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

(DAB/Permanent Red/Emerald)

NB-23-00132-1 (24ml)

NB-23-00132-2 (72ml)

NB-23-00132-3 (240ml)

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NB-23-00132-1; NB-23-00132-2; NB-23-00132-3

Storage: 2-8ºC

INTENDED USE:

The Polystain TS-GMR-Hu 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. TS-MMR-Hu 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 co-expression on a single tissue Polystain TS-GMR-Hu 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 Emerald (green). Polystain TS-GMR-Hu 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	24mL	72mL	240mL
Reagent 1	Goat HRP Polymer(RTU)	6mL	18mL	60mL
Reagent 2	Rabbit AP Polymer(RTU)	6mL	18mL	60mL
Reagent 3A	DAB Substrate(RTU)	15mL	18mLx2	120mL
Reagent 3B	DAB Chromogen (20x)	1.5mL	2mL	6mL
Reagent 4A	Permanent Red Substrate(RTU)	15mL	18mLx2	120mL
Reagent 4B	Permanent Red Activator(5x)	3mL	7.2mL	12mLx2
Reagent 4C	Permanent Red Chromogen(100x)	150µL	360µL	1.2mL
Reagent 5	TS-GMR Blocker(RTU)	12mL	18mLx2	120mL
Reagent 6	Mouse HRP Polymer(RTU)	12mL	18mLx2	120mL
Reagent 7	Emerald Chromogen(RTU)	15mL	18mLx2	120mL
Reagent 8	U-Mount(RTU)	12mL	18mLx2	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.
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. NeoBiotech 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, 100% Xylene, Hematoxylin
9. Coverslip

Staining protocol NB-23-00132 protocol:

Reagent	Staining Procedure	Incubation Time (Min.)
1. Peroxidase and Alkaline Phosphatase Blocking Reagent **Not provided** Fast, easy and it will block endogenous alkaline phosphatase	a. Incubate slides in PEROXIDASE BLOCKING REAGENT we recommend NeoPure Dual Enzyme Block **NB-23-00193** b. Rinse the slide using 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-T containing 0.05% Tween-20 or **1X TBS-T (See note 9 above)**; 3 times for 2 minutes each.	
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 with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	30 min.
4. Mix **Reagent 1:** Goat HRP Polymer (RTU) **with** **Reagent 2:** Rabbit AP Polymer (RTU)	**Note:** Make sufficient polymer mixture by adding **Reagent 1** (Goat HRP Polymer) and **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. b. Incubate in moist chamber for 30 min. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	30 min
5. 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. b. Incubate for 5min. c. Rinse thoroughly with distilled water. d. Wash only with **1X TBS-T**, 3 times for 2 minutes each.	5 min.

6. Reagent 4A, 4B, 4C **Reagent 4A:** Permanent Red Substrate (RTU) **Reagent 4B:** Permanent Red Activator (5x) **Reagent 4C:** 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 4B** (Activator) into 1mL of **Reagent 4A** (Substrate buffer) and mix well. Add 10µL of **Reagent 4C** (Chromogen) into the mixture and mix well. b. **[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.**]** c. 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.** d. Rinse well with distilled water.	10 min
7. Reagent 5: TS-GMR Blocker (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 5** (TS-GMR Blocker) to cover the tissue completely. Incubate for 10min. b. Rinse slides in multiple changes of distilled water 3 times, 2min each time. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	10 min.
8. Reagent 6 **Mouse HRP Polymer(RTU)**	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. b. Rinse well in distilled or tap water for 2min.	UP to 1 hour
9. Counterstain (Optional but must be done before Emerald Chromogen step) **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. 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. b. Rinse thoroughly with tap water for 1min. c. Put slides in PBS for 5-10 seconds to blue, **DO NOT** over blue if two protein areco-localized in the nuclear. d. Rinse well in distilled or tap water for 2min. e. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	5 sec
10. Reagent 7 Emerald Chromogen (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 7** (Emerald Chromogen) to cover the tissue completely. b. Incubate slides in humid chamber for 5 minutes.	5 min.

Do hematoxylin first.	c. Wash slides in tap water for 3 times for 30 seconds! ***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.	
11.Dehydrate section **It is important to follow the protocol.**	**Note: Please wipe off extra water and air dry slides before dehydration and clear.** a. Dehydrate with 80% 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. **CAUTION: DO NOT dehydrate in xylene longer than 20 seconds! It will erase Permanent Red stain!**	2 min
12. Reagent 8 **U-Mount (RTU)**	a. Apply 1 drop (50µL) of **Reagent 8** (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.	15 min

Trouble Shooting:

PROBLEM	TIPS
Uneven stain on 3 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 non colocalized with Permanent Red. If Emerald chromogen is blue the titer on the primary antibody is not diluted 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.

PRECAUTION:

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.

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Work Sheet for NB-23-00132 Kit

We designed work sheet to help you track each step since triple color staining takes many steps. You may use this sheet for our technical support staff to review if needed.

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

NB-23-00132 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.

	Main Protocol Step	NB-23-00132 Protocol-A	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	**Step 1**	Peroxidase and phosphatase Block User supplied **NB-23-00193** is recommended.				
2	**Step 2**	HIER(Optional)				
3	**Step 3**	Goat 1°Ab, Rabbit 1°Ab& Mouse 1°Ab mix User supplied (30-60 min.)				
4	**Step 4**	**Reagent 1&Reagent 2** Goat HRP Polymer & Rabbit AP Polymer require mixing (30 min.)				
5	**Step 5**	**Reagent 3A & Reagent 3B** DAB requires mixing (5 min.) **Wash with 1xTBS-T after rinse well with distilled water.**				
6	**Step 6**	**Reagent 4A,Reagent 4B** & **Reagent 4C** Permanent Red requires mixing (10 min)				
7	**Step 7**	**Reagent 5** TS-GMR Blocker (10min)				
8	**Step 8**	**Reagent 6** Mouse HRP Polymer RTU (15 min)				

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9	**Step 9**	Counter stain(See Note) User supplied (5-10 sec)				
10	**Step 10**	**Reagent 7** Emerald Chromogen RTU (5min)				
11	**Step 11**	**It is important to follow the protocol. To maintain stain!** Dehydrate section 20seconds for each step				
12	**Step 12**	**Reagent 8** U-Mount RTU Mount & coverslip				
13	**Step 13**	**Stain pattern on controls are correct: Fill in Yes or NO**				
	Result	**Stain pattern on controls are correct: Fill in Yes or NO**				

Note1: Normal wash steps = Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes 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:

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