GBI Labs
GOLDEN BRIDGE INTERNATIONAL

ORIGENE

Polink TS-MRRt-Ms A Kit for Immunohistochemistry Staining

Polymer-HRP&AP triple staining kit to detect mouse, rabbit and rat primary antibodies on mouse tissue with DAB (Brown), GBI-Permanent Red (Red), and Emerald (Green)

Storage: 2-8ºC

Catalog No.:		
	☐ TS312A-6	*24mL (for 120 slides**)
	☐ TS312A-18	*72mL (for 360 slides**)
	☐ TS312A-60	*240mL (for 1200 slides**)

**Volume of polymer conjugate*
*** If use 100µL per slide*

Intended Use:
The **Polink TS-MRRt-Ms A** Kit is designed to use with user supplied mouse, rat and rabbit primary antibodies to detect three distinct antigens on mouse tissue, cell sample or human tissue. This kit has been tested on paraffin embedded tissue, which can be used on frozen tissue or cell smears . For frozen tissues, a lower temperature of 65ºC must be used during the Antibody Blocker step (Reagent 3) to prevent dissociation of the tissue from the slide. Please read through the entire protocol, as all steps must be performed in the defined order to achieve proper staining.
Triple immunohistostaining uses traditional methods to reveal three distinct antigens and their co-expression on a single tissue[1, 2]. The **Polink TS-MRRt-Ms A** Kit from GBI Labs (Golden Bridge International) contains the following polymer enzyme conjugates: polymer-HRP anti-mouse IgG, polymer-AP anti-rat IgG and polymer-HRP anti-rabbit IgG with three substrates/chromogens; DAB (brown), GBI-Permanent Red (Red), and Emerald (green). The **Polink TS-MRRt-Ms A** Kit is a non-biotin system, avoiding non-specific binding caused by endogenous biotin. This kit has been optimized to show no cross reaction when detecting more than two primary antibodies from the mouse and rat host species, using our unique blocking system. Simplified steps allow users to complete triple staining within 5 hours (without antigen retrieval) or 6-7 hours (with antigen retrieval). This protocol also includes a method to dehydrate, clear and permanently mount slides with coverslip.

Kit Components: 60mL Kit components are in 2 boxes labeled Box-A & Box-B

Component No.	Content	TS312A-6	TS312A-18	TS312A-60
Reagent 1	Mouse HRP Polymer (RTU)	12mL	18mLx2	120mL
Reagent 2A	DAB Substrate (RTU)	15mL	18mLx2	120mL
Reagent 2B	DAB Chromogen (20x)	1.5mL	2mL	6mL
Reagent 3	Antibody Blocker (40x)	15mLx2	50mL	100mL
Reagent 4	Rat Primer (RTU)	12mL	18mLx2	120mL
Reagent 5	Rat AP Polymer (RTU)	6mL	18mL	60mL
Reagent 6	Rabbit HRP Polymer (RTU)	6mL	18mL	60mL
Reagent 7A	GBI-Permanent Red Substrate (RTU)	15mL	18mLx2	120mL
Reagent 7B	GBI-Permanent Red Activator (5x)	3mL	7.2mL	12mLx2
Reagent 7C	GBI-Permanent Red Chromogen (100x)	150µL	360µL	1.2mL
Reagent 8	Emerald Chromogen (RTU)	15mL	18mLx2	120mL
Reagent 9	U-Mount (RTU)	12mL	18mLx2	NA

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

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. GBI sells 10xTBS-T for your convenience (B11xx)

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, Timer, Beaker
4. Wash buffer: 0.01 M PBS with 0.5% Tween20, pH7.4 or 50mM Tris HCl, 150mM NaCl, 0.05% Tween-20 pH7.6.
5. Peroxidase and alkaline phosphatase blocking buffer
6. 100% ethanol, 100% Xylene

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7. Hematoxylin
8. Coverslip

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.
- TS312A Protocol-A worksheet is suitable when all primary antibodies need pretreatment or no primary antibody needs pretreatment.
- TS312A Protocol-B worksheet is suitable when mouse primary antibody needs pretreatment or rabbit & rat primary antibodies are sensitive to pre-treatment.
- TS312A Protocol-C worksheet is suitable when rat and rabbit primary antibodies need pretreatment or mouse primary antibody is sensitive to pretreatment.
- 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 rat and mouse antibodies, and rabbit antibody requires HIER or 2 proteins are heat sensitive and detected by rabbit and mouse antibodies ,and rat antibody requires HIER.

Staining Protocol A of TS312A

Steps / Reagent	**Staining Protocol**	**Incubation Time**
1. Peroxidase and Alkaline Phosphatase Blocking Reagent Not provided We recommend using **GBI Dual Block E36xx.** Fast, easy and it will block endogenous alkaline phosphatase	a. Incubate slides in peroxidase and alkaline phosphatase blocking reagent. We recommend **GBI Dual Block E36xx**. a. Rinse the slide using distilled water at least twice.	10 minutes
2. Antigen retrieval: **Refer to primary antibody data sheet.** Supplied by user.	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 8 above)**; 3 times for 2 minutes each.	Up to 1 hour
3. Primary Antibody: **Add Mouse Primary** Supplied by user.	**Note:** Investigator needs to optimize dilution prior to triple staining. a. Apply 2 drops or enough volume of mouse primary antibody mixture to cover the tissue completely. Incubate in moist chamber for 30-60 min. 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 minutes
4. **Reagent 1:** Mouse HRP Polymer (RTU)	a. Apply 1 to 2 drops (50-100µL) of **Reagent 1** (Mouse HRP Polymer) to cover the tissue completely. b. Incubate in moist chamber for 15 min. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	15 minutes
5. **Reagent 2A&2B** **Reagent 2A:** DAB Substrate (RTU) **Reagent 2B:** DAB Chromogen (20x)	**Note:** Make enough DAB working solution by adding 1 drop of **Reagent 2B** (DAB Chromogen) in 1mL of **Reagent 2A** (DAB Substrate). Mix well. Store at 4ºC and use within 7 hours. a. Apply 1 to 2 drops (50-100µL) of your DAB working solution to cover the tissue completely. b. Incubate for 5min. c. Rinse slides in multiple changes of distilled water 2min, 3 times or under running tap water for 1minute.	5 minutes
6. **Reagent 3:** 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 3** (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 7** if not complete **step 6e** and move on to **step 8.** e. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	10 minutes
7. Antigen retrieval: **Refer to primary antibody data sheet.**	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**; 3 times for 2 minutes each.	Up to 1 hour

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8. Primary antibody mix: Add Rat and Rabbit primary antibody Supplied by user.	**Note**: Investigator needs to optimize dilution prior to triple staining. a. Apply 2 drops or enough volume of the rat and rabbit primary antibody mixture 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 to 60 minutes
9. **Reagent 4:** Rat Primer(RTU)	**Note:** This step is required for activation of RAT AP Polymer, **DO NOT skip**. a. Apply 1 to 2 drops (50-100µL) of **Reagent 4** (Rat Primer) to cover the tissue completely. b. Incubate in moist chamber for 10 min. c. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	10 minutes
10. Mix **Reagent 5:** Rat AP Polymer(RTU) with **Reagent 6**: Rabbit HRP Polymer(RTU)	**Note:** Mix **Reagent 5** (Rat AP Polymer) with **Reagent 6** (Rabbit HRP Polymer) at 1:1 ratio, do not mix more than you will need for experiment. This mixture is not stable long term for either polymer. a. Apply 1 to 2 drops (50-100µL) of Polymer mixture to cover the tissue completely. Incubate slides in moist chamber for 30 min. b. Wash with **1xTBS-T only;** 3 times for 2 minutes each.	30 minutes
11. **Reagent 7A, 7B, 7C** **Reagent 7A:** GBI-Permanent Red Substrate (RTU) **Reagent 7B:** GBI-Permanent Red Activator (5x **Reagent 7C:** GBI-Permanent Red Chromogen (100x) **To get maximum sensitivity of AP polymer, Please repeat chromogen step**	**Note**: Shake GBI-Permanent Red Activator before adding into GBI-Permanent Red Substrate. a. Add 200µL of **Reagent 7B** (Activator) into 1mL of **Reagent 7A** (Substrate) and mix well. Add 10µL of **Reagent 7C** (Chromogen) into the mixture and mix well. [**Note: For fewer slides**, Add 100µL of **Reagent 7B** (Activator) into 500µL of **Reagent 7A** (Substrate) and mix well. Add 5µL of **Reagent 7C** (Chromogen) into the mixture and mix well.] b. Apply 2 drops (100µL) or enough volume of GBI-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 GBI-Permanent Red working solution to completely cover the tissue for additional 5 to 10min.** c. Rinse well with distilled water.	10min
12. Counterstain (**Optional**) (**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. d. Rinse well in distilled or tap water for 1min. e. Wash with PBS-T containing 0.05% Tween-20 or **1X TBS-T**; 3 times for 2 minutes each.	5 seconds
13. **Reagent 8** Emerald Chromogen (RTU) Do hematoxylin first.	a. Apply 1 to 2 drops (50-100µL) of **Reagent 8** (Emerald Chromogen) to cover the tissue completely. b. Incubate slides in humid chamber for 5minutes. c. Wash slides in tap water for 30 seconds! ***Important to READ:*** Emerald Chromogen is water soluble, counter stain first. *Do not leave slides sitting in water.* Always stain Emerald chromogen **AFTER** GBI-Permanent Red stain and hematoxylin. GBI-Permanent Red removes the Emerald.	5minutes
14.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 GBI-Permanent Red stain!**	2 min
15. **Reagent 9** U-Mount (RTU)	a. Apply 1 drop (50µL) of **Reagent 9** (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 GBI-Permanent Red stain.	

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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 GBI-Permanent Red is washed away.
Emerald Chromogen is blue not green when non co-localized with GBI Permanent Red.	1. Emerald should be green when non colocalized with GBI-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	1. Missing steps or step reversed.
Green Background on the slide	1. Titer primary antibody.
GBI-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	1. Slides not completely dried before mount. Use fresh 100% Ethanol and xylene.

Precautious:
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.

Remarks:
For research use only.

References:

1. De Pasquale A, Paterlini P, Quaglino D.*Immunochemical demonstration of different antigens in single cells in paraffin-embedded histological sections.* Clin Lab Haematol. 1982;4(3):267-72.

2. Polak J. M and Van Noorden S. Introduction to Immunocytochemistry Second Edition. Bios Scientific Publishers. P41-54. 1997

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Work Sheet for TS312A Kit

We designed this work sheet to help you 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.
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

TS312A Protocol A is suitable when all primary antibodies need pretreatment or no primary antibody needs pretreatment.

	Step/ Protocol	TS312A Protocol A	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	**Step 1**	Peroxidase or Alkaline Phosphatase Block E36 is recommended. User supplied				
2	**Step 2**	HIER(Optional)				
3	**Step 3**	Mouse 1°Ab User supplied (30-60 min)				
4	**Step 4**	**Reagent 1** Mouse HRP Polymer RTU (15 min)				
5	**Step 5**	**Reagent 2A & Reagent 2B** DAB requires mixing (5 min)				
6	**Step 6**	**Reagent 3** Antibody Blocker requires mixing (10 min)				
7	**Step 8**	Rabbit 1°Ab & Rat 1°Ab (30-60 min)				
8	**Step 9**	**Reagent 4** Rat Primer RTU (10 min)				
9	**Step 10**	**Reagent 5 & Reagent 6** Rat AP Polymer & Rabbit HRP Polymer require mixing (30 min) **Wash with 1xTBS-T only.**				
10	**Step 11**	**Reagent 7A, Reagent 7B& Reagent 7C** GBI-Permanent Red requires mixing. (10min)				
11	**Step 12**	Counter stain (5-10 sec) **See Note** User supplied				
12	**Step 13**	**Reagent 8** Emerald Chromogen RTU (5min)				
13	**Step 14**	**It is important to follow the protocol. To maintain stain!** Dehydrate section 20seconds for each step				
14	**Step 15**	**Reagent 9** U-Mount RTU Mount & coverslip				
15	**Result**	**Stain pattern on controls are correct: Fill in Yes or NO**				

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

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TS312A Protocol B is suitable when mouse primary antibody needs pretreatment or rabbit & rat primary antibodies are sensitive to pre-treatment.

	Step/ Protocol	TS312A Protocol B	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	Step 1	Peroxidase or Alkaline Phosphatase Block E36 is recommended. User supplied				
2	Step 8	Rabbit 1°Ab & Rat 1°Ab (30-60 min)				
3	Step 9	**Reagent 4** Rat Primer RTU (10 min)				
4	Step 10	**Reagent 5 & Reagent 6** Rat AP Polymer & Rabbit HRP Polymer require mixing (30 min)				
5	Step 5	**Reagent 2A & Reagent 2B** DAB requires mixing (5 min)				
6	Step 11	**Reagent 7A, Reagent 7B& Reagent 7C** GBI-Permanent Red requires mixing. (10min)				
7	Step 7	HIER Supplied by user				
8	Step 3	Mouse 1°Ab (30-60 min)				
9	Step 4	**Reagent 1** Mouse HRP Polymer RTU (15 min)				
10	Step 12	Counter stain (5-10 sec) **See Note** User supplied				
11	Step 13	**Reagent 8** Emerald Chromogen RTU (5min)				
12	Step 14	**It is important to follow the protocol. To maintain stain!** Dehydrate section 20seconds for each step				
13	Step 15	**Reagent 9** U-Mount RTU Mount & coverslip				
14	Result	**Stain pattern on controls are correct: Fill in Yes or NO**				

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

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TS312A Protocol C is suitable when rat and rabbit primary antibodies need pretreatment or mouse primary antibody is sensitive to pretreatment.

	Step/ Protocol	TS312A Protocol C	Experiment 1 Date:	Experiment 2 Date:	Experiment 3 Date:	Experiment 4 Date:
1	**Step 1**	Peroxidase or Alkaline Phosphatase Block E36 is recommended. User supplied				
2	**Step 3**	Mouse 1°Ab User supplied (30-60 min)				
3	**Step 4**	**Reagent 1** Mouse HRP Polymer RTU (15 min)				
4	**Step 5**	**Reagent 2A & Reagent 2B** DAB requires mixing (5 min)				
5	**Step 7**	HIER Supplied by user				
6	**Step 8**	Rabbit 1°Ab & Rat 1°Ab (30-60 min)				
7	**Step 9**	**Reagent 4** Rat Primer RTU (10 min)				
8	**Step 10**	**Reagent 5 & Reagent 6** Rat AP Polymer & Rabbit HRP Polymer require mixing (30 min)				
9	**Step 11**	**Reagent 7A, Reagent 7B& Reagent 7C** GBI-Permanent Red requires mixing. (10min)				
10	**Step 12**	Counter stain (5-10 sec) **See Note** User supplied				
11	**Step 13**	**Reagent 8** Emerald Chromogen RTU (5min)				
12	**Step 14**	**It is important to follow the protocol. To maintain stain!** Dehydrate section 20seconds for each step				
13	**Step 15**	**Reagent 9** U-Mount RTU Mount & coverslip				
14	**Result**	**Stain pattern on controls are correct: Fill in Yes or NO**				

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

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