

## Tumor Phenotyping and Immune Cell Regulation using Multiplex IHC

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**MULTIVIEW® PLUS (MOUSE-HRP/RABBIT-AP) IHC Kit (ENZ-KIT181)**

**POLYVIEW® PLUS (RABBIT-HRP) Reagent (ENZ-ACC103)**

**HIGHDEF® Black IHC Chromogen (HRP) (ADI-950-171)**

**HIGHDEF® Blue IHC Chromogen (HRP) (ADI-950-151)**

**HIGHDEF® Red IHC Chromogen (HRP) (ADI-950-210)**

**HIGHDEF® Yellow IHC Chromogen (HRP) (ADI-950-170)**

**HIGHDEF® IHC Mount (ADI-950-261)**

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### INTRODUCTION

For over a decade, Histalim has provided services in histology, cytology, and molecular biology with extensive experience in immuno-oncology and immune therapies. With broad experience in neurosciences, toxicity assessment, therapeutic antibodies characterization, evaluation of implantable medical devices, and more. Histalim focuses on providing each client with the highest quality service. Histalim's scientific experts and European network of histopathology experts along with top of the line histological and image processing techniques and analysis capabilities enable Histalim to efficiently meet each client's specific need.

Immunohistochemistry (IHC) is important for pathologists and researchers in the diagnosis of tumors and analysis of the tissue environment. A technique which allows for maximal data per tissue section is very appealing when patient tissue is limited. Multiplex IHC allows for the evaluation of co-expression and spatial organization of multiple targets within preserved tissue architecture. This is particularly important in tumor immunology where it is critical to phenotype the immune and cancer cells in the context of the tumor microenvironment.

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 markers on human tissue sections. To that effect, two panels composed of four markers each were chosen. The first panel includes phenotypic markers commonly used in the diagnosis of lung cancer (CK5/6, Napsin A, p63, and TTF-1) while the second panel focusses on the characterization of the immune cell response and its regulation (CD3, CD8, PD-1, and PD-L1). The intensity, sensitivity, and specificity obtained with both panels demonstrated the capacity of Enzo's detection reagents and chromogens in multiplex protocols.

## MATERIALS AND METHODS

Primary antibodies used in Panel 1 were:

- CK5/6 monoclonal antibody from rabbit (Novus)
- Napsin A monoclonal antibody from rabbit (Cell Signaling)
- p63 monoclonal antibody from mouse (Biocare Medical)
- TTF-1 monoclonal antibody from rabbit (Abcam)

Primary antibodies used in Panel 2 were:

- CD3 monoclonal antibody from rabbit (Roche)
- CD8 monoclonal antibody from rabbit (Abcam)
- PD-1 monoclonal antibody from rabbit (Abcam)
- PD-L1 monoclonal antibody from rabbit (Cell Signaling)

Enzo's MULTIVIEW® PLUS (mouse-HRP/rabbit-AP) IHC Kit contains:

- Antigen Retrieval Reagent, pH 9 (10X)
- ISH/IHC Peroxidase Block
- Antibody Blocker/Diluent
- MULTIVIEW® PLUS HRP (anti-mouse) reagent
- MULTIVIEW® PLUS AP (anti-rabbit) reagent
- HIGHDEF® DAB Chromogen/Substrate set
- HIGHDEF® Green AP Chromogen/Substrate Set
- Hematoxylin
- IHC Wash Buffer salts

IHC kits reagents used in this study were:

- POLYVIEW® PLUS HRP (anti-rabbit) Reagent (ENZ-ACC103)
- HIGHDEF® Black IHC Chromogen (HRP) (ADI-950-171)
- HIGHDEF® Blue IHC Chromogen (HRP) (ADI-950-151)
- HIGHDEF® Red IHC Chromogen (HRP) (ADI-950-210)
- HIGHDEF® Yellow IHC Chromogen (HRP) (ADI-950-170)
- HIGHDEF® IHC Mount (ADI-950-261)

## WORKING SOLUTIONS

- MULTIVIEW® PLUS HRP (anti-mouse) reagent, MULTIVIEW® PLUS AP (anti-rabbit) reagent, POLYVIEW® PLUS HRP (anti-rabbit) reagent, ISH/IHC Peroxidase Block, Antibody Blocker/Diluent, and Hematoxylin are provided in a ready-to-use format.
- 1X Antigen Retrieval Solution: 10 mL of 10X Antigen Retrieval Solution were added to 90 mL of deionized water. The solution was thoroughly mixed. For optimal results, the solution should be used immediately.
- HIGHDEF® Black IHC chromogen (HRP): 1 mL of the HIGHDEF® Black IHC chromogen (HRP) substrate buffer was added to a mixing bottle and 20 µL of concentrated HIGHDEF® Black IHC chromogen (HRP) solution were added. The solution was mixed and allowed to reach RT before using. At room temperature, this solution is stable for up to 24 hours.

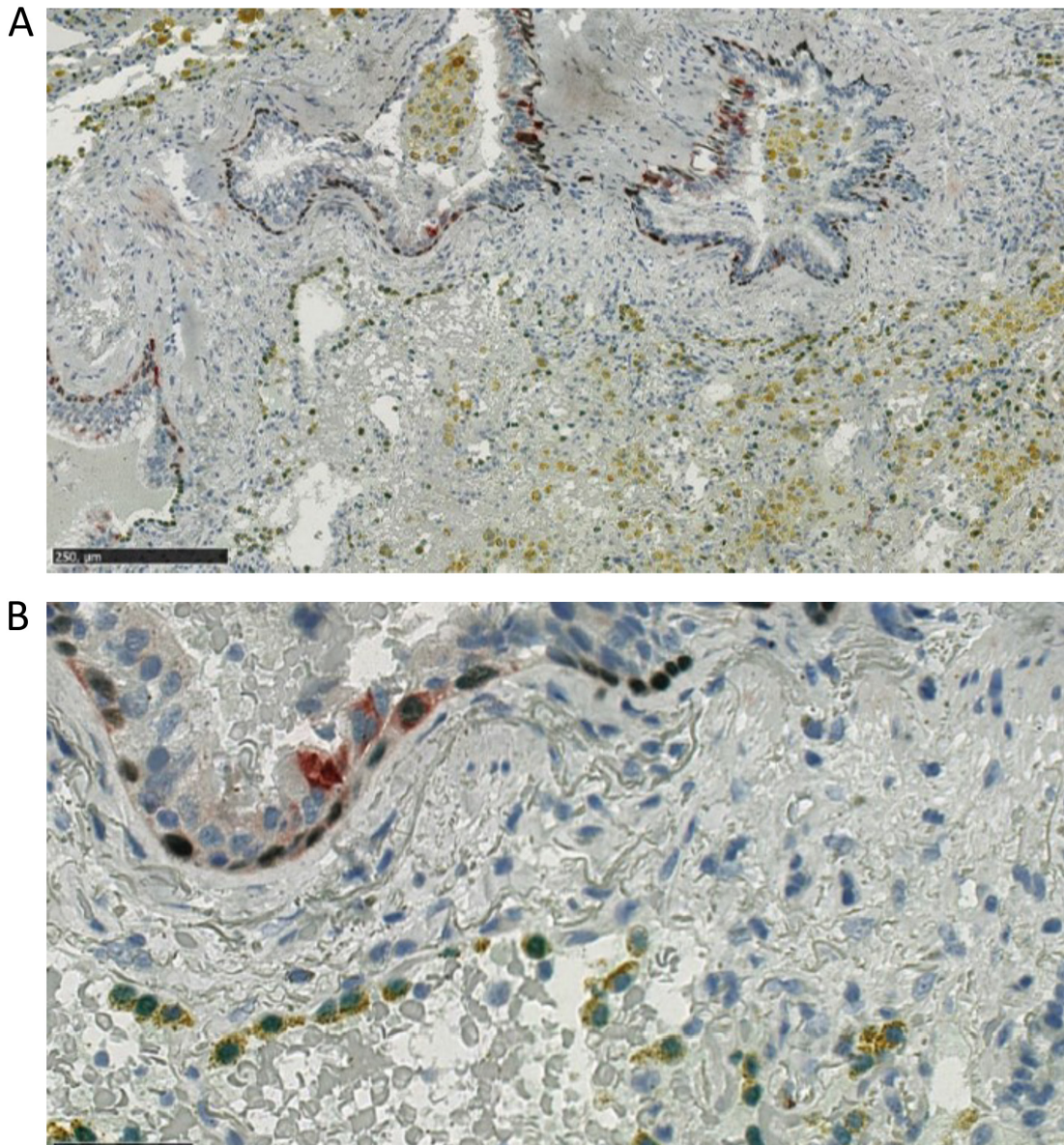
- **HIGHDEF® Blue IHC chromogen (HRP):** 1 mL of the HIGHDEF® Black IHC chromogen (HRP) substrate buffer was added to a mixing bottle and 20 µL of concentrated HIGHDEF® Black IHC chromogen (HRP) solution were added. The solution was mixed and allowed to reach RT before using. For optimal staining, the solution should be made fresh.
- **HIGHDEF® Green AP chromogen/substrate:** A 1 mL solution was prepared containing 920 µL of HIGHDEF® Green AP Substrate buffer and 80 µL of HIGHDEF® Green AP Chromogen. The solution was mixed well and protected from bright light. At room temperature, this solution is stable for up to 6 hours.
- **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.

## FOUR-COLOR IHC STAINING PROTOCOL

Consecutive FFPE tissue sections 3 µm in thickness were prepared and left for a minimum of one hour in an oven set at 60° C. Staining was performed on a Leica Bond RX automatic slide stainer at room temperature. Leica's wash buffer was used throughout the protocol. Sections were dewaxed in xylene, rehydrated through graded ethanol solutions, and washed in deionized water. Antigen retrieval was performed at pH 9.0 with the Antigen Retrieval Solution for 20 minutes at 99° C. Quenching of endogenous peroxidases was achieved with the ISH/IHC Peroxidase Block for 5 minutes at room temperature. Sequential immunostaining was then performed as follows: 30-minute incubation with the primary antibody, 20-minute incubation with the MULTIVIEW® PLUS or POLYVIEW® PLUS detection reagent, and 10-minute incubation with the HIGHDEF® IHC chromogen. Antibody removal was executed in between each staining cycle. Finally, tissue sections were counterstained for one minute using Enzo's hematoxylin, cleared, and mounted with HIGHDEF® IHC mount without dehydration. Images were captured on a fully automated large capacity slide scanner.

## RESULTS

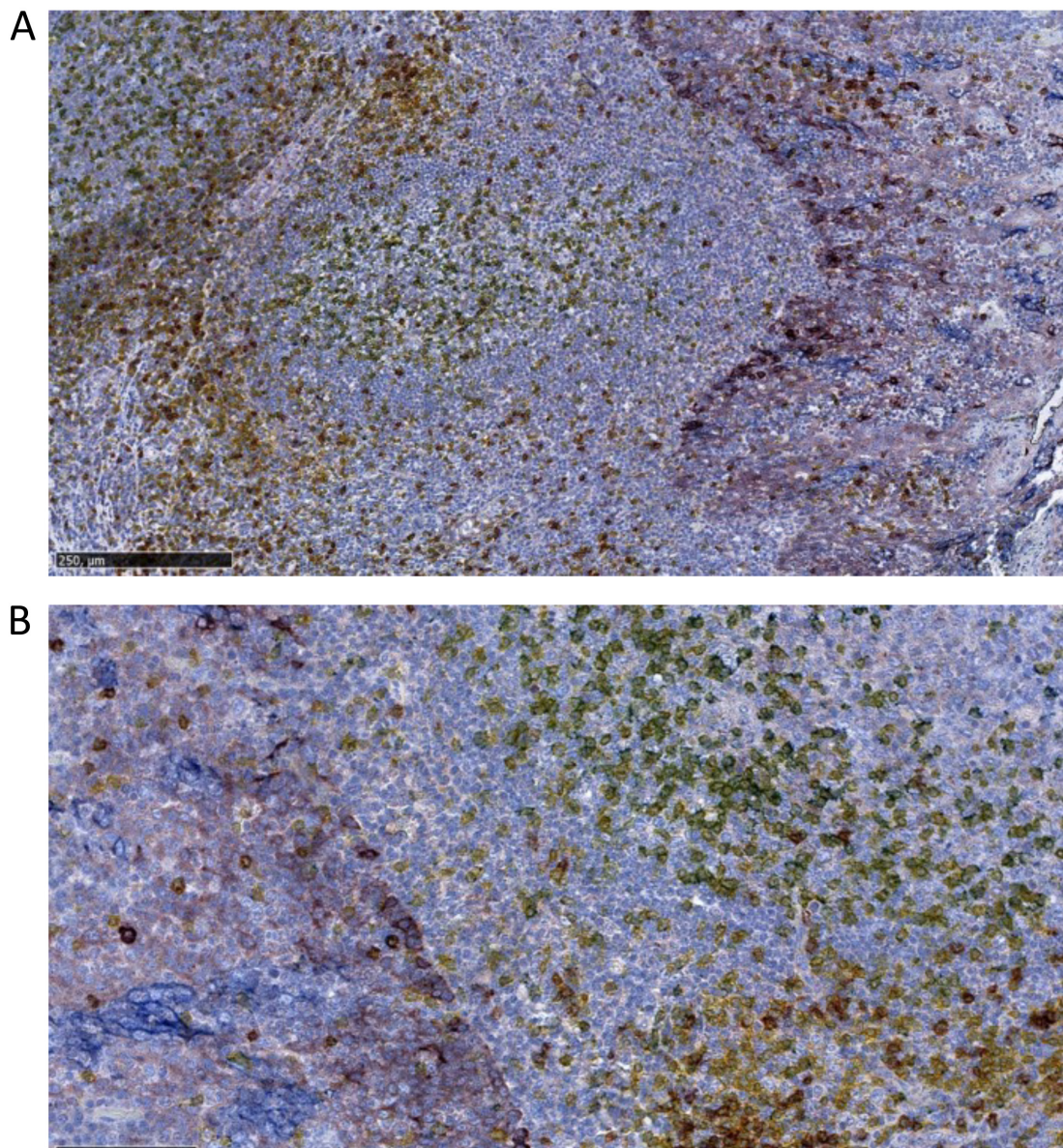
The first panel was composed of phenotypic markers useful in the diagnosis of lung cancers: CK5/6, Napsin A, p63, and TTF-1 (Sethi *et al.*, 2012). **Figure 1A** demonstrates the utility of the multiplex in spatial organization of the targets. The higher magnification used in **Figure 1B** illustrates the advantage of a multiplex IHC approach in co-localization analysis. For example, cells co-expressing Napsin A in the cytoplasm (yellow) and TTF-1 in the nucleus (green) can be easily identified.



**Figure 1:** PreMultiplex IHC staining on tissue section from human lung adenocarcinoma using panel 1. Simultaneous detection by IHC of CK5/6 (red), Napsin A (yellow), p63 (black), and TTF-1 (green) (A: 250µm scale bar; B: 50µm scale bar).

# APPLICATION NOTE

The second panel included CD3 and CD8 to identify cytotoxic T lymphocytes and the checkpoint inhibitors, programmed cell death protein-1 (PD-1) and its ligand (PD-L1) (Zou *et al.*, 2016). **Figure 2A** demonstrates the utility of the multiplex in spatial organization of the targets. The higher magnification in **Figure 2B** illustrates the advantage of this approach in co-localization analysis. For example, cells co-expressing CD3 (yellow) and CD8 (red) on the membrane of cytotoxic T cells can be easily identified.



**Figure 2:** Multiplex IHC staining on tissue section from human tonsil using panel 2. Simultaneous detection by IHC of CD3 (yellow), CD8 (red), PD-1 (green), and PD-L1 (blue) (A: 250µm scale bar; B: 100µm scale bar).

## CONCLUSION

The purpose of this work was to design panels, which would aid in the diagnosis and phenotyping of tumors with Enzo's chromogenic kits in a multiplex protocol. The two protocols were successful in specifically detecting the targets with strong intensity and limited background. With Enzo's chromogenic tools, Histalim can now offer pathological analysis of an automated multiplex chromogenic IHC to its customers.

## Testimonial

"A multiplex with Enzo's detection reagents and chromogens allows for all of the targets and their distribution in the complex tumor context to be contemporarily assessed easily by the observer on the same slide without any need for expensive digital imaging. Its utility is particularly demonstrated in Figure 1 where a lung adenocarcinoma biopsy shows specific staining for TTF-1 and Napsin A. The co-localization of these markers in parallel with p63 and CK5/6 staining allows for a differential diagnosis correlating with an adenocarcinoma phenotype (TTF-1+/Napsin A+; p63-/CK5/6-) versus lung squamous cell carcinoma (TTF-1-/Napsin A -; p63+/CK5/6+)" – Domenico Lazzaro, MD, PhD, Histalim Pathologist.

## References

Sethi S, *et al.* (2012) Dual color multiplex TTF-1 + Napsin A and p63 + CK5 immunostaining for subcategorizing of poorly differentiated pulmonary non-small carcinomas into adenocarcinoma and squamous cell carcinoma in fine needle aspiration specimens. *Cytojournal*. 9, 10.

Zou W, *et al.* (2016) PD-L1 (B7-H1) and PD-1 pathway blockade for cancer therapy: Mechanisms, response biomarkers, and combinations. *Sci. Transl. Med.* 8, 328.

Visit [www.enzolifesciences.com/IHC](http://www.enzolifesciences.com/IHC) for more information including:

- References
- Cited Samples
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# APPLICATION NOTE

## NOTES



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