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

(Emerald/Permanent Red)

NB-23-00096- 3(120 ml)

NB-23-00096- 2(36 ml)

NB-23-00096- 1(12 ml)

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NB-23-00096-1; NB-23-00096-2; NB-23-00096-3

Storage: 2-8ºC

INTENDED USE:

The PolyStain DS Kit is designed to use with user supplied mouse and rabbit primary antibody to detect two distinct antigens on mouse tissue or cell samples. NB-23-00090 kits can be used on frozen or paraffin embedded tissues, and freshly prepared monolayer cell smears. Our system is designed not give background on most mouse strains however there may be some mouse strains especially when using frozen that require additional blocking; we recommend PureStain Mouse-on-Mouse Kit Blocking A & B solutions (NB-23-00076) to improve specificity of the mouse primary antibody on mouse tissue. Double staining is one of most common methods used in immunohistostaining that allows for revealing two distinct antigens in a single tissue. The PolyStain DS Kit from Neobiotech Labs supplies two polymer enzyme conjugates: Mouse AP Polymer and Rabbit HRP Polymer with two distinct substrates/chromogens, BCIP/NBT (purple color, use with the Mouse AP Polymer) and AEC (green color, use with the Rabbit HRP Polymer). A Primer step is used to increase specificity of antibody staining. Both enzyme conjugates are applied to the specimen at the same time and mixed on the slide. This kit offers simplified steps that make for a quicker and easier protocol than that used in a sequential procedure. The 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	Mouse Primer (RTU)	12ml	18mlx2	120ml
Reagent 2	Mouse HRP Polymer (RTU)	6ml	18ml	60ml
Reagent 3	Rabbit AP Polymer (RTU)	6ml	18ml	60ml
Reagent 4A	Permanent Red Substrate (RTU)	15ml	18mlx2	120ml
Reagent 4B	Permanent Red Activator (5x)	3ml	7.2ml	12mlx2
Reagent 4C	Permanent Red Chromogen (100x)	150µL	360µL	1.2ml
Reagent 5	Emerald Chromogen (RTU)	15ml	18mlx2	120ml
Reagent 6	U-Mount (RTU)	12ml	18mlx2	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 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. We recommend **NeoPure Dual Enzyme Block NB-23-00193**. b. Rinse the slide using distilled water.	10 min.
2. HIER Pretreatment: Refer to Ab data sheet.	a. Heat Induced Epitope Retrieval (HIER) may be required for primary antibody suggested by vendor. 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. PureStain Mouse-on-Mouse Kit Blocking **A** & B solutions (NB-23-00076) **(optional see protocol note 2)**	a. Add 2 drops (100µl) or enough volume of PureStain Mouse-on-Mouse Kit Blocking **A** & B solutions to cover the tissue section and Incubate. b. Rinse with PBS with 0.05% Tween-20 for 2 min., 3 times	30 min.
4. PureStain Mouse-on-Mouse Kit Blocking A & **B** solutions (NB-23-00076) **Not provided (optional see protocol note 2)**	a. Add 2 drops (100µl) or enough volume of PureStain Mouse-on-Mouse Kit Blocking A & **B** solutions to cover the tissue section and Incubate. Do not exceed 5min. b. Rinse with PBS with 0.05% Tween-20 for 2 min., 3 times.	5 min.

5. Mouse antibody 1 and Rabbit antibody 2: **Supplied by user**	**Note**: Investigator needs to optimize dilution and incubation times prior to double staining, as both Permanent Red and Emerald Chromogen are very strong. a. Apply 2 drops or enough volume of both Mouse Primary Antibody and Rabbit 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
6. Reagent 1 Mouse Primer (RTU)	a. Add 2 drops (100µl) or enough volume of **Reagent 1** (Mouse Primer) to cover the tissue section and Incubate Room Temperature for 10-15minutes. b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.	10-15min
7. Reagent 2&3 **Reagent 2 :** Mouse HRP Polymer (RTU) **Reagent 3:** Rabbit AP Polymer (RTU)	**Note**: Make sufficient polymer mixture by adding **Reagent 2** (Mouse HRP Polymer) and **Reagent 3** (Rabbit AP Polymer) at 1:1 ratio, mix well. Do Not mix more than you need for the experiment because the polymer mixture is not stable for long term storage. a. Apply 1 to 2 drops (50-100µL) of the mixture to cover the tissue completely. b. Incubate in moist chamber for 30 min. c. Wash with 1X TBS-T only; 3 times for 2 minutes each	30 min
8. Reagent 4A, 4B, 4C **Reagent 4A:** Permanent Red Substrate (RTU) **Reagent 4B:** Permanent Red Activator (5x) **Reagent 4C:** 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 4B** (Activator) into 1mL of **Reagent 4A** (Substrate) and mix well. Add 10µL of **Reagent 4C** (Chromogen) into the mixture and mix well. [Note: For fewer slides, Add 100µL of **Reagent 4B** (Activator) into 500µL of **Reagent 4A** (Substrate) and mix well. Add 5µL of **Reagent 4C** (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.	10 min

9. 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 those two 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 with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each..	5 seconds.
10. Reagent 5 Emerald Chromogen (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 5** (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 steps because Permanent Red removes the Emerald Chromogen.	5 min.
11. 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 for 20seconds. b. Dehydrate with 95% ethanol for 20seconds. c. Dehydrate with 100% ethanol for 20seconds. d. Dehydrate with 100% ethanol for 20seconds. e. Dehydrate with 100% ethanol for 20seconds. f. Dehydrate with xylene for 20seconds. **CAUTION**: DO NOT dehydrate with xylene longer than 20 seconds! It will erase Permanent Red stain!	30 min. in 40-50°C oven Or: overnight at room temperature
12. Reagent 6 U-Mount (RTU)	a. Apply 1 drop (50µL) of **Reagent 6** (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 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

PROTOCOL NOTES:

1. The fixation, tissue slide thickness, antigen retrieval and primary antibody dilution and incubation time affect results significantly. Investigator needs to consider all factors and determine optimal conditions when interpreting the result.
2. Mouse-on-Mouse Kit Blocking (sample provided) the anti-mouse secondary has been absorbed to rat serum resulting in most mouse strains having no background, however some mouse strains may need additional blocking. Mouse-on-Mouse Kit Blocking (NB-23-00076) works very well on frozen tissue.

PRECAUTIONS:

Please wear gloves and take other necessary precautions.

FOR RESEARCH USE

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Work Sheet for NB-23-00096 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-23-00096 Protocol is suitable when both mouse and rabbit primary antibodies need or do not need pre-treatment step

Protocol Step	NB-23-00096 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	HIER if needed				
Step 3 Optional	PureStain Mouse-on-Mouse Kit Blocking A 30 min. NB-23-00076				
Step 4 Optional	PureStain Mouse-on-Mouse Kit Blocking B 5 min. NB-23-00076				
Step 5	Ms 1°Ab & Rb 1°Ab mix (30-60 min.)				

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Step 6	**Reagent 1** Mouse Primer (15 min.)				
Step 7	**Reagent 2 & Reagent 3** Mouse HRP Polymer & Rabbit AP Polymer require mixing (30 min) **Wash only with 1xTBS-T.**				
Step 8	**Reagent 4A, Reagent 4B& Reagent 4C** Permanent Red requires mixing (10min)				
Step 9	Counter stain (Do not over counter stain) Hematoxylin User supply Wash with PBS/ 0.05% Tween20 for 2 min, 3 times.				
Step 10	**Reagent 5** Emerald Chromogen RTU (5min)				
Step 11	Dehydrate section 20seconds for each step **It is important to follow the protocol.**				
Step 12	**Reagent 6** U-Mount RTU Mount & coverslip				
Result	Stain pattern on controls are correct: Fill in Yes or NO				

Testing result:

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