



PolyStain TS Kit - for Goat, Mouse Rabbit antibody on Human tissue

(DAB/Permanent Red/Ni-DAB)

NB-23-00133- 3(240 ml)

NB-23-00133- 2(72 ml)

NB-23-00133- 1(24 ml)

**PolyStain TS Kit - for Goat, Mouse Rabbit antibody on Human tissue
(DAB/Permanent Red/Ni-DAB)**

NB-23-00133-1; NB-23-00133-2; NB-23-00133-3

Storage: 2-8°C

INTENDED USE:

The PolyStain DS Kit is designed to use with user supplied goat/mouse/rabbit primary antibodies to detect three distinct antigens on a single human tissue or cell samples. This Kit has been tested on paraffin embedded tissue only; however it may be used on frozen or freshly prepared monolayer cell smears. Please read through entire protocol as this protocol requires many steps to be done in their defined order. Triple staining uses traditional and non-traditional methods in immunohistostaining to reveal three distinct antigens and their coexpression on a single tissue. PolyStain DS Kit from NeoBiotech Labs supplies polymer enzyme conjugates: Polymer-HRP anti-goat, Polymer-AP anti-rabbit and Polymer-HRP anti-mouse with three chromogens, DAB (brown); Permanent Red (red); and DAB-Ni (black). PolyStain DS Kit is a non-biotin system, avoiding non-specific binding caused by endogenous biotin. This kit has been optimized to have no cross reaction when detecting three different primary antibodies using our unique blocking system. Simplified steps allow users to complete triple staining within 3 hours (without antigen retrieval) or 4 hours (with antigen retrieval). The well tested protocol provides user to permanently mount slides with coverslip.

KIT COMPONENTS:

Component No.	Content	NB-23-00133-1	NB-23-00133-2	NB-23-00133-3
Reagent 1	Goat HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 2	Rabbit AP Polymer (RTU)	6mL	18mL	60mL
Reagent 3A	DAB Substrate (RTU)	15mL	18mL x 2	120mL
Reagent 3B	DAB Chromogen (20x)	1.5mL	2mL	6mL
Reagent 4A	Permanent Red Substrate (RTU)	15mL	18mL x 2	120mL
Reagent 4C	Permanent Red Chromogen (100x)	150µL	360µL	1.2mL
Reagent 5	TS-GMR Blocker (RTU)	12mL	18ml x 2	120mL
Reagent 6	Mouse HRP Polymer (RTU)	12mL	18mLx2	120mL
Reagent 7A	DAB-Ni Substrate (20x)	1mL	2mL	6mL
Reagent 7B	Hydrogen Peroxide (20X)	1mL	2mL	6mL
Reagent 7C	Nickel Solution (7x)	3mL	6mL	18mL
Reagent 8	U-Mount (RTU)	3mL	9mL	NA

HRP = Horseradish Peroxidase AP = Alkaline Phosphatase Ms = Mouse Rb = Rabbit

PROTOCOL NOTES:

1. Proper 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 deparaffinize 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. 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. This will generate false positive and/or false negative signal.
7. The fixation, tissue section thickness, antigen retrieval and primary antibody dilution and incubation time effect results significantly. Investigator needs to consider all factors and determine optimal conditions when interpreting results.

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

Reagent	Staining Procedure	Incubation Time (Min.)
1. Peroxidase and phosphatase Blocking Reagent Supplied by user	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 slides using 2 changes of distilled water.	10 min.
2. Antigen retrieval (optional): Refer to primary antibody data sheet.	Note: Investigator needs to do antigen retrieval only one time during protocol see staining protocol a. Refer to primary antibody data sheet for antigen retrieval methods b. Wash with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	
3. Primary Antibody Mix: Mix one Goat, one Mouse and one Rabbit primary antibody Supplied by user.	Note: Investigator needs to optimize dilution prior to triple staining. DO NOT combine the same host species primary antibodies together at this step. a. Apply 2 drops or enough volume of goat, mouse and rabbit primary antibody mixture to cover the tissue completely. Incubate in moist chamber for 30-60min. Recommend 30min to shorten total protocol time. b. Wash slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	30 min
4. Mix Reagent 1 with Reagent 2 Reagent 1: Goat HRP Polymer (RTU) Reagent 2: Rabbit AP Polymer (RTU)	Note: Make sufficient polymer mixture by mixing Reagent 1 (Goat HRP Polymer) with Reagent 2 (Rabbit AP Polymer) at 1:1 ratio, mix well. Do Not mix more than you need for the experiment because the polymer mixture may not be as stable as non-mixed polymer. a. Apply 1 to 2 drops (50-100µL) of the mixture to cover the tissue completely. Incubate in moist chamber for 30 min. b. Wash slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times.	30 min.
5. Reagent 3A & 3B: Reagent 3A: DAB Substrate (RTU) Reagent 3B: DAB Chromogen (20x)	Note: Make enough DAB mix by adding 1 drop of Reagent 3B (DAB Chromogen) in 1mL of Reagent 3A (DAB Substrate). Mix well. Use within 7 hours. Prepare AP-Red Plus at this time (see step 6). a. Apply 1 to 2 drops (50-100µL) of your DAB working solution to cover the tissue completely. Incubate for 5min. b. Rinse slides in multiple changes of distilled water 3 times, 2min each time or under running tap water for 2minute	3 - 10 min

