Product Manual

MULTIVIEW® PLUS (mouse-HRP/rabbit-AP) IHC Kit (Brown/Green)

Catalog #: ENZ-KIT181-0150

150 Tests

NOTE: This version contains a change to instructions for the preparation of HIGHDEF® DAB Chromogen/Substrate.

For the latest product information, including support documentation, visit us online:
www.enzolifesciences.com

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Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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INTRODUCTION

The MULTIVIEW® PLUS (mouse-HRP/rabbit-AP) IHC Kit (Brown/Green) provides reagents and materials for ultrasensitive multiplex detection of mouse and rabbit antibodies used in immunohistochemistry (IHC) staining. The kit is a non-biotin detection system suitable for identifying antigens in formalin-fixed paraffin-embedded tissues. This kit may also be used with blood smears, cytosmears, and cell preparations.

MULTIVIEW® PLUS (mouse-HRP/rabbit-AP) IHC Kit (Brown/Green) contains both MULTIVIEW® PLUS HRP (Anti-Mouse) Reagent and MULTIVIEW® PLUS AP (Anti-Rabbit) Reagent, which have been developed by directly labeling anti-mouse or anti-rabbit immunoglobulins with enzymes using a proprietary labelling technology in a ready-to-use format. This ensures consistent and reproducible immunodetection of mouse and rabbit antibodies against nuclear, cytoplasmic, and membrane antigens in different types of tissues and cells. The MULTIVIEW® PLUS HRP (Anti-Mouse) Reagent and MULTIVIEW® PLUS AP (Anti-Rabbit) Reagent enable faster staining procedures compared to traditional two-step methods using biotin and avidin/streptavidin conjugates.

Enzo's HIGHDEF® DAB Chromogen, HIGHDEF® Green AP Chromogen, and Hematoxylin, coupled with the peroxidase and antibody blockers included in the kit, provide strong signal with minimal background.

The MULTIVIEW® PLUS (mouse-HRP/rabbit-AP) IHC Kit (Brown/Green) is suitable for use with all mouse and rabbit IgG antibodies, both monoclonal and polyclonal. The reagents from this kit can be used for manual staining or with automated staining instruments and are well suited for multiplex immunohistochemical staining assays.

MATERIALS SUPPLIED

Reagent Supplied	Volume
Antigen Retrieval Reagent, pH 9 (10X)	100 mL
ISH/IHC Peroxidase Block	22.5 mL
Antibody Blocker/Diluent	45 mL
MULTIVIEW® PLUS HRP (Anti-Mouse) Reagent	11.25 mL
MULTIVIEW® PLUS AP (Anti-Rabbit) Reagent	11.25 mL
HIGHDEF® DAB Chromogen	1 mL
HIGHDEF® DAB Substrate	22.5 mL
HIGHDEF® Green AP Chromogen	2 mL
HIGHDEF® Green AP Substrate	22.5 mL

Hematoxylin	22.5 mL
Empty Mixing Bottle	1 bottle
IHC Wash Buffer Salts	2 packs

STORAGE

Store all of the kit components at 2-8°C. Do not freeze. The Hematoxylin may be stored at room temperature away from direct bright light.

STABILITY

When used and stored as directed, the kit is stable for 1 year from date of receipt.

ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED

- IHC PAP Pen (Product No. ADI-950-233)
- Slide holder
- Xylene (or Xylene substitute) for dewaxing
- Xylene substitute for clearing prior to mount (either Shandon Xylene Substitute, ThermoFisher Scientific Cat. No. 9990505 or Citrus Clearing Solvent, ThermoFisher Scientific Cat. No. 8301)
- 50%, 70%, 90%, and 100% Ethanol (Reagent Grade)
- Distilled or deionized water (dH_2O)
- Tween-20®
- Specimen slides and coverslips
- HIGHDEF® Mount (Product No. ADI-950-261)
- Heating Oven, set at ≥60°C
- Pressure Cooker, set at ≥100°C
- Heating blocks for slides
- Absorbent wipes
- Timer
- Also available: Antigen Retrieval Reagent, pH 6 (10X) (Product No. ENZ-ACC112)
- Additional IHC Wash Buffer Salts, if necessary

PRECAUTIONS

1. **For Research Use Only.** Not to be used for *in vitro* diagnostic purposes.
2. Use a safety pipetting device for all pipetting. Never pipet by mouth.
3. Interpretation of the results is the sole responsibility of the user.
4. Wear appropriate personal protective apparel. Avoid contact with clothes and exposed skin. In case of accidental skin exposure, flush with water immediately. Consult a physician if required.

REAGENT PREPARATION

1X Antigen Retrieval Solution:

To prepare 100 mL of 1X Antigen Retrieval Solution, add 10 mL of Antigen Retrieval Reagent (10X) to 90 mL of deionized water. Mix well, and keep protected from direct bright light. For best results, use immediately.

Note: *Also available is Antigen Retrieval Reagent, pH 6 (10X) (Product No. ENZ-ACC112).*

Wash Buffer:

To prepare one liter of Wash Buffer, add 1 pack of IHC Wash Buffer Salts to 1 liter of deionized water. Mix well, and add 500 µL of Tween-20®. Store buffer at 4°C.

HIGHDEF® DAB Chromogen/Substrate:

This reagent must be prepared shortly before use. Per 1 mL of HIGHDEF® Substrate Buffer, add 40 µL of HIGHDEF® DAB Chromogen. Mix well, and keep protected from direct bright light. At room temperature, this solution is stable for one day. When refrigerated and protected from light, this solution is stable for up to seven days.

HIGHDEF® Green AP Chromogen/Substrate:

This reagent must be prepared shortly before use. Per 920 µL of HIGHDEF® Green AP Substrate Reagent, add 80 µL of HIGHDEF® Green AP Chromogen to make 1 mL of solution. Mix well, and keep protected from bright light. At room temperature, this solution is stable for up to 6 hours. When refrigerated and protected from light, this solution is stable for up to 1 day.

STAINING PROTOCOL – MULTIPLEX IMMUNOHISTOCHEMISTRY

NOTE:

(1) Do not allow the slides to dry between steps during the entire procedure. Add sufficient amounts of reagents to specimens during incubation steps and cover the slides while incubating to prevent slides from drying out.

(2) If weak signal is noticed, increase the incubation times for primary antibodies and the MULTIVIEW® PLUS HRP (Anti-Mouse) and MULTIVIEW® PLUS AP (Anti-Rabbit) Reagents*.*

I. SPECIMEN SLIDE PREPARATION AND PRETREATMENT

The MULTIVIEW® PLUS (mouse-HRP/rabbit-AP) IHC Kit (Brown/Green) can be used on formalin-fixed, paraffin embedded (FFPE) biopsy sections. It can also be used on fixed cells. No special preparative materials are required for use of the system on fixed cells.

A. BIOPSY SLIDES

Note: *Paraffin-embedded biopsy specimen slides must be deparaffinized and treated with an Antigen Retrieval Reagent prior to immunohistochemistry procedure.*

1. Apply one to three FFPE sections (4-6 microns thick) of each biopsy specimen to a charged specimen slide.

   Bake tissue-mounted slides vertically for at least 2 hours (up to 18 hours) at 60-80°C to fix the slides. Store fixed slides at room temperature.

2. Arrange the tissue specimen mounted slides in a slide holder.

   Note: *(1) If the slides will be used immediately after the sections are fixed on the slides by baking, proceed to step 4.*

   (2) If the wax on the slides had solidified on storage, proceed to step 3.

3. Transfer the slides in drying oven at 55-60°C for 20 minutes to melt the wax.

4. Deparaffinize the specimen slides by soaking them sequentially in the following solutions for the time indicated:

Soak Number	Reagent	Duration of Soak
1	Xylene (or Xylene substitute)	2 x 5 minutes
2	100% Ethanol	2 x 3 minutes
3	90% Ethanol	1 minute
4	70% Ethanol	1 minute
5	50% Ethanol	1 minute
6	Deionized Water	1 minute

Caution: *Xylene saturates rapidly with paraffin. Replace the solutions for each batch of slides.*

5. After the final soak, wipe the excess liquid around the section on the glass slide.
6. Retrieve antigen with 1X Antigen Retrieval Solution (see REAGENT PREPARATION section) for 20 minutes at 99°C.

   Note: *Antigen retrieval methods may vary depending on the antibody in use. The user should try different solutions, times, and temperatures to determine the optimal method for their chosen antibody.*
7. Wash the slides with dH_2O for 5 minutes.
8. Wipe the excess liquid around the section on the glass slide and encircle the tissue section with a PAP pen. Dry for 10 seconds.
9. Incubate each specimen in a sufficient amount of IHC/ISH Peroxidase Block to cover the tissue section (~150 µL) for 5 minutes at room temperature. Wash the slides with Wash Buffer (see REAGENT PREPARATION section) twice for 2 minutes, tapping off excess wash buffer between each wash.

II. ANTIBODY BLOCKING AND PRIMARY ANTIBODY STAINING

Note: *If blocking is required before incubation with primary antibody, use the Antibody Blocker/Diluent provided in the kit. DO NOT use normal rabbit or mouse serum as blocking reagent.*

1. Cover each specimen slide with enough Antibody Blocker/Diluent to cover the tissue section (~150 µL) and incubate for 10 minutes. This will prevent the nonspecific binding of MULTIVIEW® PLUS HRP (Anti-Mouse) and MULTIVIEW® PLUS AP (Anti-Rabbit) Reagents.
2. Wash the slides twice in Wash Buffer for 2 minutes. Tap off excess wash solution in between the washes.
3. Add enough volume of an optimal concentration of primary mouse and rabbit antibodies to cover the specimen and incubate for 20-30 minutes at room temperature.

   Note: *Refer to primary antibody manufacturer's recommendations for optimal incubation conditions and dilutions. Dilute primary antibody in Antibody Blocker/Diluent (as provided in the kit, but can also be found as Prod. No. ENZ-ACC108-0100).*
4. Wash the slides in Wash Buffer for 5 minutes. Tap off excess wash solution. Proceed to IHC Detection section.

III. IHC DETECTION

Note: *Mix equal volumes (use ~75 µL of each reagent per slide you plan to run) of* MULTIVIEW® PLUS HRP (Anti-Mouse) and MULTIVIEW® PLUS AP (Anti-Rabbit) Reagents *in a mixing bottle or Eppendorf tube prior to use. DO NOT use normal rabbit or mouse serum as blocking reagent.*

1. To each specimen add mixture of MULTIVIEW® PLUS HRP (Anti-Mouse) and MULTIVIEW® PLUS AP (Anti-Rabbit) Reagent to cover the tissue (~150 µL). Incubate for 30 minutes at room temperature.
2. Wash the slides twice in fresh Wash Buffer for 5 minutes. Tap off excess wash solution between washings.

   Note: *First develop the color for* MULTIVIEW® PLUS AP (Anti-Rabbit) *with* HIGHDEF® Green AP Chromogen/Substrate *and then develop the color with* MULTIVIEW® PLUS HRP (Anti-Mouse) *with* HIGHDEF® DAB Chromogen/Substrate.
3. Add HIGHDEF® Green AP Chromogen/Substrate (see REAGENT PREPARATION section) to cover the tissue section (~150 µL) and allow color to develop for 10-15 minutes at room temperature.
4. Tap off excess chromogen. Wash the slides with dH_2O for two minutes. Tap off excess dH_2O.
5. Add DAB Chromogen/Substrate (see REAGENT PREPARATION section) to cover tissue section (~150 µL) and allow color to develop for 5 minutes at room temperature.
6. Tap off excess chromogen. Wash the slides with dH_2O for two minutes. Tap off excess dH_2O.
7. **[Optional]** Counterstain each specimen with Hematoxylin (~150 µL) for 1-2 minutes at room temperature.

   Note: *If a weak nuclear stain is expected, incubate with hematoxylin for 5 minutes.*
8. Tap off spent counterstain and rinse the slides with dH_2O.
9. Using water as mounting medium, view the slides using a light microscope.

   If desired, dehydrate the slides in 70% alcohol for 1 minute followed by 100% alcohol for 1 minute. Then, clear sections in xylene substitute for 1 minute and then mount using permanent histological mounting medium.

   IMPORTANT: Using xylene as a clearing solvent will cause the signal to dissipate. Be sure to use aliphatic hydrocarbon or D-limonene based xylene substitute instead. See ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED section (Page 2) for recommended commercially available xylene substitutes.

Multiplex IHC – Membrane and Nuclear Proteins

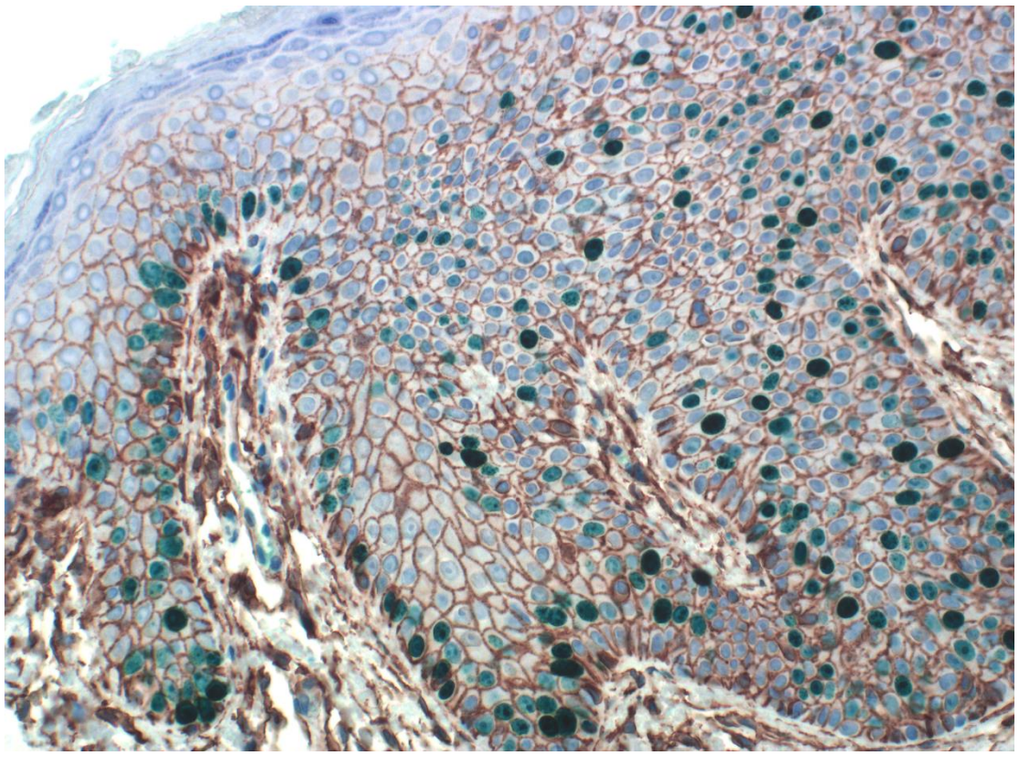

Fig 1. Mouse anti-CD44 clone SFF-304 and mouse anti-Ki-67 clone MIB-1 with Rabbit anti-Mouse Linker (Cat. No. ENZ-ACC116) was used with MULTIVIEW® PLUS (mouse-HRP/rabbit-AP) Kit (Prod. No. ENZ-KIT181) to perform multiplex immunohistochemistry staining with hematoxylin used as a counterstain.

TROUBLESHOOTING

Problem	Reason	Solution
No signal noticed on any slide	Wrong reagent used	Carefully follow instructions as described in this manual. Do not substitute kit reagents.
	Sodium azide contamination	Use sodium azide free buffers.
Weak signal	Primary antibody concentrations may be low.	Increase the primary antibody concentration and/or incubate it longer.
	Inefficient removal of excess wash solutions	Gently tap off wash solutions after each wash. Wipe off excess washings around the specimen, if necessary.
	Incubations with the detection reagents may be too short.	Increase incubation times.
	Paraffin or pap pen covered the section	When paraffin is noticed on sections, expose slides to 100% ethanol for 5 minutes and wash with dH_2O. Try not to come into contact with the tissue when using the pap pen.
	No green signal observed	Xylene was used to wash slides instead of xylene substitute
Moderate background	Sections dried during the procedure.	Process 4-5 slides at a time (when processing manually). Cover the slides during incubations to avoid rapid evaporation of reagents.
	Nonspecific binding of reagents to tissue	Increase incubation time with blocking reagent.
	Primary antibody used may be too concentrated.	Further dilute the primary antibody.
	Paraffin not removed completely.	Incubate section 5 more minutes in 100% ethanol followed by dH_2O. Replace de-waxing reagents more frequently.
High background	Slides are not properly washed.	Use only the Wash Buffer recommended in the protocol.
	Substrate incubation is too long.	Shorten the incubation time in HIGHDEF® DAB Chromogen/Substrate.
	Antibody concentration is too high.	Dilute the primary antibody.
	High level of endogenous peroxidase.	Increase the peroxidase block incubation from 5 minutes to 10 minutes.
Tissue falling off slide	Antigen retrieval is too harsh.	Use a different antigen retrieval method, or reduce time or temperature.
Only brown staining on slide	HRP was developed first	Develop green (AP) first, then develop brown color (HRP).

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