PolyStain TS Kit - for 1 Mouse and 2 Rabbit antibody on Rodent tissue

(DAB/Permanent Red/Emerald)

NB-23-00136- 3(240 ml)

NB-23-00136- 2(72 ml)

NB-23-00136- 1(24 ml)

Neo-Biotech
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PolyStain TS Kit - for 1 Mouse and 2 Rabbit antibody on Rodent tissue (DAB/Permanent Red/Emerald)

NB-23-00136-1; NB-23-00136-2; NB-23-00136-3

Storage: 2-8ºC

INTENDED USE:

The PolyStain DS Kit is designed to use with user supplied one mouse and two rabbit primary antibodies to detect three distinct antigens on a single mouse/rat tissue or cell samples. Kit has been tested on tissue specimens that are paraffin embedded. For frozen tissue a lower temperature of 65ºC must be used for Antibody Blocker (Reagent 6) to prevent tissue from dissociating from slide. Please read through entire protocol as this protocol requires many step to be done in the defined order. Triple staining uses traditional methods in immunohistostaining to reveal three distinct antigens and their co-expression on a single tissue.

PolyStain DS Kit from NeoBiotech Labs supplies polymer enzyme conjugates: polymer-HRP anti-mouse IgG, polymerHRP anti-rabbit IgG and polymer-AP anti-rabbit IgG with three substrates/chromogens; DAB (brown), Emerald (green), and Permanent Red (Red). 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 detection when detecting more two primary antibodies from the same host species using unique blocking system.

Simplified steps allow users to complete triple staining within 5 hours (without antigen retrieval) or 6-7 hours (with antigen retrieval). The well tested protocol provides user a method to permanently mount slides with coverslip.

KIT COMPONENTS:

Component No.	Content	NB-23-00136-1	NB-23-00136-2	NB-23-00136-3
Reagent 1	Mouse Primer (RTU)	12mL	36mL	120mL
Reagent 2	Mouse HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 3	Rabbit AP Polymer (RTU)	6mL	18mL	60mL
Reagent 4A	DAB Substrate (RTU)	15mL	18mL x 2	120mL
Reagent 4B	DAB Chromogen (20x)	1.5mL	2mL	6mL
Reagent 5A	Permanent Red Substrate (RTU)	15mL	18mL x 2	120mL
Reagent 5B	Permanent Red Activator (5x)	3mL	7.2mL	12mL x 2
Reagent 5C	Permanent Red Chromogen (100x)	150µL	360µL	1.2mL
Reagent 6	Antibody Blocker (40x)	15mL x 2	50mL	100mL

Reagent 7A	TS-MRR Blocker A (RTU)	12mL	18mL x 2	120mL
Reagent 7B	TS-MRR Blocker B (RTU)	12mL	18mL x 2	120mL
Reagent 8	Rabbit HRP Polymer (RTU)	12mL	18mLx2	120mL
Reagent 9	Emerald Chromogen (RTU)	15mL	18mLx2	120mL
Reagent 10	U-Mount (RTU)	12mL	18mL x 2	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 Isotope 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. **Important**: Never combine two antibodies from the same host species in one incubation step. Incubate 1st rabbit primary antibody with mouse antibody.
8. 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.
9. 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.

Staining protocol selection and limitation of the kit:

- Most antigens will not be destroyed by heat. However, users need to check if there are proteins on the tissue that are heat sensitive before proceeding with the staining.
- NB-23-00136 Protocol-2 worksheet is suitable for one Mouse & one Rabbit primary Abs need pre-treatment, the other Mouse primary Ab is sensitive to pre-treatment.
- NB-23-00136 Protocol-3 worksheet is suitable when one Mouse & one Rabbit primary antibody are sensitive to pre-treatment but the second Mouse primary antibody needs pre-treatment.
- Please read the following table carefully before you start the experiment to ensure the result.
- This kit is not suitable for the following condition: 2 proteins are heat sensitive and detected by 2 mouse antibodies and one rabbit antibody requires HIER.

Staining protocol NB-23-00136 protocol-1:

Reagent	Staining Procedure	Incubation Time (Min.)
1. Peroxidase and phosphatase Blocking Reagent Fast, easy and it will block endogenous alkaline phosphatase 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 slide using distilled water at least twice.	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 or 1xTBS-T **(See note 9 above)** 3 times for 2 minutes each	
3. Primary Antibody Mix**: Mix 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 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.	30 min

	b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each	
4. Reagent 1 Mouse Primer (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 1 (Mouse Primer)** to cover the tissue completely. Incubate slides in moist chamber for 15 min. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	10 min.
5. Mix **Reagent 2:** Mouse HRP Polymer (RTU) with **Reagent 3:** Rabbit AP Polymer (RTU)	**Note**: Make sufficient polymer mixture by adding **Reagent 2** (Mouse HRP Polymer) and **Reagent 3** (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 nonmixed 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
6. Reagent 4A&4B **4A:** DAB Substrate(RTU) **4B:** DAB Chromogen (20x)	**Note**: Make enough DAB mix by adding 1 drop of **Reagent 4B** (DAB Chromogen) in 1mL of **Reagent 4A** (DAB Substrate). Mix well. Use within 7 hours store at 4°C. a. Apply 1 to 2 drops (50-100µL) of your DAB mixture to cover the tissue completely. b. Incubate for 5min. c. Rinse thoroughly with distilled water. d. Wash with **1xTBS-T** 3 times for 2 minutes each.	5 min
7. Reagent 5A, 5B, 5C **Reagent 5A:** Permanent Red Substrate (RTU) **Reagent 5B:** Permanent Red Activator (5x) **Reagent 5C:** Permanent Red Chromogen (100x) **To get maximum sensitivity of AP polymer, Please always use fresh made Permanent Red and repeat chromogen step.**	**Note**: Shake Permanent Red Activator before adding into Permanent Red Substrate. a. Add 200µL of **Reagent 5B** (Activator) into 1mL of **Reagent 5A** (Substrate) and mix well. Add 10µL of **Reagent 5C** (Chromogen) into the mixture and mix well. [**Note**: For fewer slides, Add 100µL of **Reagent 5B** (Activator) into 500µL of **Reagent 5A** (Substrate) and mix well. Add 5µL of **Reagent 5C** (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 working solution to completely cover the tissue for additional 5 to 10min. c. Rinse well with distilled water.	10 min

8. Reagent 6 Antibody Blocker (40x)	**Note**: This step will block antibodies of previous step so no cross reaction will occur in this protocol. HIER can be done immediately after Antibody Blocker step if the primary antibodies requires antigen retrieval. For frozen tissues, a lower temperature of 65ºC must be used during the Antibody Blocker step to prevent dissociation of the tissue from the slide. a. Use hot plate or water bath to heat diluted **Reagent 6** (**Antibody Blocker**) to 1x solution (1 part of Antibody Blocker in 39 parts of distilled water) to 80°C. Make enough volume to cover the tissue in beaker. b. Put slides in heated Antibody Blocker for 10 minutes at 80°C. c. Remove slides from the **Antibody blocker**; cool slides 5 seconds. d. Rinse slides in multiple changes of distilled water. If antigen retrieval step is required go directly to **step 9** if not complete **step 8e** and move on to **step 10.** e. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each...	10 min
9. Antigen retrieval: **Refer to primary antibody data sheet.**	a. Refer to primary antibody data sheet for antigen retrieval methods. b. Wash with PBS/ 0.05% Tween20 or **1xTBS-T** 3 times for 2 minutes each.	UP to 1 hour
10. Reagent 7A TS-MRR Blocker A (RTU)	a. Apply 2 drops or enough volume of **Reagent 7A** (DS-MRR Blocker A) to cover the tissue completely. Mix well on the slide and incubate in moist chamber for 30 min. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each	30 min
11. Reagent 7B TS-MRR Blocker B (RTU**)**	a. Apply 2 drops or enough volume of **Reagent 7B** (DS-MRR Blocker B) to cover the tissue completely. Mix well on the slide and Incubate in moist chamber for 5 min. b. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	5 min
12. 2nd Mouse primary antibody Supplied by user.	**Note**: Investigator needs to optimize dilution prior to triple staining. a. Apply 2 drops or enough volume of the 2nd rabbit primary antibody to cover the tissue completely. Incubate in moist chamber for 30-60 min. Recommend 30 minutes 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
13. Reagent 8 Mouse HRP Polymer	a. Apply 1 to 2 drops (50-100µL) of **Reagent 8** (Mouse HRP Polymer) to cover the tissue completely. Incubate slides in moist chamber for 15 min.	15 min

(RTU)	b. Wash with PBS/ 0.05% Tween20 or 1xTBS-T 3 times for 2 minutes each.	
14. Counterstain **(Optional but must be done before Emerald Chromogen step)** Not provided	**Note**: If two antigens are co-localized in the nucleas 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 colocalization 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. d. Rinse well in distilled or tap water for 1min. e. Wash with PBS/ 0.05% Tween20 or **1xTBS-T** 3 times for 2 minutes each.	5 sec
15. Reagent 9 Emerald Chromogen (RTU) **Do hematoxylin first.**	a. Apply 1 to 2 drops (50-100µL) of **Reagent 9** (Emerald Chromogen) to cover the tissue completely. b. Incubate slides in humid chamber for 5 minutes. c. Wash slides in tap water for 1 minute. d. Rinse with distilled water. ***Important to READ**: Emerald Chromogen is water soluble, counter stain first. Do not leave slides sitting in water. Always stain Emerald chromogen **AFTER** Permanent Red stain and hematoxylin. Permanent Red removes the Emerald*	5 min
16.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 85% 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
17. Reagent 10 **U-Mount (RTU)**	a. Apply 1 drop (50µL) of **Reagent 10** (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.	

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 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. 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, 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-23-00136 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-23-00136 Protocol-1 is suitable when all primary antibodies need pre-treatment or all primary antibodies do not need pre-treatment

Protocol Step	NB-23-00136 Protocol	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase or Alkaline Phosphatase Block Recommend NB-23-00193 User supplied				
Step 2	HIER(Optional)				
Step 3	Mouse 1°Ab &Rabbit 1°Ab mix User supplied (30-60min)				
Step 4	**Reagent 1** Mouse primer RTU 15min				
Step 5	**Reagent 2&Reagent 3** Mouse AP Polymer & Rabbit HRP Polymer require mixing (30min)				
Step 6	**Reagent 4A& Reagent 4B** DAB requires mixing. (5min)				

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Step 7	**Reagent 5A, Reagent 5B Reagent 5C** Permanent Red requires mixing. (10min)				
Step 8	**Reagent 6** Antibody Blocker requires mixing. (10min)				
Step 10	**Reagent 7A** DS-MMR Blocker A RTU (30min)				
Step 11	**Reagent 7B** DS-MMR Blocker B RTU (5min)				
Step 12	Mouse 1°Ab User supplied (30-60 min)				
Step 13	**Reagent 8** Mouse HRP Polymer RTU (15 min)				
Step 14	Counter stain(**Note 2**) User supplied (5-10 sec)				
Step 15	**Reagent 9** Emerald Chromogen RTU (5min)				
Step 16	**It is important to follow the protocol. To maintain stain!** Dehydrate section 20seconds for each step				
Step 17	**Reagent 10** U-Mount RTU Mount & coverslip				
Result	**Stain pattern on controls are correct: Fill in Yes or NO**				

Note: 1. Normal wash steps = Wash with PBS containing 0.05% Tween-20 or 1x TBS-T for 3 times for 2 min each.
2.*Using as a co-localization staining kit,
If antigens are co-localized in nucleus 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:

NB-23-00136 Protocol-2 is suitable when one Mouse & one Rabbit primary antibodies need pre-treatment, but the second Rabbit primary antibodies is sensitive to pre-treatment.

Protocol Step	NB-23-00136 Protocol-2	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase or Alkaline Phosphatase Block Recommend NB-23-00193 User supplied				
Step 12	Mouse 1°Ab (sensitive to HIER) User supplied (30-60min)				
Step 13	**Reagent 8** (RTU) Mouse HRP Polymer RTU (15min)				
Step 6	**Reagent 4A&4B** DAB requires mixing (5 min)				
Step 8	**Reagent 6** Antibody Blocker requires mixing (10min)				
Step 9	HIER (DAB will not be removed)				
Step 10	**Reagent 7A (RTU)** DS-MRR Blocker A RTU (30min)				
Step 11	**Reagent 7B (RTU)** DS-MRR Blocker B RTU (5min)				
Step 3	Mouse 1°Ab & Rabbit 1°Ab mix (Abs requires HIER) User supplied (30-60 min)				

Step 4	**Reagent 1** Mouse primer RTU 15min				
Step 5	**Reagent 2&Reagent 3** Mouse HRP Polymer & Rabbit AP Polymer require mixing (30min) Wash with 1x TBS-T				
Step 7	**Reagent 5A, Reagent 5B& Reagent 5C** Permanent Red requires mixing. (10min)				
Step 14	Counter stain(**Note 2**) User supplied (5-10 sec)				
Step 15	**Reagent 9** Emerald Chromogen RTU (5min)				
Step 16	**It is important to follow the protocol. To maintain stain!** Dehydrate section 20seconds for each step				
Step 17	**Reagent 10** U-Mount RTU Mount & coverslip				
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 nucleus 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:

NB-23-00136 Protocol-3 is suitable when one Mouse & one Rabbit primary antibodies are sensitive to pre-treatment but the second Rabbit primary antibody needs pre-treatment

Protocol Step	NB-23-00136 Protocol-3	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
Step 1	Peroxidase or Alkaline Phosphatase Block Recommend NB-23-00193 User supplied				
Step 3	Mouse 1°Ab (sensitive to HIER) User supplied (30-60min)				
Step 4	**Reagent 1** Mouse primer RTU 15min				
Step 5	**Reagent 2&Reagent 3** Mouse HRP Polymer & Rabbit AP Polymer require mixing. (30min))				
Step 6	**Reagent 3A&Reagent 3B** DAB require mixing. (5min)				
Step 7	**Reagent 5A, Reagent 5B& Reagent 5C** Permanent Red requires mixing. (10min)				
Step 8	**Reagent 6** Antibody Blocker required mixing. (10min)				
Step 9	HIER Refer to antibody datasheet.				
Step 10	**Reagent 7A** DS-MRR Blocker A RTU (30min)				
Step 11	**Reagent 7B** DS-MRR Blocker B RTU (5min)				

Step 12	Mouse 1°Ab (Not sensitive to HIER) User supplied (30-60min.)				
Step 13	**Reagent 8** Rabbit HRP Polymer (RTU) (15min.)				
Step 14	Counter stain(**Note 2**) User supplied (5-10 sec)				
Step 15	**Reagent 9** Emerald Chromogen RTU (5min)				
Step 16	**It is important to follow the protocol. To maintain stain!** Dehydrate section 20seconds for each step				
Step 17	**Reagent 10** U-Mount RTU Mount & coverslip				
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 nucleus 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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