<p>6. Reagents 4A, 4B & 4C</p> <p>Reagent 4A: Permanent Red Substrate (RTU)</p> <p>Reagent 4B: Permanent Red Activator (5x)</p> <p>Reagent 4C: Permanent Red Chromogen (100x)</p>	<p>a. Add 200µL of Reagent 4B (Activator) into 1mL of Reagent 4A (Substrate buffer) 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 buffer) and mix well. Add 5µL of Reagent 4C (Chromogen) into the mixture and mix well.]</p> <p>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.</p> <p>c. Rinse well with distilled water.</p>	<p>10 min</p>
<p>7. Reagent 5: TS-GMR Blocker (RTU)</p>	<p>a. Apply 1 to 2 drops (50-100µL) of Reagent 5 (TS-GMR Blocker) to cover the tissue completely. Incubate for 10min.</p> <p>b. Rinse slides in multiple changes of distilled water 3 times, 2min each time.</p> <p>c. Wash with PBS/ 0.05% Tween20 for 2 minutes, 3 times.</p>	<p>10 min</p>
<p>8. Reagent 6 Mouse HRP Polymer(RTU)</p>	<p>a. Apply 1 to 2 drops (50-100µL) of Reagent 6 (Mouse HRP Polymer) to cover the tissue completely. Incubate slides in moist chamber for 15 min.</p> <p>b. Wash slides with PBS/ 0.05% Tween20 for 2 minutes, 3 times.</p>	<p>15 min</p>
<p>9. Reagent 7A,3B,7B,7C&7D</p> <p>Reagent 7A: DAB-Ni Substrate(20x)</p> <p>Reagent 3B: DAB Chromogen(20x)</p> <p>Reagent 7B: Hydrogen Peroxide(20x)</p> <p>Reagent 7C: Nickel Solution(7x)</p>	<p>a. Prepare 1mL of distilled water. Add 1 drop of DAB-Ni Substrate Buffer (Reagent 7A) into 1mL of distilled water. Mix well.</p> <p>b. Add 1 drop of DAB Chromogen (Reagent 3B) and 1 drop of concentrated Hydrogen Peroxide (Reagent 7B) to the diluted Reagent. Mix well.</p> <p>c. Add 3 drops of Nickel Solution (Reagent 7C) to the mixture. Mix well.</p> <p>d. Add about 100µL (2 drops) of DAB-Ni working solution to each slide and incubate in an enclosed chamber at room temperature for about 5 minutes. When appropriate color is developed, rinse under tap water gently for about 1-2 minutes.</p> <p>e. Keep away from light during operation and use the prepared DAB-Ni mixture within 7 hours at 4°C.</p>	
<p>10. Counterstain (Optional) Not provided</p>	<p>a. Counterstain with 2 drops (100µL) or enough volume of counterstain solution to completely cover tissue. Incubate for 10-15sec.</p> <p>b. Rinse thoroughly with tap water for 2-3min.</p> <p>c. Rinse well in distilled water.</p>	<p>10-15 sec</p>

<p>11. Dehydrate section</p> <p>It is important to follow the protocol.</p>	<p>Note: Please wipe off extra water and air dry slides before dehydration and clear.</p> <ol style="list-style-type: none"> Dehydrate with 80% ethanol 20seconds. Dehydrate with 95% ethanol 20seconds. Dehydrate with 100% ethanol 20seconds. Dehydrate with 100% ethanol 20seconds. Dehydrate with 100% ethanol 20seconds. Dehydrate with xylene 20seconds. <p>CAUTION: DO NOT dehydrate in xylene longer than 20 seconds! It will erase Permanent Red stain!</p>	<p>2 min</p>
<p>12. Reagent 8</p> <p>U-Mount (RTU)</p>	<ol style="list-style-type: none"> Apply 1 drop (50µL) of Reagent 8 (U-Mount) to cover the tissue section and apply glass coverslip. 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 SHOOTING:

PROBLEM	TIPS
Uneven stain on 3 primary antibodies	<ol style="list-style-type: none"> Need to adjust the titer of each antibody. The amount of each protein expressed on tissue may be different. Set slides in Xylene too long so that Permanent Red is washed away.
No stain on 1 or 2 antibodies	Missing steps or step reversed.
Permanent Red is leaching	<ol style="list-style-type: none"> Use fresh 100% ethanol and xylene. 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 xylen

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 ONLY

Work Sheet for NB-23-00133 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-23-00133 Protocol-A is suitable for all primary antibodies need pre-treatment, all primary antibodies do not need pre-treatment or all primary antibodies are not sensitive to pre-treatment

Protocol Step	NB-23-00133 Protocol	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase and phosphatase Block Recommend NB-23-00193 User supplied				
Step 2	HIER(Optional)				
Step 3	Goat 1°Ab, Rabbit 1°Ab& Mouse 1°Ab mix User supplied (30-60 min.)				
Step 4	Reagent 1&Reagent 2 Goat HRP Polymer & Rabbit AP Polymer require mixing (30 min.)				
Step 5	Reagent 3A&Reagent 3B DAB requires mixing (5 min.)				
Step 6	Reagent 4A, Reagent 4B&Reagent 4C Permanent Red requires mixing (10 min)				

Step 7	Reagent 5 TS-GMR Blocker (10min)				
Step 8	Reagent 6 Mouse HRP Polymer RTU (15 min)				
Step 9	Reagent 7A, Reagent 3B, Reagent 7B & Reagent 7C DAB-Ni requires mixing RTU (5min)				
Step 10	Counter stain(10-15sec) User supplied				
Step 11	It is important to follow the protocol. To maintain stain! Dehydrate section 20seconds for each step				
Step 12	Reagent 8 U-Mount RTU Mount & coverslip				
Result :	Stain pattern on controls are correct: Fill in Yes or NO				

Note1: Normal wash steps = Wash with PBS containing 0.05% Tween-20 for 3 times for 2 min each.

Note2: *Using as a co-localization staining kit,

If antigens are co-localized in nuclear counter stain and blue should be for 5 seconds to blue.

If antigens are co-localized in cytoplasm and membrane or in different cells counter stain using normal protocol time.

Testing result: