PolyStain DS Kit - for Goat and Mouse antibody on Human tissue

For co-localization (Emerald/Permanent Red)

NB-23-00118- 3(120 ml)

NB-23-00118- 2(36 ml)

NB-23-00118- 1(12 ml)

Neo-Biotech
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NB-23-00118-1; NB-23-00118-2; NB-23-00118-3

Storage: 2-8ºC

INTENDED USE:

The PolyStain DS Kit is designed to use with user supplied goat and mouse antibodies to detect two distinct antigens on human tissue or cell samples. This kit has been tested in paraffin tissue. However, this kit can be used on frozen specimen and freshly prepared monolayer cell smears.

Double staining is a common method used in immunohistochemistry for the detection of two distinct antigens in a single tissue. The PolyStain DS Kit from NeoBiotech Labs supplies two polymer enzyme conjugates: AP polymer anti-Goat IgG and HRP polymer anti-Mouse IgG with two distinct substrates/chromogens, Emerald (Green) and Permanent Red (Red). Simplified steps offer a convenient protocol as the enzyme conjugates are applied to the specimen sequentially. A second advantage of Kit, it allows the researcher to visualize when two proteins are co-localized because of the color change when the chromogens overlap that can be semi-quantitative. For example, if the area of co-localization stains blue, the antigen indicated by Emerald is expressed at higher concentration in the cell and if the color is purple, the antigen indicated by Permanent-Red is expressed at higher concentrations. The PolyStain DS Kit is non-biotin system avoiding endogenous biotin non-specific binding.

KIT COMPONENTS:

Component No.	Content	6mL Kit	36mL Kit	120mL Kit
Reagent 1	Goat AP Polymer (RTU)	6mL	18mL	60mL
Reagent 2	DS-GM Blocker (RTU)	6mL	18mL	60mL
Reagent 3	Mouse HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 4A	Permanent Red Substrate (RTU)	15mL	18mL x 2	70mL
Reagent 4B	Permanent Red Activator (5x)	3mL	7.2mL	14mL
Reagent 4C	Permanent Red Chromogen (100x)	150µl	360 µl	0.7mL
Reagent 5	Emerald Chromogen(RTU)	7mL	18mL	70mL
Reagent 6	U-Mount (RTU)	12mL	18mL	NA

Gt=Goat Ms=Mouse

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. Tissues must be adhered to the slide properly to ensure maximum quality staining.
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 and negative tissue controls, reagent control (slides treated with Isotype control reagent).
6. Proceed with IHC staining: **DO NOT** let specimens or tissues dry from this point on.
7. 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 results.
8. **Note**: 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. 1X TBS-T =50mM Tris HCl, 150mM NaCl, 0.05% Tween-20 pH7.6.

Equipment or material needed but not provided:

1. Equipment and material for deparaffinization, such as fume absorbing hood, etc.
2. Heat source (microwave or hot plate) for HIER and antigen retrieval buffers.
3. Thermometer
4. Beaker
5. Timer
6. Wash buffer: 0.01 M pH7.4 PBS with 0.5% Tween20
7. Peroxidase and alkaline phosphatase blocking buffer
8. 100% ethanol 9. 100% Xylene 10. Hematoxylin

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 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/0.05% Tween20 for 2 minutes, 3 times.	Up to 1 hour
3. Primary Antibody Mix: **one Goat and one Mouse antibody** Supplied by user	**Note**: Investigator needs to optimize dilution prior to double staining. a. Apply 2 drops or enough volume of goat and mouse 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-T containing 0.05% Tween-20 or 1X TBST; 3 times for 2 minutes each	30-60min
4. Reagent 1 Goat AP Polymer (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 1** (Goat AP Polymer) to cover each section. b. Incubate in moist chamber for 15 min. c. Wash with 1X TBS-T only; 3 times for 2 minutes each.	15 min.
5. Reagent 2 DS-GM Blocker (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 2** (DS-GM Blocker) to cover each section. b. Incubate in moist chamber for 10 min. c. Blot off solution. Rinse once with PBS-T containing 0.05% Tween-20 for 5sec.	10 min
6. Reagent 3 Mouse HRP(AEC) Polymer (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 3** (Mouse HRP Polymer) to cover each section. b. Incubate in moist chamber for 15 min. c. Wash with 1X TBS-T only; 3 times for 2 minutes each.	15 min
7. Reagent 4A, 4B, 4C **Reagent 4A:** Permanent Red Substrate (RTU)	**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**	10 min

Reagent 4B: Permanent Red Activator (5x) **Reagent 4C:** Permanent Red Chromogen (100x) **(To get maximum sensitivity of AP polymer, Please repeat chromogen step)**	(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.	
8. 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 TBST**; 3 times for 2 minutes each.	5 sec
9. 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, do counter stain first. Do not leave slides sitting in water. Always stain Emerald chromogen **AFTER** Permanent Red stain and hematoxylin because Permanent Red removes the Emerald.*	5 min.
10.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
11. 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

PRECAUTIONS:

Please wear gloves and take other necessary precautions.

FOR RESEARCH USE

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Work Sheet for NB-23-00118 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-23-00118 Protocol	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase & levamisole Block **NB-23-00193** is recommended. User supplied				
Step 2 Optional	HIER if needed User supplied (up to 60 min)				
Step 3	Mix one Goat and one Mouse primary antibodies User supplied (30-60 min)				
Step 4	**Reagent 1** Goat AP Polymer RTU (15min)				
Step 5	**Reagent 2** DS-GM Blocker RTU (10min) Rinse with PBS then Go immediately to step 6				
Step 6	**Reagent 3** Mouse HRP Polymer RTU (15min) **Wash with 1xTBS-T only.**				

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

Testing result: