and mouse tissue or cell samples. This kit has been tested on paraffin embedded tissue. However, this kit can be used to stain frozen specimen and/or freshly prepared monolayer cell smears. Double staining is a common method used in immunohistostaining, allowing for the detection of two distinct antigens in a single tissue. PolyStain DS-Rb+Rt/Hu+Ms Kit supplies the user with two polymer enzyme conjugates: HRP polymer anti-Rat IgG (minimal cross reaction to mouse) and AP polymer anti-Rabbit IgG with two distinct substrates/chromogen, Emerald and Permanent Red. Emerald chromogen reacts with the anti-Rat HRP polymer conjugate to produce a green color. Permanent Red reacts with anti-Rabbit AP polymer to produce the subsequent red color. When two proteins are co-localized a blue/ purple color will develop depending which antigen is stronger. If only the rat antigen is present only the Emerald chromogen will be present and if the rabbit antigen is present only the Permanent Red chromogen will be present. PolyStain DS-Rb+Rt/Hu+Ms Kit is a non-biotin system avoiding the extra steps involved in blocking non-specific binding due to endogenous biotin.

KIT COMPONENTS:

Component No.	Content	12mL Kit	36mL Kit	120mL Kit
Reagent 1	Rat (No Ms) HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 2	Rabbit AP Polymer (RTU)	6mL	18mL	60mL
Reagent 3A	Permanent Red Substrate (RTU)	15mL	18mLx2	120mL
Reagent 3B	Permanent Red Activator (5x)	3mL	7.2mL	12mLx2
Reagent 3C	Permanent Red Chromogen (100x)	150µL	360µL	1.2mL
Reagent 4	Emerald Chromogen (RTU)	15mL	18mLx2	60mL
Reagent 5	U-Mount (RTU)	3mL	9mL	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 prepared as close to a monolayer as possible to obtain satisfactory results.
5. Three control slides are recommended for interpretation of results: positive, reagent (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. 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 interpreting results.

Reagent	Staining Procedure	Incubation Time (Min.)
1. Peroxidase and alkaline phosphatase Blocking Reagent Supplied by user	a. Incubate slides in peroxidase and alkaline phosphatase blocking reagent (NeoPure Dual Enzyme Block NB-23-00193 is Recommended) for 10 minutes. b. Rinse the slides using 2 changes of 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	Up to 1 hour
3. Primary Antibody Mix: one Rat and one Rabbit antibody **Supplied by user**	**Note:** Investigator needs to optimize primary antibody titer prior to double staining as both Permanent Red and Emerald Chromogen are very strong. a. Apply 2 drops or enough volume of rat and rabbit primary antibody mixture to cover the tissue completely. Incubate in moist chamber for 30-60 min. Recommend 30min to shorten total protocol time. b. Wash with PBS/0.05% Tween20 for 2 minutes, 3 times.	30-60 min
4. Polymer mixture: **Reagent 1:** Rat (No Ms) HRP Polymer (RTU)	**Note**: Make sufficient polymer mixture by adding **Reagent 1** Rat (No Ms) HRP Polymer and **Reagent 2** Rabbit AP Polymer at 1:1 ratio, mix well. a. Apply 1 to 2 drops (50-100µl) of the mixture to cover each section. b. Incubate in moist chamber for 30 min.	30 min

Reagent 2: Rabbit AP Polymer (RTU)	c. Wash with PBS/ 0.05% Tween20 for 2 minutes, 3 times. **Make enough mixture for the experiment. Do not make extra volume as mixture is not stable for long term storage.**	
5. Reagent 3A, 3B, 3C **Reagent 3A:** Permanent Red Substrate (RTU) **Reagent 3B:** Permanent Red Activator (5x) **Reagent 3C:** Permanent Red Chromogen (100x)	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. c. Rinse well with distilled water.	10 min
6. Counterstain (Optional) (Optional but must be done before Emerald Chromogen step) Not provided	**Note:** If two antigens are co-localized in nuclear you want less counter stain to optimize the visualization in the nucleus; however you can counter stain using normal protocol time if antigens are co-localized in cytoplasm or membrane or the three antigens are localized in different cells. a. Counterstain dip in diluted hematoxylin for 5 seconds for nuclear co-localization or 30 seconds for cytoplasmic or membrane co-localization. DO NOT over stain with hematoxylin. b. Rinse thoroughly with tap water for 1min. c. Put slides in PBS for 5-10 seconds to blue, **DO NOT** over blue. d. Rinse well in distilled or tap water for 1min. e. Wash slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	5 sec
7. Reagent 4 Emerald Chromogen (RTU)	a. Apply 1 to 2 drops (50-100µl) of **Reagent 4** Emerald Chromogen to cover the tissue completely. b. Incubate in moist chamber for 5 minutes. c. Wash slides in tap water for 1minute. 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.
8.Dehydrate section It is important to follow the protocol.	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!**	30-60 min.

9.Reagent 5 U-Mount (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 5** 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.	

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

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Work Sheet for NB-23-00127 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-23-00127 Protocol is suitable when both Rabbit and rat primary antibodies need or do not need pre-treatment step.

Protocol Step	Protocol NB-23-00127	**Experiment 1 Date:**	**Experiment 2 Date:**	**Experiment 3 Date:**	**Experiment 4 Date:**
Step 1	Peroxidase & Alkaline Phosphatase Block **NB-23-00193** is recommend User supplied				
Step 2	HIER if needed User supplied (up to 60 min)				
Step 3	Rabbit 1°Ab & Rat 1°Ab mix (30-60 min.)				
Step 4	**Reagent 1 & Reagent 2** Rat (No Ms) HRP Polymer & Rabbit AP Polymer require mixing (30 min.)				
Step 5	**Reagent 3A,Reagent 3B &Reagent 3C** Permanent Red requires mixing (5min)				
Step 6	Counter stain (5seconds) (Do not over counter stain) Hematoxylin User supply Wash with PBS/0.05% Tween20 for 2 min, 3 times				
Step 7	**Reagent 4** Emerald Chromogen RTU (5min)				

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Step 8	It is important to follow the protocol to maintain stain! Dehydrate section 20 seconds for each step.				
Step 9	**Reagent 5** U-Mount RTU Mount & coverslip				
Result	**Stain pattern on controls are correct: Fill in Yes or NO**				

Testing result: