Navinci

APPLICATION NOTE

High-throughput fully automated *in situ* proximity ligation assays for spatial interactomics

NaveniBright™ and Naveni® PD1/PD-L1 meet BOND RX/RXᵐ

Introduction

The Naveni® *in situ* proximity ligation assays (isPLAs) are well-established tools for the study of protein-protein interactions (PPIs), post-translational modifications (PTMs) and the ultrasensitive detection of single proteins in tissue samples, which together form the foundations of spatial interactomics. isPLA is an immuno-based technology which detects closely located epitopes via antibodies conjugated to oligonucleotides that generate amplified fluorescent or chromogenic signal. Here, we introduce the fully automated version of the NaveniBright™ and Naveni® PD1/PD-L1 kits with HRP-based readout, optimized and validated for use on BOND RX and RXᵐ. The power of spatial interactomics provided by Navinci meets the speed, reliability, and robustness of the Leica autostainers, thus shortening the workflow and increasing throughput without compromising quality.

NaveniBright BOND RX HRP

NaveniBright BOND RX is a flexible isPLA kit for use on FFPE tissue or cell sections with an HRP-based chromogenic readout on the BOND RX/RXᵐ Fully Automated Research Stainer (Fig. 1). Two unlabeled primary antibodies (supplemented by user) against different epitopes of the same protein or two potentially interacting proteins are incubated on FFPE samples. Navenibodies – secondary antibodies conjugated to proprietary oligonucleotide arms – hybridize to the target-specific antibodies. Only if the detected epitopes are within approximately 10-40 nm will the oligo arms

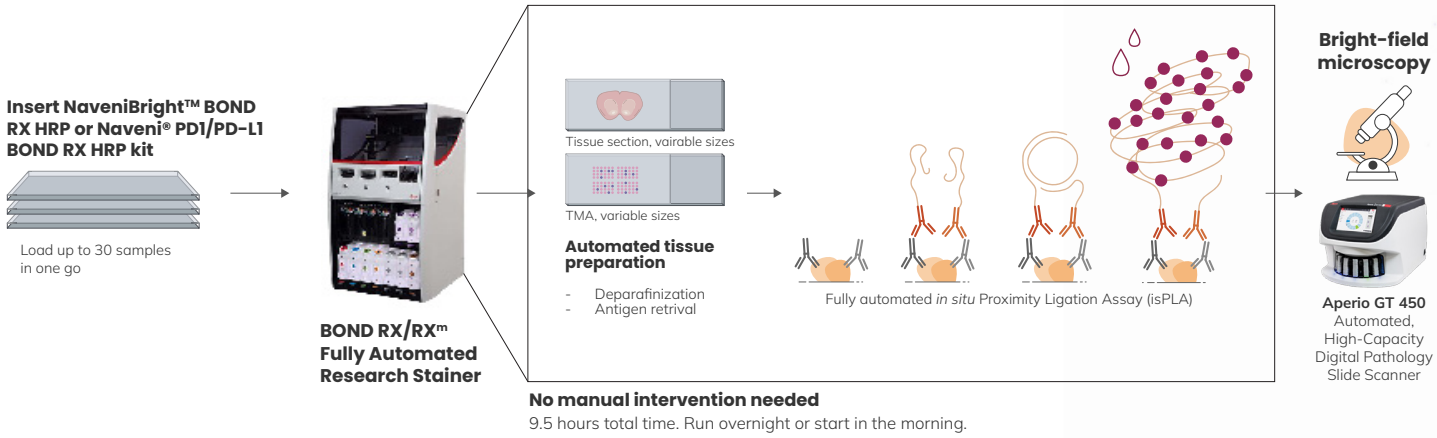
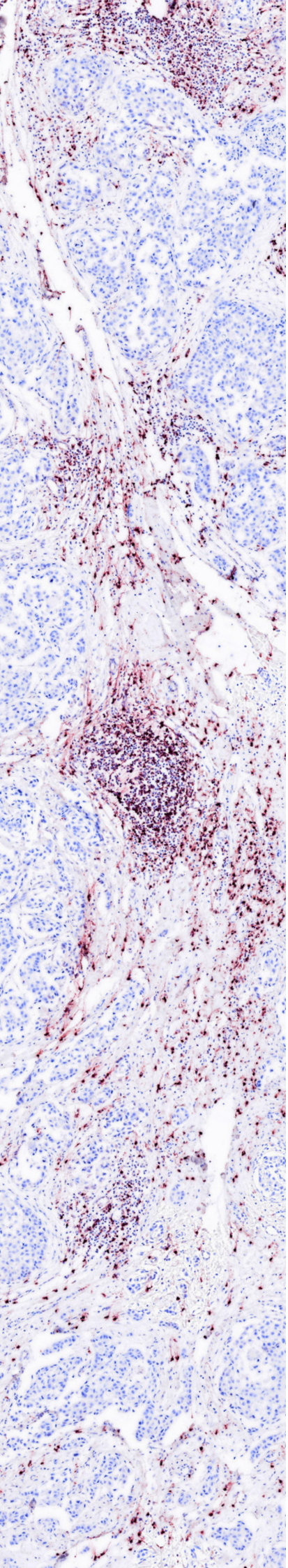


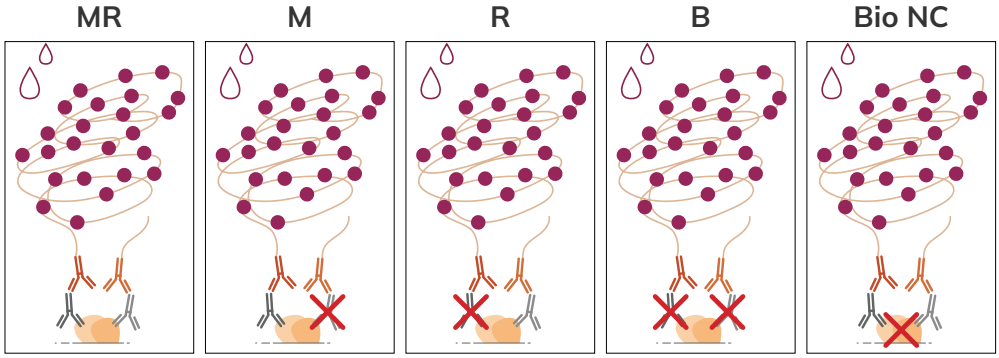
Fig. 1. NaveniBright BOND RX HRP usage on the BOND RX/RXᵐ Fully Automated Research Stainers. Multiple samples and assays can be run in the instrument simultaneously, saving time and eliminating the need for manual intervention. A standard template for NaveniBright is now available as part of the BOND RX/RXᵐ software, which allows for an effortless, fully automated execution of the isPLA protocol.

Version 1.0
Uppsala June 4, 2024



interact with one another through enzyme-driven reactions to form a circular DNA complex. The circle sequence is then enzymatically amplified hundreds of times, resulting in a long ssDNA “nest”. This repetitive sequence is visualized via hybridization with labeled detection oligos, producing a strong HRP-based chromogenic signal. All steps can be performed in a fully automated fashion in the BOND RX/RX^{m} Fully Automated Research Stainer. The NaveniBright BOND RX HRP kit contains reagents sufficient to stain 30 slides, at a maximum of 4 runs. The stained slides can be imaged with any brightfield microscope or scanner.

Controls and abbreviations used in this application note



MR One mouse and one rabbit primary antibody are present in the isPLA reaction. Each one targets one of the respective interaction partners, resulting in isPLA signals in a tissue positive for the assayed interaction, single protein or PTM.

M Only the mouse primary antibody is present in the isPLA reaction, resulting in no signal, independent of tissue and the presence or absence of the targeted protein(s).

R Only the rabbit primary antibody is present in the isPLA reaction, resulting in no signal, independent of tissue and the presence or absence of the targeted protein(s).

B No primary antibodies are present in the isPLA reaction, resulting in no signal, independent of tissue.

Bio NC One mouse and one rabbit primary antibody are present in the isPLA reaction in a tissue negative for the interaction (biological negative control), resulting in no signal.

Validation of isPLA on BOND RX/RX^{m}

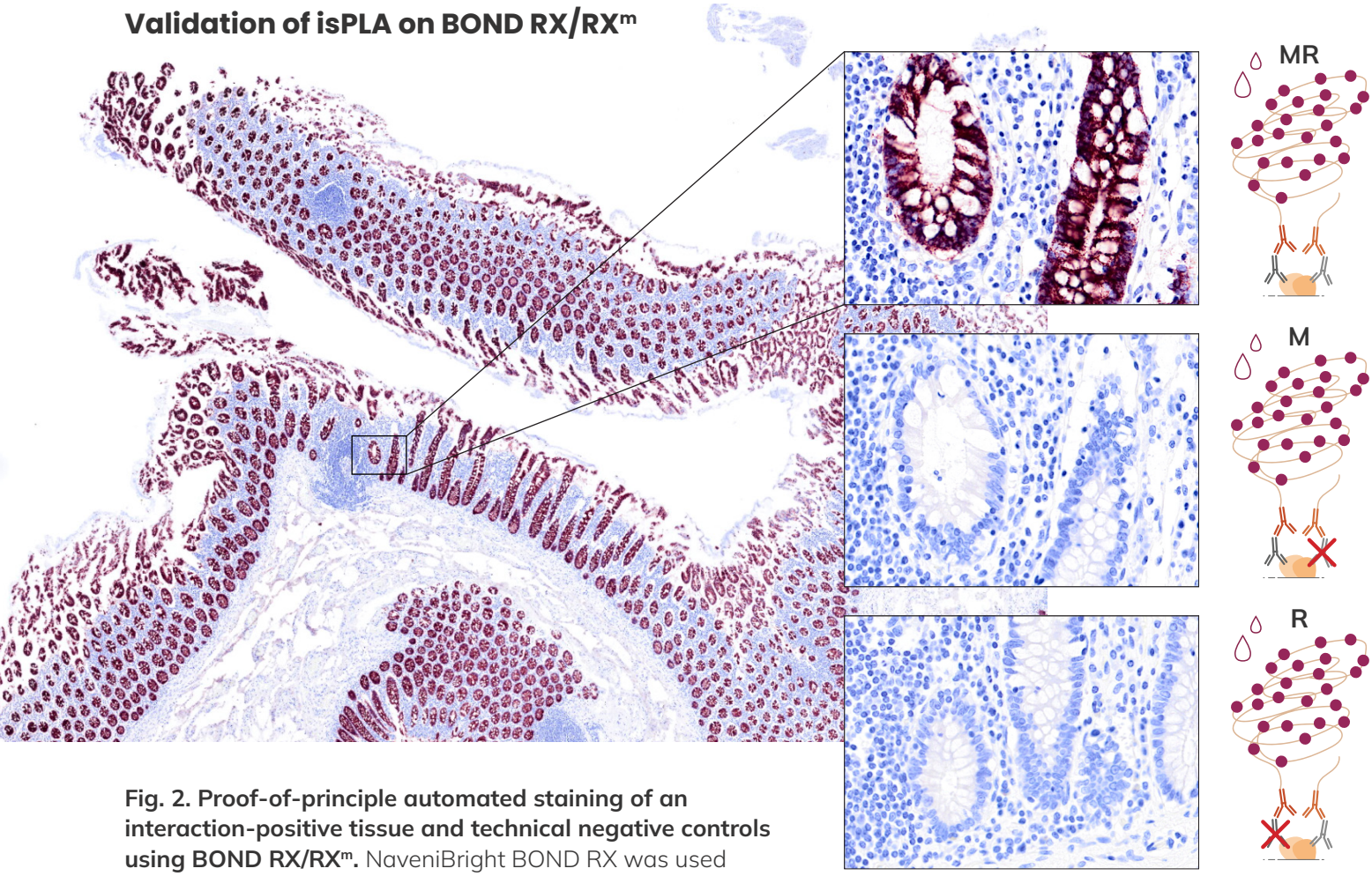


Fig. 2. Proof-of-principle automated staining of an interaction-positive tissue and technical negative controls using BOND RX/RX^{m}. NaveniBright BOND RX was used to detect the E-cadherin/β-catenin interaction which is highly abundant in human colon FFPE tissue. Specificity and background were demonstrated by removing one of the primary antibodies (zoom-in MR vs technical negative controls M and R) in consecutive colon tissue sections. Strong and specific staining was observed in the MR reaction, whereas negative controls were blank.

