# NeoStain Poly DS Kit - for Goat and Rabbit antibody on Human/Rodent tissue

For co-localization (Emerald/Permanent Red)

**NB-23-00111**

# NeoStain Poly DS Kit - for Goat and Rabbit antibody on Human/Rodent tissue For co-localization (Emerald/Permanent Red)

**#Cat : NB-23-00111-3** **Size: 120 ml**
**#Cat : NB-23-00111-2** **Size: 36 ml**
**#Cat : NB-23-00111-1** **Size: 12 ml**

**Storage: 2-8ºC**

**Intended use:**

NeoStain Poly DS Kit is designed to use with user supplied goat and rabbit primary antibodies, to detect two distinct antigens on human and mouse 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 commonly methods used in immunohistostaining for revealing two distinct antigens in a single tissue.

NeoStain Poly DS Kit from NeoBiotech Labs supplies two polymer enzyme conjugates: HRP Polymer anti-Goat IgG and AP Polymer anti-Rabbit IgG with two substrates/chromogens, Emerald (Green) and Permanent Red (Red). Simplified steps offer a convenient protocol as the enzyme conjugates are applied to the specimen simultaneously. Permanent Red reacts with anti-rabbit AP polymer conjugate to produce the red color. Emerald chromogen reacts with anti-Goat HRP polymer conjugate to produce the green color. When two proteins are co-expressed in the same location, the area of colocalization shows blue color if more Emerald is present and purple blue if more Permanent Red is present.

NeoStain Poly DS Kit is a non-biotin system, avoiding blocking steps for endogenous biotin non-specific binding.

**Kit components:**

| Component No. | Content | 6mL Kit | 36mL Kit | 120mL Kit |
|---|---|---|---|---|
| **Reagent 1** | Goat HRP Polymer (RTU) | 6ml | 18ml | 60ml |
| **Reagent 2** | Rabbit AP Polymer (RTU) | 6ml | 18ml | 60ml |
| **Reagent 3A** | Permanent Red Substrate (RTU) | 15ml | 18ml x 2 | 120ml |
| **Reagent 3B** | Permanent Red Activator (5x) | 3ml | 7.2ml | 12ml x 2 |
| **Reagent 3C** | Permanent Red Chromogen (100x) | 150µl | 360µL | 1.2mL |
| **Reagent 4** | Emerald Chromogen (RTU) | 15ml | 18ml x 2 | 120ml |
| **Reagent 5** | U-Mount (RTU) | 12ml | 18ml x 2 | NA |



**Neo Biotech**
**74 rue des Suisses – 92000 Nanterre**

**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 deparffinized with xylene and rehydrated with a graded series of alcohols before staining.
4. Cell smear samples should be prepared as close to a monolayer as possible to obtain satisfactory results.
5. Three control slides are recommended for interpretation of results: positive, reagent (slides treated with Isotype control reagent), and negative control.
6. **DO NOT** let specimen or tissue dry during protocol.
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. 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.

| Reagent | Staining Procedure | Incubation Time (Min.) |
|---|---|---|
| **1. Peroxidase and Alkaline Phosphatase Blocking Reagent**<br>Not provided | a. Incubate slides in peroxidase and alkaline phosphatase blocking reagent (NeoPure Dual Enzyme Block **NB-23-00193** was Recommended)<br>b. Rinse the slide using distilled water at least twice. | 10 – 20 min. |
| **2. HIER Pretreatment:**<br>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-T containing 0.05% Tween-20 or 1X TBST (**See note 8 above**); 3 times for 2 minutes each. | |
| **3.** Primary Antibody Mix**: one Goat and one Rabbit antibody**<br><br>Supplied by user | **Note**: Investigator needs to optimize dilution prior to double staining.<br>a. Apply 2 drops (100µL) or enough volume of goat and rabbit primary antibodies mixture to cover the tissue completely. Incubate in moist chamber for 30-60min. Recommend 30min to shorten total protocol time.<br>b. Wash with PBS-T containing 0.05% Tween-20 or 1X TBST; 3 times for 2 minutes each. | 30-60min |

| | | |
|---|---|---|
| **4.**Mix<br>**Reagent 1:**<br>Goat HRP Polymer (RTU) with<br>**Reagent 2** Rabbit AP Polymer (RTU) | **Note**: Only make enough mixture for the experiment performed. Mixture is not stable for long term storage. Make sufficient polymer mixture by adding **Reagent 1** Goat HRP Polymer and **Reagent 2** Rabbit AP Polymer at 1:1 ratio, mix well.<br>a. Apply 2 drops (100µL) or enough volume of the mixture to cover each section.<br>b. Incubate in moist chamber for 30min.<br>c. Wash with 1X TBS-T only; 3 times for 2 minutes each | 30min. |
| **5. Reagent 3A, 3B, 3C**<br><br>**Reagent 3A:**<br>Permanent Red Substrate (RTU)<br>**Reagent 3B:**<br>Permanent Red Activator (5x) **Reagent 3C:** Permanent Red Chromogen (100x)<br><br>**To get maximum sensitivity of AP polymer, Please repeat chromogen step** | **Note**: Shake Permanent Red Activator before adding into Permanent Red Substrate.<br>a. Add 200µL of **Reagent 3B** (Activator) into 1mL of **Reagent 3A** (Substrate buffer) and mix well. Add 10µL of **Reagent 3C** (Chromogen) into the mixture and mix well. (Note: For fewer slides, Add 100µL of **Reagent 3B** (Activator) into 500µL of **Reagent 3A** (Substrate buffer) and mix well. Add 5µL of **Reagent 3C** (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. 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.<br>c. Rinse well with distilled water. | 10 min |
| **6. Counterstain (Optional) (Optional but must be done before Emerald Chromogen step)**<br><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 10-30 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 with PBS-T containing 0.05% Tween-20 or **1X TBST**; 3 times for 2 minutes each. | 5 seconds |
| **7. Reagent 4** Emerald Chromogen (RTU) | a. Apply 1 to 2 drops (50-100µL) of **Reagent 4** (Emerald Chromogen) to cover the tissue completely.<br>b. Incubate in moist chamber for 5 minutes.<br>c. Wash slides in tap water for 1 minute.<br>d. Rinse with distilled water. | 5 min |

| | | |
|---|---|---|
| | ***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.* | |
| 8.Dehydrate section **It is important to follow the protocol.** | **<u>Note</u>: 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>**<u>CAUTION</u>: DO NOT dehydrate with xylene longer than 20 seconds! It will erase Permanent Red stain!** | 2 min |
| **9. Reagent 5**<br>U-Mount (RTU) | a. Apply 1 drop (50µL) of Reagent 5 (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 | |

**Protocol notes:**

| 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 | Titer primary antibody |
| Permanent Red is leaching | 1. Use fresh 100% ethanol and xylene.<br>**2.** Slide sat too long in xylene. **Do not** go over **20 seconds!** |
| Artifacts on slides | Slides not completely dried before mount. Use fresh 100% Ethanol and xylene. |

**Precautions:**

Please wear gloves, eye protection and take other necessary precautions. If any of the reagent come in contact with skin wash area completely with plenty of water and soap. If irritation develops seek medical attention

**FOR RESEARCH USE**

# Work Sheet for NB-23-00111 Kit

We designed this work sheet to help you keep 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.

- Used for tester to check "√ "each step during the experiment
- Steps follow after de-paraffinization
- Refer to insert for details of each step

**NB-23-00111** Protocol is suitable when both rabbit and rabbit primary antibodies need or do not need pre- treatment step

| **Protocol Step** | **NB-23-00111 Protocol** | **Experiment 1 Date:** | **Experiment 2 Date:** | **Experiment 3 Date:** | **Experiment 4 Date:** |
|---|---|---|---|---|---|
| **Step 1** | Peroxidase& alkaline phosphatase Block User supplied | | | | |
| **Step 2** | HIER if needed User supplied | | | | |
| **Step 3** | Gt 1°Ab & Rb 1°Ab mixture (30-60 min.) | | | | |
| **Step 4** | **Reagent 1 & Reagent 2** Goat HRP Polymer (RTU)& Rabbit AP Polymer (RTU) require mixing 30min | | | | |
| **Step 5** | **Reagent 3A, Reagent 3B & Reagent 3C** Permanent Red Requires mixing! 10min | | | | |
| **Step 6** | Counter stain (5seconds) (Do not over counter stain) Hematoxylin User supply Wash with PBS/0.05% Tween20 for 2 min, 3 times. | | | | |

| | | | | | |
|---|---|---|---|---|---|
| **Step 7** | **Reagent 4**<br>Emerald Chromogen (RTU) (5min) | | | | |
| **Step 8** | **It is important to follow the protocol to maintain stain!**<br>Dehydrate section 20seconds for each step | | | | |
| **Step 9** | **Reagent 5**<br>U-Mount (RTU) Mount & coverslip | | | | |
| **Result** | Stain pattern on controls are correct: Fill in Yes or NO | | | | |

Testing result: