APPLICATION NOTE

Three-color IHC Staining On Free-floating Sections From Human Brain

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MULTIVIEW® PLUS (MOUSE-HRP/RABBIT-AP) IHC KIT (ENZ-KIT181)
HIGHDEF® BLUE IHC CHROMOGEN (AP) (ADI-950-150)
HIGHDEF® RED IHC CHROMOGEN (HRP) (ADI-950-210)
HIGHDEF® YELLOW IHC CHROMOGEN (HRP) (ADI-950-170)

INTRODUCTION

Immunohistochemistry (IHC) on human brain tissue sections is widely used for diagnosis by anatomopathologists and researchers. Indeed this technique allows the visualization of specific antigens and thus their distribution into the cells and their compartments. To handle this type of tissue can be very challenging due to the condition of the brain removal and the quality of the tissue. There is a growing interest on multiplexing IHC and being able to perform this technique on free-floating human brain sections as good as formalin-fixed paraffin-embedded sections on slide could be a step forward to analyze larger and thicker specimen (i.e. 50 µm).

The MULTIVIEW® PLUS (mouse-HRP/rabbit-AP) IHC Kit from Enzo Life Sciences is a non-biotin, one-step detection system suitable for demonstrating the expression of multiple antigens on tissue sections. It enables faster and more consistent staining procedures than traditional two-step methods using biotin and avidin/streptavidin conjugates. Indeed, mouse antibodies are directly tagged with HRP and rabbit antibodies with AP at the same time, all with significantly lower background staining.

The main objective of this study was to validate the use of the MULTIVIEW® PLUS (mouse-HRP/rabbit-AP) IHC Kit and the HIGHDEF® IHC chromogens to detect multiple staining on human free-floating brain sections. The intensity, sensitivity, and specificity obtained for all staining demonstrate the capacity of the Enzo Life Sciences kit and their chromogen to achieve multiplexing IHC on free-floating tissue sections.

MATERIALS

- 50 µm free-floating tissue sections from human brain
- Antigen retrieval solution: 10 mM sodium citrate buffer pH 6.0
- Peroxidase block: 20% (v/v) methanol, 3% (v/v) H_2O_2 in 0.1 M PBS
- IHC Wash Buffer salts (included in ENZ-KIT181)
- Antibody Blocker/Diluent (included in ENZ-KIT181)
- Glutamate decarboxylase 65/67 (GAD65/67) polyclonal antibody from rabbit
- Tyrosine hydroxylase (TH) monoclonal antibody from mouse
- Vesicular glutamate transporter-2 (vGLUT2) monoclonal antibody from mouse
- MULTIVIEW® PLUS (mouse-HRP/rabbit-AP) IHC kit (ENZ-KIT181)
- HIGHDEF® Blue IHC chromogen (AP) (ADI-950-150)
- HIGHDEF® Red IHC chromogen (HRP) (ADI-950-210)
- HIGHDEF® Yellow IHC chromogen (HRP) (ADI-950-170)

REAGENT PREPARATION

- HIGHDEF® Blue IHC chromogen (AP): 1 mL of the HIGHDEF® Blue IHC chromogen (AP) substrate buffer was added to a mixing bottle and 20 µL of concentrated HIGHDEF® Blue IHC chromogen (AP) solution were added. The solution was mixed and allowed to reach room temperature (RT) before using. For optimal staining, the solution should be made fresh.

- HIGHDEF® Red IHC chromogen (HRP): HIGHDEF® Red IHC chromogen (HRP) is a single, highly stable, AEC chromogen/substrate working solution supplied ready-to-use.

- HIGHDEF® Yellow IHC chromogen (HRP): 1 mL of the HIGHDEF® Yellow IHC chromogen (HRP) substrate buffer was added to a mixing bottle and 20 µL of concentrated HIGHDEF® Yellow IHC chromogen (HRP) solution were added. The solution was mixed and allowed to reach RT before using. For optimal staining, the solution should be made fresh.

- IHC Wash Buffer (1 L): One pack of IHC Wash Buffer Salts was added to 1000 mL of deionized water. The IHC Wash Buffer was mixed well and 500 µL of Tween-20® was added. The IHC Wash Buffer was stored at 4° C.

SINGLE-COLOR IHC STAINING PROTOCOL

1. Antigen Retrieval

- Antigen retrieval was performed with antigen retrieval solution for 20 minutes at 85° C.
- Free-floating sections were left to cool for 30 minutes at RT.
- Free-floating sections were washed in dH_2O for 5 minutes at RT.

2. Quenching Endogenous Peroxidase

- Each specimen was incubated in a sufficient amount of peroxidase block to cover the tissue section for one hour at RT.
- Free-floating tissue sections were washed with IHC Wash Buffer twice for 2 minutes at RT.

3. Antibody Blocking

- Each specimen was incubated in a sufficient amount of Antibody Blocker/Diluent to cover the tissue section for 10 minutes at RT. This will prevent the non-specific binding of MULTIVIEW® PLUS HRP (anti-mouse) and MULTIVIEW® PLUS AP (anti-rabbit) reagents.
- Free-floating tissue sections were washed with IHC Wash Buffer twice for 2 minutes at RT.

4. Incubation with the Primary Antibody

- Primary antibody against either GAD65/67, TH or vGLUT2 was diluted in Antibody Blocker/Diluent at the optimal concentration previously determined. Enough volume was used to cover the specimen for one hour at RT.
- Free-floating sections were washed with IHC Wash Buffer for 5 minutes at RT.

5. Incubation with the Secondary Antibody

- The appropriate MULTIVIEW® PLUS reagent (mouse-HRP or rabbit-AP) was added to cover the tissue section and left for 30 minutes at RT.
- Free-floating tissue sections were washed with IHC Wash Buffer twice for 2 minutes at RT.

6. Color Development

- HIGHDEF® Red IHC chromogen (HRP) was used to cover the tissue section and color was allowed to develop for 10 minutes at RT.
- Free-floating tissue sections were washed with dH_2O twice for 2 minutes at RT.

7. Mounting

- Free-floating sections were mounted on 2x gelatin slides, air-dried and cover-slipped in aqueous-based mounting medium.

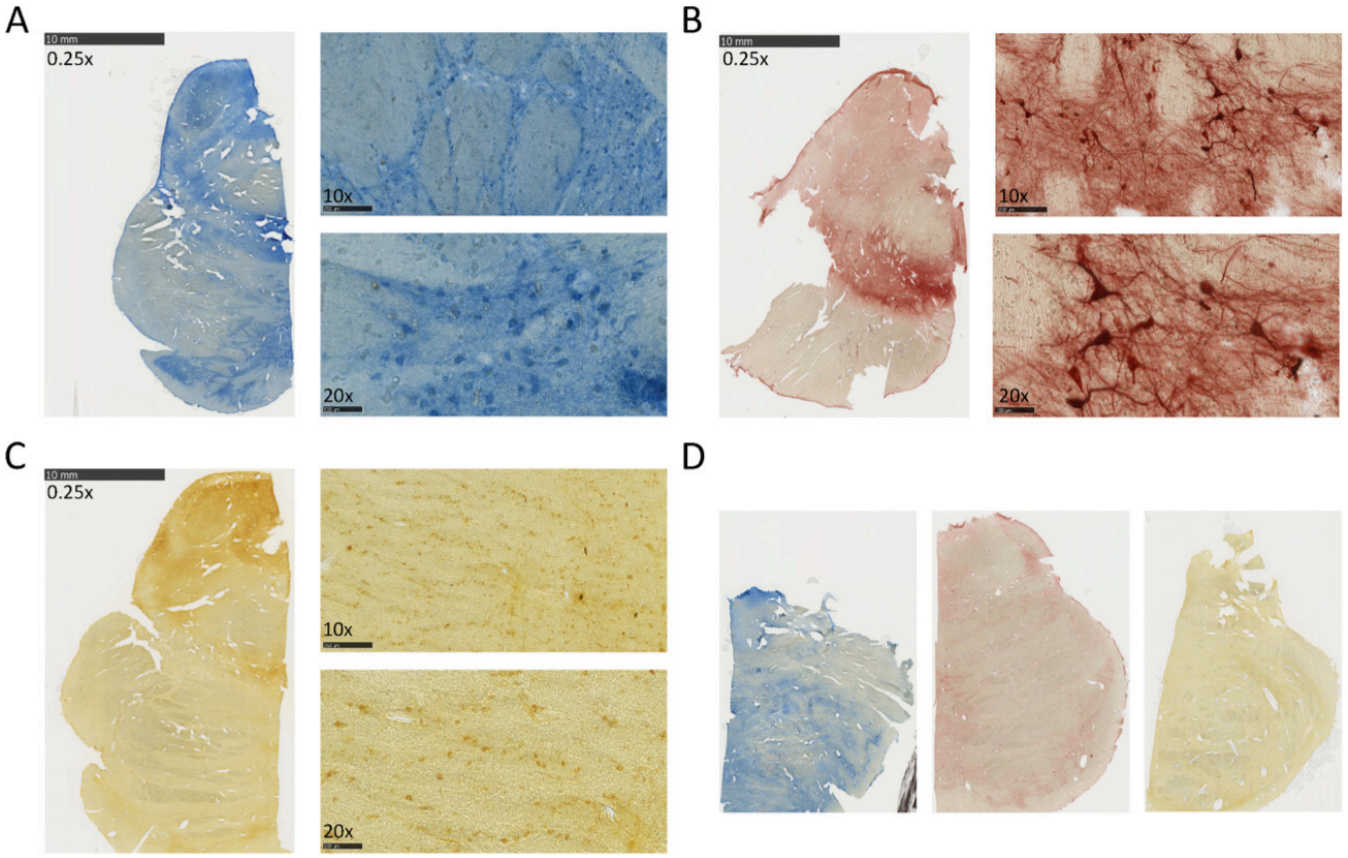

Figure 1. Single IHC staining on free-floating sections from human brain. Immunohistochemistry staining of GAD65/67 (A), TH (B), and vGLUT2 (C) on free-floating sections from human brain using MULTIVIEW® PLUS IHC detection reagents and HIGHDEF® IHC chromogens from Enzo Life Sciences. Negative controls were performed for each staining by omitting the primary antibody (D).

THREE-COLOR IHC STAINING PROTOCOL

1. Antigen Retrieval

- Antigen retrieval was performed with antigen retrieval solution for 20 minutes at 85° C.
- Free-floating sections were left to cool for 30 minutes at RT.
- Free-floating sections were washed in dH_2O for 5 minutes at RT.

2. Quenching Endogenous Peroxidase

- Each specimen was incubated in a sufficient amount of peroxidase block to cover the tissue section for one hour at RT.
- Free-floating sections were washed with IHC Wash Buffer twice for 2 minutes at RT.

3. Antibody Blocking

- Each specimen was incubated in a sufficient amount of Antibody Blocker/Diluent to cover the tissue section for 10 minutes at RT. This will prevent the non-specific binding of MULTIVIEW® PLUS HRP (anti-mouse) and MULTIVIEW® PLUS AP (anti-rabbit) reagents.
- Free-floating tissue sections were washed with IHC Wash Buffer twice for 2 minutes at RT.

4. Incubation with two Primary Antibodies

- Primary antibodies against GAD65/67 and vGLUT2 were diluted in Antibody Blocker/Diluent at the optimal concentration previously determined. Enough volume was used to cover the specimen for one hour at RT.
- Free-floating tissue sections were washed with IHC Wash Buffer for 5 minutes at RT.

5. Incubation with Secondary Antibodies

- Equal volumes of MULTIVIEW® PLUS HRP (anti-mouse) and MULTIVIEW® PLUS AP (anti-rabbit) reagents were mixed in a mixing bottle prior to use. Specimens were covered with the mixture and incubated for 30 minutes at RT.
- Free-floating sections were washed with IHC Wash Buffer twice for 2 minutes at RT.

6. Color Development

- HIGHDEF® Blue IHC chromogen (AP) working solution was added first to cover the tissue section and color was allowed to develop for 10 minutes at RT.
- Free-floating sections were washed with IHC Wash Buffer three times for 1 minute at RT.
- HIGHDEF® Yellow IHC chromogen (HRP) working solution was then added to cover the tissue section and color was allowed to develop for 10 minutes at RT.
- Free-floating sections were washed with IHC Wash Buffer three times for 1 minute at RT.

7. Incubation with a Third Primary Antibody

- Monoclonal antibody against TH was diluted in Antibody Blocker/Diluent at the optimal concentration previously determined. Enough volume was used to cover the specimen for one hour at RT.
- Free-floating sections were washed with IHC Wash Buffer for 5 minutes at RT.

8. Incubation with Secondary Antibody

- MULTIVIEW® PLUS HRP (anti-mouse) reagent was added to cover the tissue section and left for 30 minutes at RT.
- Free-floating sections were washed with IHC Wash Buffer twice for 2 minutes at RT.

9. Color Development

- HIGHDEF® Red IHC chromogen (HRP) was used to cover the tissue section and color was allowed to develop for 10 minutes at RT.
- Free-floating sections were washed with dH_2O twice for 2 minutes at RT.

10. Mounting

- Free-floating sections were mounted on 2x gelatin slides, air-dried and cover-slipped in aqueous-based mounting medium.

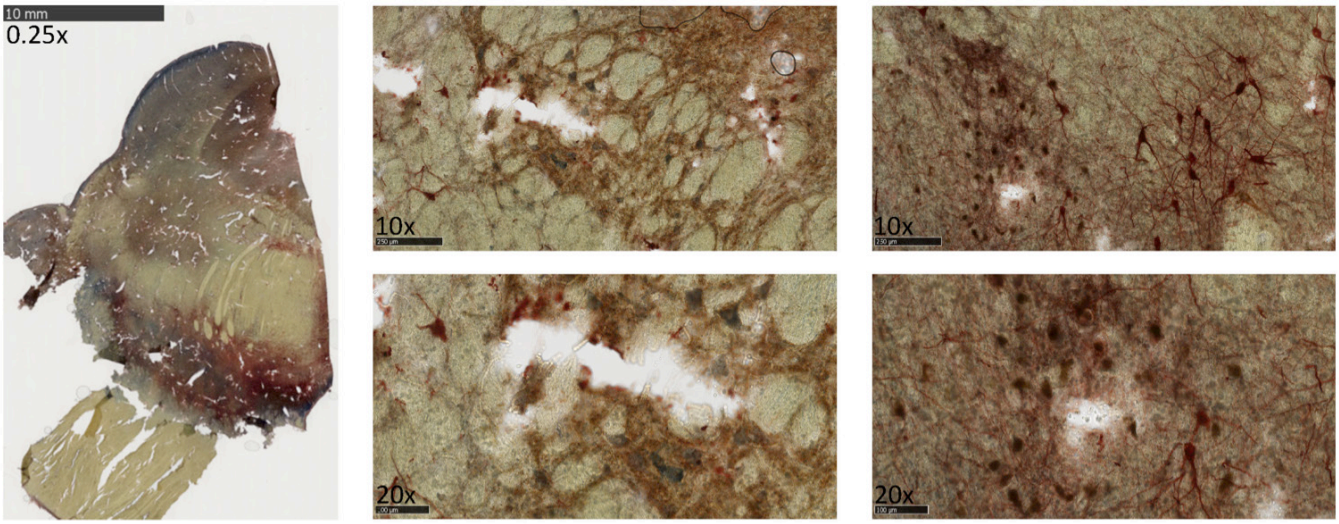

Figure 2: Three-color IHC staining on free-floating sections from human brain. Simultaneous detection by immunohistochemistry of GAD65/67 (blue), TH (red), and vGLUT2 (yellow) on free-floating sections from human brain using MULTIVIEW® PLUS IHC detection reagents and HIGHDEF® IHC chromogens from Enzo Life Sciences.

CONCLUSION

The purpose of this work was to ascertain the compatibility of Enzo's MULTIVIEW® PLUS (mouse-HRP/rabbit-AP) IHC kit and HIGHDEF® IHC chromogens with multiplexing immunohistochemistry on human free-floating tissue sections. A successful simultaneous detection of GAD65/67, TH, and vGLUT2 was observed with a good intensity of the signal and a low residual background. Thus the multiplex staining on human free-floating sections can be achieved with Enzo life Sciences' reagents.

Visit www.enzolifesciences.com/IHC for more information.

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