# PolyStain DS Kit - for 2 Rabbit antibody on Human/Rodent tissue

For co-localization (Emerald/Permanent Red)

**NB-23-00107- 3(120 ml)**

**NB-23-00107- 2(36 ml)**

**NB-23-00107- 1(12 ml)**

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NB-23-00107-1; NB-23-00107-2; NB-23-00107-3

**Storage: 2-8ºC**

## INTENDED USE:

The PolyStain DS 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. PolyStain DS Kit from NeoBiotech Labs 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 NeoBiotech 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. 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** | AP Polymer anti-Rabbit(RTU) | 6ml | 18ml | 60ml |
| **Reagent 2A** | Permanent Red AP Substrate(RTU) | 7ml | 18ml | 60ml |
| **Reagent 2B** | Permanent Red Activator (5x) | 1.4ml | 3.6ml | 12ml |
| **Reagent 2C** | Permanent Red Chromogen(100x) | 70µL | 180µL | 0.6ml |
| **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) | 6mL | 18mL | 60mL |
| **Reagent 6** | 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 need to be adhered to the slide tightly to avoid tissue falling off.
3. Paraffin embedded section must be deparffinized 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.

## 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 Blocking Reagent Not provided** | a. Incubate slides in peroxidase blocking reagent (Ready-to-use 3% $H_2O_2$ solution) for 10 minutes.<br>b. Rinse the slide using distilled water | 10 min. |
| **2. HIER Pretreatment:** Refer to antibody data sheet. | a. Heat Induced Epitope Retrieval (HIER) may be required for primary antibody suggested by vendor.<br>b. Wash with PBS for 2 min., 3 times | UP to 1h |
| **3.** Preblock (optional) | a. For paraffin section, improved formula saves the need for a preblock step.<br>b. For frozen tissue, preblock may or may not be required depending on fixative. (Preblock catalogue No. **NB-23-00169** was recommended.) | |
| **4. Rabbit Antibody 1:** Supplied by user | **Notes**: Investigator needs to optimize dilution and incubation times prior to double staining.<br>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.<br>b. Rinse with PBS containing 0.05% Tween-20 for 2 min., 3 times. | 30 - 60 min. |
| **5.Reagent 1** AP Polymer anti-Rabbit(RTU) | a. Apply 2 drops (100µl) of Reagent 1 AP Polymer antiRabbit to cover each section.<br>b. Incubate in moist chamber for 20-30 min.<br>c. Rinse with PBS containing 0.05% Tween-20 for 2 min., 3 times.<br>d. Rinse well with tap water. | 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) | 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.]<br>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.<br>c. Rinse well with distilled water. | 10 min |
| **7. Reagent 3A:** DS-RR Blocker A | 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.<br>b. Rinse with PBS containing 0.05% Tween-20 for 2 min., 3 times. | 30 min |
| **8. Reagent 3B:** DS-RR Blocker B | 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.<br>b. Rinse with PBS containing 0.05% Tween-20 for 2 min., 3 times. | 5 min |

| | | |
|---|---|---|
| **9. Rabbit antibody 2:**<br>Supplied by user | **Notes**: Investigator needs to optimize dilution and incubation times prior to double staining.<br>a. Apply 2 drops (100µl) or enough volume of rabbit primary antibody 2 to cover the tissue completely.<br>b. Rinse with PBS containing 0.05% Tween-20 for 2 min., 3 times | 30 - 60 min. |
| **10. Reagent 4:**<br>HRP Polymer anti-Rabbit(RTU) | a. Apply 2 drops (100µl) of Reagent 4 HRP Polymer antiRabbit to cover each section.<br>b. Incubate in moist chamber for 15-20 min.<br>c. Rinse with tap water for 2 min., 3 times | 15- 20 min. |
| **11.** Counterstain **(Optional but must be done before Emerald Chromogen step)**<br>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.<br>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.<br>b. Rinse thoroughly with tap water for 1min.<br>c. Put slides in PBS for 5-10 seconds to blue, **DO NOT** over blue.<br>d. Rinse well in distilled or tap water for 1min.<br>e. Wash slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times. | 5 sec |
| **12. Reagent 5:**<br>Emerald Chromogen (RTU)<br><br>**Do hematoxylin first.** | a. Apply 1 to 2 drops (50-100µL) of **Reagent 5** Emerald Chromogen to cover the tissue completely.<br>b. Incubate slides in humid chamber for 5 minutes.<br>c. Wash slides in tap water for 10 seconds!<br>***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 |
| **13.Dehydrate section** | **Note**: Please wipe off extra water and air dry slides before dehydration and clear.<br>a. Dehydrate with 85% ethanol 20seconds.<br>b. Dehydrate with 95% ethanol 20seconds.<br>c. Dehydrate with 100% ethanol 20seconds.<br>d. Dehydrate with 100% ethanol 20seconds.<br>e. Dehydrate with 100% ethanol 20seconds.<br>f. Dehydrate with xylene 20seconds.<br>**CAUTION: DO NOT dehydrate with xylene longer than 20 seconds! It will erase Permanent Red stain!** | 2 min |
| **14. Reagent 6**<br>U-Mount(RTU) | a. Apply 1 to 2 drops (50-100µL) of Reagent 6 (U-Mount) to cover the tissue section and apply glass coverslip.<br>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.<br>2. The amount of each protein expressed on tissue may be different.<br>3. Set slides in water too long so that Emerald is washed away.<br>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 | 1. Titer primary antibody.<br>2. Use 10% Donkey serum, goat or horse serum as a preblock. |
| Permanent Red is leaching | 1. Use fresh 100% ethanol and xylene.<br>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 ONLY**

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# 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 "√ "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

| **Protocol Step** | **NB-23-00107 Protocol** | **Experiment 1 Date:** | **Experiment 2 Date:** | **Experiment 3 Date:** | **Experiment 4 Date:** |
|---|---|---|---|---|---|
| **Step 1** | Peroxidase Block | | | | |
| **Step 2** | HIER if needed | | | | |
| **Step 3** | Preblock(Optional) | | | | |
| **Step 4** | 1st 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) | | | | |

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| | | | | | |
|---|---|---|---|---|---|
| **Step 7** | **Reagent 3A**<br>DS-RR Blocker A(RTU)<br>30min | | | | |
| **Step 8** | **Reagent 3B**<br>DS-RR Blocker B(RTU)<br>5min | | | | |
| **Step 9** | 2nd Rabbit 1°Antibody<br>Supplied by user | | | | |
| **Step 10** | **Reagent 4**<br>HRP Polymer anti-Rabbit<br>(RTU) 15min | | | | |
| **Step 11** | Counterstain<br>**(Optional but must be done before Emerald Chromogen step)**<br>Not provided | | | | |
| **Step 12** | **Reagent 5**<br>Emerald Chromogen<br>(RTU) 5min Do<br>hematoxylin first. | | | | |
| **Step 13** | Dehydrate section<br>20seconds for each step<br><br>**It is important to follow the protocol.** | | | | |
| **Step 14** | **Reagent 6**<br>U-Mount (RTU) Mount<br>& coverslip | | | | |
| **Result** | Stain pattern on controls are correct: Fill in Yes or NO | | | | |

Testing result:

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