



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

For colocalization (Emerald/Permanent Red)

NB-23-00115- 3(120 ml)

NB-23-00115- 2(36 ml)

NB-23-00115- 1(12 ml)

**PolyStain DS Kit - for Goat and Rat antibody on Human/Mouse tissue –
For colocalization (Emerald/Permanent Red)**

NB-23-00115-1; NB-23-00115-2; NB-23-00115-3

Storage: 2-8°C

INTENDED USE:

The PolyStain DS-GRt-Hu/Ms C Kit is designed to use with user supplied goat and rat primary antibodies to detect two distinct antigens on human/mouse tissue or cell samples. The kit has been tested on paraffin-embedded human and mouse tissues. Double staining is one of most common methods used in immunohistostaining that allows for the detection of two distinct antigens in a single tissue. The design of PolyStain DS-GRt-Hu/Ms C Kit from NeoBiotech Labs supply two polymer enzyme conjugates: AP polymer anti-Goat IgG and HRP polymer anti-Rat IgG with two distinct substrates/chromogen, HRP/Emerald (Green) and AP/Permanent Red (red). With this protocol the goat and rat primary antibodies will be applied to the tissue at the same time but the user will apply the polymer enzyme conjugate to the specimen sequentially. When two proteins are present in the same location a blue or purple color will develop depending on quantity of each antigen screened. If only the anti-goat antigen is present only the Permanent Red chromogen will be present and if the Rat antigen is present only the Emerald chromogen will be present in the cell. The PolyStain DS-GRt-Hu/Ms C 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 2A	Permanent Red Substrate (RTU)	15mL	18mLx2	70mL
Reagent 2B	Permanent Red Activator (5x)	3mL	7.2mL	14mL
Reagent 2C	Permanent Red Chromogen (100x)	150µL	360µL	0.7mL
Reagent 3	DS-Gt/Rt Blocker (RTU)	6mL	18mL	60mL
Reagent 4	Rat Primer (RTU)	6mL	18mL	60mL
Reagent 5	Rat HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 6	Emerald Chromogen (RTU)	7mL	18mL	70mL
Reagent 7	U-Mount (RTU)	12mL	18mL	NA

Gt=Goat, Rt =Rat

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. NeoBiotech sells 10xTBS-T for your convenience (NB-23-00201)

Equipment or chemicals/buffers needed but not provided:

fume absorbing hood, etc.	Peroxidase and alkaline phosphatase blocking buffer
Heat source (microwave or hot plate) for antigen retrieval buffers.	100% ethanol
Thermometer	100% Xylene
Beaker	Hematoxylin
Timer	Wash buffer: 0.01M PBS with 0.5% Tween20, pH7.4
coverslip	

Reagent	Staining Procedure	Incubation Time (Min.)
<p>1. Peroxidase and Alkaline Phosphatase Blocking Reagent Not provided Fast, easy and it will block endogenous alkaline phosphatase</p>	<p>a. Incubate slides in peroxidase and alkaline phosphatase blocking reagent (NeoPure Dual Enzyme Block NB-23-00193 was Recommended) for 10 minutes b. Rinse the slide using 2 changes of distilled water.</p>	10 min.
<p>2. HIER Pretreatment: Refer to antibody data sheet.</p>	<p>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 8 above); 3 times for 2 minutes each.</p>	Up to 1 hour
<p>3. Primary Antibody Mix: one Goat and one Rat antibody Supplied by user</p>	<p>Note: Investigator needs to optimize dilution prior to double staining. a. Apply 2 drops or enough volume of goat and rat primary antibodies 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 TBS-T; 3 times for 2 minutes each.</p>	30-60min
<p>4. Reagent 1 Goat AP Polymer</p>	<p>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 only with 1X TBS-T, 3 times for 2 minutes each</p>	15 min.
<p>5. Reagent 2A, 2B, and 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)</p>	<p>Note: Shake Permanent Red Activator before adding into Permanent Red Substrate. a. Add 200μL of Reagent 2B (Activator) into 1mL of Reagent 2A (Substrate buffer) 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 buffer) 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.</p>	10 min

<p>6. Reagent 3 DS-GRt Blocker (RTU)</p>	<p>a. Apply 1 to 2 drops (50-100µL) of Reagent 3 (DS-GRt Blocker) to cover each section. b. Incubate in moist chamber for 10 min. c. Tap off solution. Wipe away excess but DO NOT Rinse</p>	<p>10 min</p>
<p>7. Reagent 4 Rat Primer (RTU)</p>	<p>a. Add 2 drops (100µL) or enough volume of Reagent 4 (Rat Primer) to cover the tissue section and Incubate at Room Temperature for 10-15 minutes. b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each.</p>	<p>15 min</p>
<p>8. Reagent 5: Rat HRP Polymer (RTU)</p>	<p>a. Apply 1 to 2 drops (50-100µL) of Reagent 5 (Rat HRP Polymer) to cover each section. b. Incubate in moist chamber for 10-15 min. c. Wash with distilled water.</p>	<p>15min</p>
<p>9. Counterstain (Optional) (Optional but recommend to be done before Emerald Chromogen step) Not provided</p>	<p>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 two antigens are localized in different cells. a. Counterstain dip in diluted hematoxylin for 5 seconds for nuclear colocalization 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. Wash with PBS-T containing 0.05% Tween-20 or 1X TBS-T; 3 times for 2 minutes each. Do not leave longer than 30 seconds in counter stain as the HRP enzyme will start to fail.</p>	<p>10 to 30sec.</p>
<p>10. Reagent 6 Emerald Chromogen (RTU)</p>	<p>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.</p>	
<p>11. Dehydrate section It is important to follow the protocol.</p>	<p>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.</p>	

CAUTION: DO NOT dehydrate with xylene longer than 20 seconds! It will erase Permanent Red stain!

12. Reagent 7 U-Mount (RTU)	a. Apply 1 (50 μ L) of Reagent 7 (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.
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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	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:

Standard laboratory personal protective equipment should be worn: i.e. gloves, eye protection and appropriate lab coat.

FOR RESEARCH USE

Work Sheet for NB-23-00115 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-23-00115 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	HIER if needed				
Step 3	Gt 1°Ab & Rat 1°Ab mix (30-60 min.)				
Step 4	Reagent 1 Goat AP Polymer RTU (15min) Wash with 1xTBS-T only.				
Step 5	Reagent 2A, 2B, 2C Permanent Red requires mixing. (10min)				
Step 6	Reagent 3 DS-GRt Blocker RTU (10min) Do Not Rinse Tap off & go directly to step 7				
Step 7	Reagent 4 Rat Primer RTU (10-15 min.)				

Step 8	Reagent 5 Rat HRP Polymer RTU (10-15min)				
Step 9	Counter stain with Hematoxylin Supplied by user				
Step 10	Reagent 6 Emerald Chromogen RTU (5 min)				
Step 11	Dehydrate section 20seconds for each step It is important to follow the protocol.				
Step 12	Reagent 7 U-mount RTU Mount & coverslip				
Result	Stain pattern on controls are correct: Fill in Yes or NO				

Testing result: