

Ne  *Biotech*

NeoStain DS-RR-Hu/Ms C

Kit for

immunohistochemistry

staining

NB-23-00107- 1

NB-23-00107- 2

NB-23-00107- 3

NeoStain DS-RR-Hu/Ms C Kit - for immunohistochemistry staining

#Cat: NB-23-00107-1 Size: 12ml (60 slides)

#Cat: NB-23-00107-2 Size: 36ml (180 slides)

#Cat: NB-23-00107-3 Size: 120ml (600 slides)

Storage: 2-8°C**Intended use:**

The **NeoStain DS-RR-Hu/Ms C Kit** is designed to use with user supplied two rabbit 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 one of most common methods used in immunohistostaining that allow revealing two distinct antigens in a single tissue. **NeoStain DS-RR-Hu/Ms C Kit** from NeoBiotech supplies two polymer enzyme conjugates: HRP Polymer anti - Rabbit IgG and AP Polymer anti-Rabbit IgG with two distinct substrates/chromogens, Permanent Red (Red color, use with AP polymer anti-Rabbit IgG) and Emerald chromogen (Green color, use with HRP polymer anti-Rabbit IgG). A second advantage of c- 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 than the antigen indicated by Permanent-Red is expressed at higher concentrations. **NeoStain DS-RR-Hu/Ms C 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	AP Polymer anti-Rabbit (RTU)	6ml	18ml	60ml
Reagent 2A	Permanent Red AP Substrate (RTU)	15ml	18mlx2	120ml
Reagent 2B	Permanent Red Activator (5x)	3ml	7.2ml	12mlx2
Reagent 2C	Permanent Red Chromogen(100x)	150µL	360µL	1.2ml
Reagent 3A	DS-RR Block A (RTU)	6mL	18mL	60mL
Reagent 3B	DS-RR Block B (RTU)	6mL	18mL	60mL
Reagent 4	HRP Polymer anti-Rabbit (RTU)	6mL	18mL	60mL
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 need to be adhered to the slide tightly to avoid tissue falling off.
3. Paraffin embedded section must be deparaffinized with xylene and rehydrated with a graded series of ethanol before staining.
4. Cell smear samples should be made as much 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. **Important:** Never combine two antibodies from the same host species in one incubation step.
7. Proceed IHC staining: **DO NOT** let specimen or tissue dry from this point on.
8. 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. Neo Biotech sells 10xTBS-T for your convenience (NB-23-00201)

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. Timer
5. Beaker
6. Wash buffer: 0.01 M PBS with 0.5% Tween20, pH7.4
7. Peroxidase and alkaline phosphatase blocking buffer
8. 100% ethanol
9. 100% Xylene
10. Hematoxylin
11. Coverslip

Reagent	Staining Procedure	Incubation Time (Min.)
1. Peroxidase and Alkaline Phosphatase Blocking Reagent Not provided We recommend using Neopure Dual Enzyme Block NB-23-00193 Fast, easy and it will block	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
2. HIER Pretreatment: Refer to antibody 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 TBST (See note 8 above) ; 3 times for 2 minutes each.	UP to 1h
3. Neoblock (optional)	a. For paraffin section, improved formula saves the need for a Neoblock step. b. For frozen tissue, Neoblock may or may not be required depending on fixative. Neoblock catalogue No. NB-23-00169 as recommended.)	
4. Rabbit Antibody 1: Supplied by user	Notes: Investigator needs to optimize dilution and incubation times prior to double staining. a. Apply 2 drops or enough volume of rabbit primary antibody 1 to cover the tissue completely. Incubate in moist chamber for 30-60 min. b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBST; 3 times for 2 minutes each.	30 – 60 min.
5. Reagent 1 AP Polymer anti-Rabbit (RTU)	a. Apply 2 drops (100µl) of Reagent 1 AP Polymer anti- Rabbit to cover each section. b. Incubate in moist chamber for 20-30 min. c. Wash with 1X TBS-T only ; 3 times for 2 minutes each.	15 - 20 min
6. 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 buffer) and mix well. Add 10µL of Reagent 2C (Chromogen) into the mixture and mix well. [Note: For fewer slides, Add 100µL of Reagent 2 (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	10

	<p>working solution to completely cover the tissue for additional 5 to 10min.</p> <p>c. Rinse well with distilled water.</p>	
<p>7. Reagent 3A: DS-RR Blocker A</p>	<p>a. Apply 2 drops (100µl) or enough volume of Reagent 3A DS-RR Blocker A to cover the tissue completely. Mix well on the slide and Incubate in moist chamber for 30 min.</p> <p>b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBST; 3 times for 2 minutes each.</p>	30
<p>8. Reagent 3B: DS-RR Blocker B</p>	<p>a. Apply 2 drops (100µl) or enough volume of Reagent 3B DS-RR Blocker B to cover the tissue completely. Mix well on the slide and Incubate in moist chamber for 5 min.</p> <p>b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBST; 3 times for 2 minutes each</p>	5
<p>9. Rabbit antibody 2: Supplied by user</p>	<p>Notes: Investigator needs to optimize dilution and incubation times prior to double staining.</p> <p>a. Apply 2 drops (100µl) or enough volume of rabbit primary antibody 2 to cover the tissue completely.</p> <p>b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBST; 3 times for 2 minutes each.</p>	30 – 60
<p>10. Reagent 4: HRP Polymer anti- Rabbit(RTU)</p>	<p>a. Apply 2 drops (100µl) of Reagent 4 HRP Polymer anti Rabbit to cover each section.</p> <p>b. Incubate in moist chamber for 15-20 min.</p> <p>c. Rinse with tap water for 2 min., 3 times</p>	15- 20
<p>11. Counterstain (Optional but must 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 three antigens are localized in different cells.</p> <p>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.</p> <p>b. Rinse thoroughly with tap water for 1min.</p> <p>c. Put slides in PBS for 5-10 seconds to blue, DO NOT over blue.</p> <p>d. Rinse well in distilled or tap water for 1min.</p> <p>e. Wash with PBS-T containing 0.05% Tween-20 or 1X TBST; 3 times for 2 minutes each.</p>	5 sec

<p>12. Reagent 5: Emerald Chromogen (RTU)</p> <p>Do hematoxylin first.</p>	<p>a. Apply 1 to 2 drops (50-100µL) of Reagent 5 Emerald Chromogen to cover the tissue completely.</p> <p>b. Incubate slides in humid chamber for 5 minutes.</p> <p>c. Wash slides in tap water for 10 seconds!</p> <p>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.</p>	<p>5</p>
<p>13. Dehydrate section</p>	<p>Note: Please wipe off extra water and air-dry slides before dehydration and clear.</p> <p>a. Dehydrate with 85% ethanol 20seconds.</p> <p>b. Dehydrate with 95% ethanol 20seconds.</p> <p>c. Dehydrate with 100% ethanol 20seconds.</p> <p>d. Dehydrate with 100% ethanol 20seconds.</p> <p>e. Dehydrate with 100% ethanol 20seconds.</p> <p>f. Dehydrate with xylene 20seconds.</p> <p>CAUTION: DO NOT dehydrate with xylene longer than 20 seconds! It will erase Permanent Red stain!</p>	<p>2</p>
<p>14. Reagent 6 U-Mount (RTU)</p>	<p>a. Apply 1 to 2 drops (50-100µL) of Reagent 6 (U-Mount) to cover the tissue section and apply glass coverslip.</p> <p>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.</p>	

Protocol Notes:

Problem	Tips
Uneven stain on 2 primary antibodies	<ol style="list-style-type: none">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.	<ol style="list-style-type: none">1. 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	<ol style="list-style-type: none">1. Missing steps or step reversed.
Green Background on the slide	<ol style="list-style-type: none">1. Titer primary antibody.2. Use 10% goat serum, donkey serum, or Horse serum as Neoblock.
Permanent Red is leaching	<ol style="list-style-type: none">1. Use fresh 100% ethanol and xylene.2. Slide sat too long in xylene. Do not go over 20 seconds!
Artifacts on slides	<ol style="list-style-type: none">1. Slides not completely dried before mount. Use fresh 100% Ethanol and xylene.

Precautions:

Please wear gloves and take other necessary precautions.

Remarks:

For research use only.

References:

1. De Pasquale A , Paterlini P , Quaglino D. Immunochemical demonstration of different antigens in single cells in paraffin-embedded histological sections. Clin Lab Haematol. 1982;4(3):267-72.
2. Polak J. M and Van Noorden S. Introduction to Immunocytochemistry Second Edition. Bios Scientific Publishers. P41-54. 1997

Work Sheet for NB-23-00107 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 “V “each step during the experiment
- Steps follow after de-paraffinization
- Refer to insert for details of each step

NB-23-00107 Protocol is suitable when both rabbit and rabbit primary antibodies need or do not need pre-treatment step

Step / Protocol	NB-23-00107 Protocol Reaction time	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase Block				
Step 2	HIER if needed				
Step 3	Neoblock (Optional)				
Step 4	1 st Rabbit 1°Antibody Supplied by user 30-60 min				
Step 5	Reagent 1 AP Polymer anti- Rabbit (RTU) 15min				
Step 6	Reagent 2A, Reagent 2B& Reagent 2C Permanent Red requires mixing (10min)				
Step 7	Reagent 3A DS-RR Blocker A(RTU) 30min				
Step 8	Reagent 3B DS-RR Blocker B(RTU) 5min				
Step 9	2 nd Rabbit 1°Antibody Supplied by user				
Step 10	Reagent 4 HRP Polymer anti-Rabbit (RTU) 15min				
Step 11	Counterstain (Optional but must be done before Emerald Chromogen step) Not provided				
Step 12	Reagent 5 Emerald Chromogen (RTU) 5min Do hematoxylin first.				

Step 13	Dehydrate section 20 seconds 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: