



TANDEM™ IHC STAINING AND DETECTION KITS USERMANUAL

EPITOMICS TANDEM™ IHC STAINING AND DETECTION KIT USERMANUAL

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2.0 PURPOSE

Epitomics Tandem™ IHC Staining and Detection Kits enable researchers to pair Epitomics rabbit monoclonal antibodies (RabMAbs) with any mouse monoclonal of their choice. This kit is intended for *in vitro research purposes* and has been optimized for paraffin embedded formalin fixed tissues. Each kit provides enough reagents to perform up to 50 staining reactions.

3.0 BACKGROUND

Double staining is a technique used to evaluate the expression of two distinct antigens in the same tissue sample. The advent of Rabbit Monoclonal antibodies (RabMAbs) has opened up the possibility of performing reliable and clean dual IHC staining of formalin fixed, paraffin-embedded tissue samples.

Until now, the majority of staining protocols involved biotinylated primary antibodies or using a combination of monoclonal and polyclonal antibodies. These procedures generally provide inconsistent staining with high background. Using RabMAbs can easily rectify this poor outcome due to these advantages: diverse epitope recognition, high specificity and affinity, and better antigen detection. Additionally, RabMAbs can be easily paired with mouse monoclonal antibodies in a straightforward procedure that results in superior antigen recognition with more precise and consistent results as well as clean dual labeled samples.

The **Tandem™ IHC Staining Kits** include a primary Rabbit Monoclonal antibody and provide the necessary reagents to perform dual color staining using polymer based detection antibodies for a stronger clean signal. The **Tandem™ IHC Detection Kits** provide the same reagents as the Staining Kits but without the primary RabMAb to allow customers to pair any suitable RabMAb with a Mouse mAb of their choice.

4.0 MATERIALS AND REAGENTS PROVIDED

Table 1

Reagent	Form provided	Amount provided
Antigen Retrieval solution (1X)	ready-to-use	500 ml
Peroxidase Block solution	ready-to-use	12 ml
Blocking solution	ready-to-use	12 ml
Primary antibody diluent	ready-to-use	15 ml
Negative control rabbit IgG (1X)	ready-to-use	5 ml
Primary Rabbit mAb (provided with Staining Kits)		100 ul
Secondary anti-Rabbit (AP' or HRP)	ready-to-use	10 ml
Secondary anti-Mouse (AP' or HRP)	ready-to-use	10 ml
DAB A	ready-to-use	350 ul
DAB B	ready-to-use	10 ml
Fast Red tablets		2 tablets
Fast Red solution	ready-to-use	10 ml

Note: Storage conditions; please remove and store primary antibody at -20°C and store all other reagents at 4°C.

4.1 NOTES ON MATERIALS

- DAB
 - i. Add 32ul of DAB A to 1ml of DAB B and mix.
 - ii. The DAB working solution is stable for 5 days in 4°C.
- Fast Red Solution
 - i. Add 1 Fast Red tablet to 5 ml of Fast Red solution and shake until fully dissolved
 - ii. Immediately apply solution to tissue section

4.2 MATERIALS AND REAGENTS REQUIRED

- Xylene
- Ethanol, anhydrous denatured, histological grade (100%, 95%, 70%, 50%)
- Washing buffer:
 - i. TBST washing buffer: (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween-20) – TBST is Tris Base Saline buffer with 0.1% Tween-20
- Distilled water (dH2O)
- Hematoxylin QS
- Permanent Mounting medium
- Aqueous Mounting medium
- Mouse Monoclonal Antibody
- Rabbit Antibody (or other species) - required when using Detection Kit.

5.0 EQUIPMENT REQUIRED

- Slide Rack
- Aluminum Slide holder
- Rice cooker
- Pipettors and pipet tips of various sizes
- Glass coverslips

6.0 STAINING CONSIDERATIONS

When planning your dual IHC staining it is important to choose the appropriate stain for each target antigen. In order to make the subsequent visualization of your staining easier it is recommended that antigens expected to be localized to the nucleus be stained with HRP and antigens that are targeted to the cytoplasm or membrane be labeled with AP. Therefore it is important to consider the species of the primary antibody and choose the appropriate labeled secondary to match.

The detection reagents provided with each Tandem™ IHC Kits (supplied with a Rabbit Monoclonal) have been chosen with this detection scheme in mind and as such the appropriate secondary antibodies have been provided with each kit.

When using the Tandem™ IHC Detection kits and pairing two antibodies of your choice, it is helpful to consider the expected staining patterns of the two antigens and choose the appropriate secondary antibodies and detection reagents to match the recommendations outlined in Table 2.

Table 2 – Recommended Dual Color Staining Scheme

Expected Staining Pattern/Location of the Target Antigen	Recommended Stain
Nuclear	Brown (HRP-labeled secondary)
Membrane, Cytoplasm or Organelle	Red (AP-labeled secondary)

Example 1.

p53-RabMAb (nuclear staining) – use anti-Rabbit-HRP secondary
Bcl-2 mouse mAb (cytoplasmic staining) – use anti-Mouse-AP secondary

Example 2.

AIF RabMAb (cytoplasmic/mitochondrial staining) – use anti-Rabbit-AP secondary
p53 mouse mAb (nuclear staining) – use anti-mouse-HRP secondary

7.0 PROCEDURE (Please read through entire procedure before beginning.)

1. *Deparaffinization/Rehydration*

- a. Heat slides in an oven at 65 °C for 1 hour.
- b. De-paraffinize/hydrate using the following series of 5 minute washes: two Xylene washes followed by two 100% ethanol rinses, followed by 95% ethanol, 70% ethanol, 50% ethanol and distilled water wash for 5 min on a shaker.

2. *Antigen Retrieval*

- a. Immerse slides into staining dish containing Antigen Retrieval Solution.
- b. Place covered staining dish into the rice cooker. Add 120 mL dH₂O and press “cook”.
- c. When “cook” is turned off (about 20–30 min), unplug the cooker and remove the staining dish to the bench top.
- d. Allow to cool down, without cover, for 20 min.

3. *Staining*

- a. Wash slides with TBST for 5 min on a shaker.
- b. Inactivate endogenous peroxidase by covering tissue with the peroxide block solution for 10 min.
- c. Wash slides three times with TBST (3 min each on a shaker).
- d. Block slides with the blocking solution for 1 hour.
- e. Dilute mouse monoclonal and rabbit monoclonal primary antibodies in primary antibody diluent to half the recommended dilution (e.g. if recommended dilution is 1:100, make 1:50 dilution (final volume of 100ul each)).
- f. Apply primary antibodies to each tissue section at a ratio of 1:1 volume (200ul total) and incubate overnight in the humidified chamber (4 °C).
NOTE: 100-200ul can be use to cover one slide
- g. Wash slides three times with TBST (3 min each on a shaker).
- h. Apply to each section secondary HRP-conjugated anti-mouse antibody and AP-conjugated anti-rabbit antibody at a ratio of 1:1; or secondary AP-conjugated anti-mouse antibody and HRP-conjugated anti-rabbit antibody at a ratio of 1:1, incubate for 45 minutes at room temperature.**

- i. Wash slides three times with TBS* (3 min each on a shaker).
- j. Add freshly prepared Fast Red solution to the sections
- k. Incubate tissue sections with substrate for 10-25 minutes at room temperature
- l. Wash slides three times with TBS* (3 min each on a shaker).
- m. Add freshly prepared DAB substrate to the sections.
- n. Incubate tissue sections with the substrate at room temperature until suitable staining develops (generally 1-2 min).
- o. Rinse sections with water.
- p. Counterstain with Hematoxylin.
- q. Rinse sections well with water.
- r. Cover tissue completely with Crystal/Mount medium and bake for 1-2 hrs until solution becomes solid.
- s. Dip slides 3 times into xylene to clear.
- t. Mount coverslips on slides using Permount medium.

4. Notes

* Detergents, such as Tween-20 or TritonX100, will accelerate fast red decomposition and dual stains.

** Red cytoplasm/membrane staining combined with brown nuclear staining is recommended, as red nuclear staining is difficult to visualize. Select one of the two combinations of secondary antibodies to match this template. (See table 1)

8.0 TROUBLESHOOTING

Table 3 – Troubleshooting

Problem	Possible Cause
No staining	-Steps of the protocol or reagents not used in proper order -Antigens are destroyed -Tissue is incorrectly fixed/ or processed
Weak Staining	-Incomplete deparaffinization (weak staining with high background) -Inadequate epitope retrieval -Inadequate reagents incubation time -Inappropriate tissue fixation method used -Incorrect substrates preparation
High Background	-Incomplete deparaffinization -Incomplete blocking of peroxidase activity -Slides not thoroughly washed

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- Inappropriate tissue fixation method used
 - Sections dried during staining procedure
 - Excessive incubation with substrates
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9.0 Epitomics Tandem™ IHC Staining Kits

Each Tandem™ Staining kit includes one primary rabbit monoclonal antibody which can be paired with any mouse monoclonal of choice. Each kit contains the corresponding labeled secondary antibodies.

Catalog number	Primary RabMAb	Detection
4015-1	AIF	Anti-rabbit AP '+ Anti-mouse HRP
4016-1	BAX	Anti-rabbit AP '+ Anti-mouse HRP
4019-1	BCL-2	Anti-rabbit AP '+ Anti-mouse HRP
4020-1	BCL-X	Anti-rabbit AP '+ Anti-mouse HRP
4017-1	BID	Anti-rabbit AP '+ Anti-mouse HRP
4018-1	BIM	Anti-rabbit AP '+ Anti-mouse HRP
4021-1	ER-alpha phospho	Anti-rabbit HRP '+ Anti-mouse AP
4025-1	HER-2	Anti-rabbit AP '+ Anti-mouse HRP
4022-1	Keratin-8	Anti-rabbit AP '+ Anti-mouse HRP
4023-1	Keratin-18	Anti-rabbit AP '+ Anti-mouse HRP
4024-1	p53	Anti-rabbit HRP '+ Anti-mouse AP

Epitomics Tandem™ IHC Detection Kits (w/o Rabbit primary antibody)

Two different kits are provided allowing customers to choose the appropriate detection scheme based on their primary antibodies (see Sec. 6.0).

Catalog number	Description
4000-1	Epitomics Tandem™ IHC Detection kit-I (Anti-Rabbit-HRP, Anti-Mouse-AP)
4001-1	Epitomics Tandem™ IHC Detection kit-II (Anti-Rabbit-AP, Anti-Mouse-HRP)