Fig. 3A

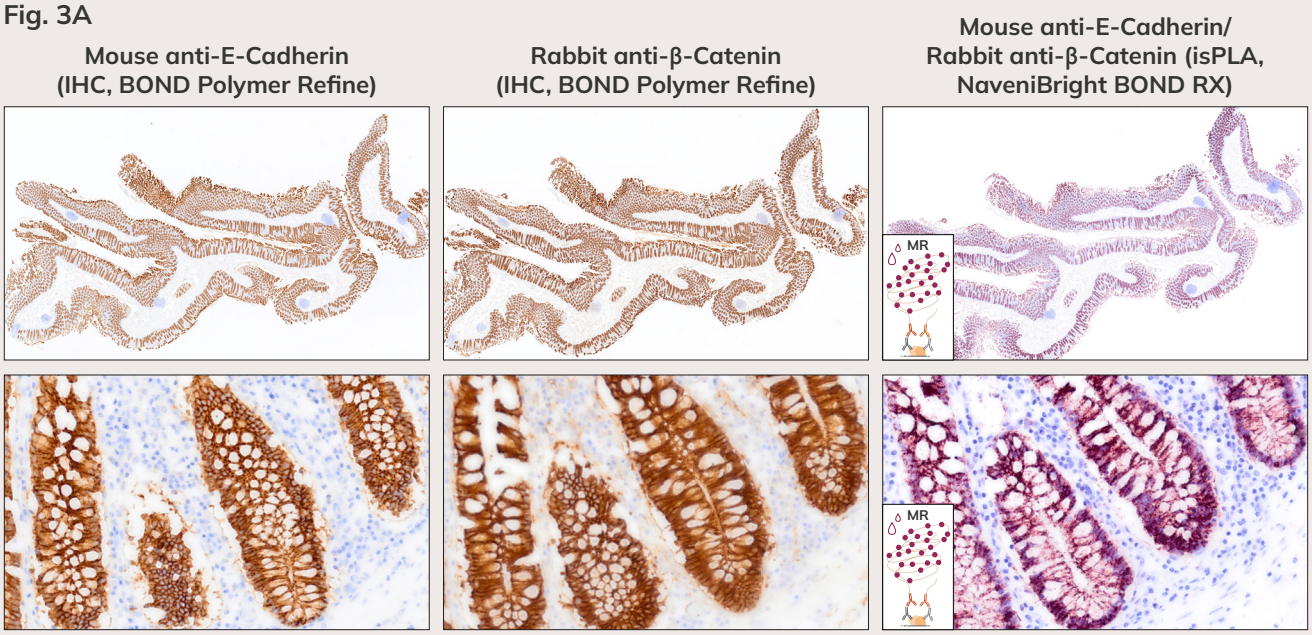


Fig. 3B

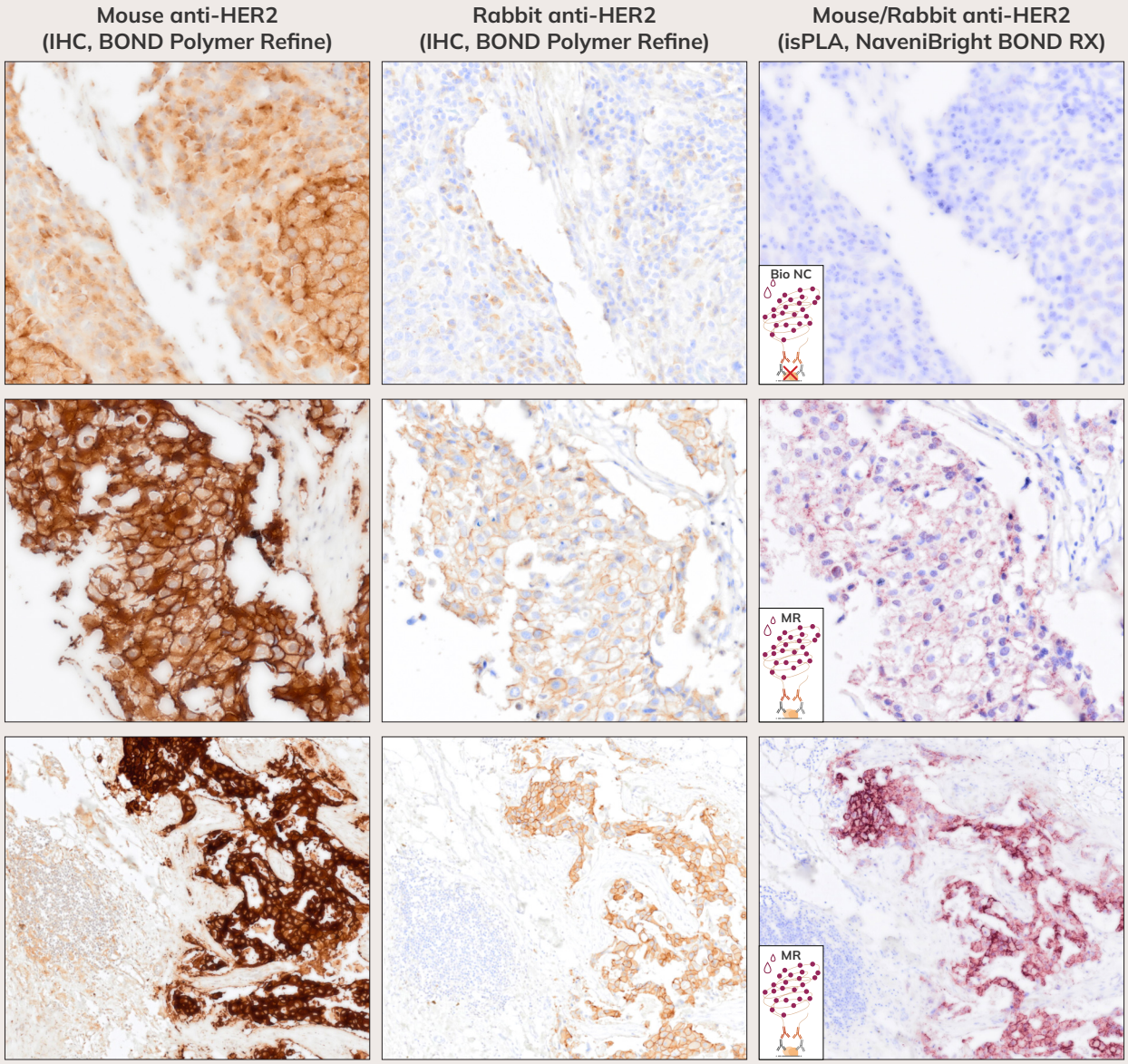
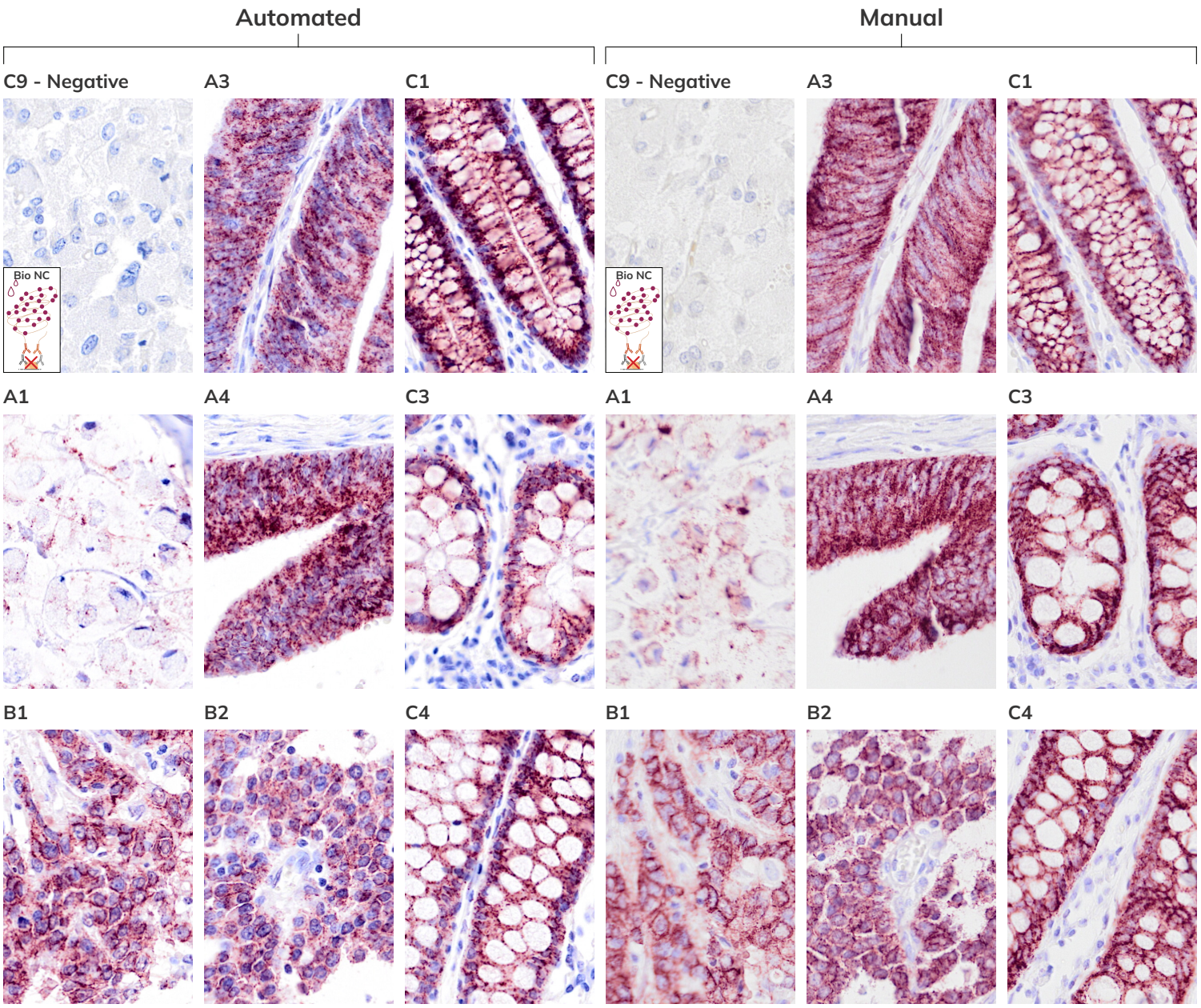


Fig. 3. Performance comparison of BOND RX/RX^m-automated isPLA for detection of interactions and single proteins with immunohistochemistry (IHC).

(A) IHC (BOND Polymer Refine) and isPLA (NaveniBright BOND RX) were performed on consecutive sections of colon FFPE tissue positive for E-cadherin and β-catenin using the BOND RX/RX^m Fully Automated Research Stainer. The interaction was captured with the isPLA kit and compared to the individual expression of E-cadherin and β-catenin. Antibody concentrations and exposure times were matched across experiments and samples. Strong and specific staining was observed with both methods, with similar distribution patterns throughout the tissue, and with particular enrichment in adherens junctions.

(B) IHC (BOND Polymer Refine) and isPLA (NaveniBright BOND RX) were performed on breast cancer (invasive ductal carcinoma) FFPE tissue cores, which were pre-scored for HER2, and on a whole slide breast cancer positive for HER2 using the BOND RX/RX^m Fully Automated Research Stainer. In this single protein detection assay, isPLA demonstrated increased sensitivity and specificity compared to IHC, evidenced by the lack of background in the HER2-negative core and by the cleaner staining in the HER2-positive samples. Signal localization was consistent between the isPLA and the IHC stains.



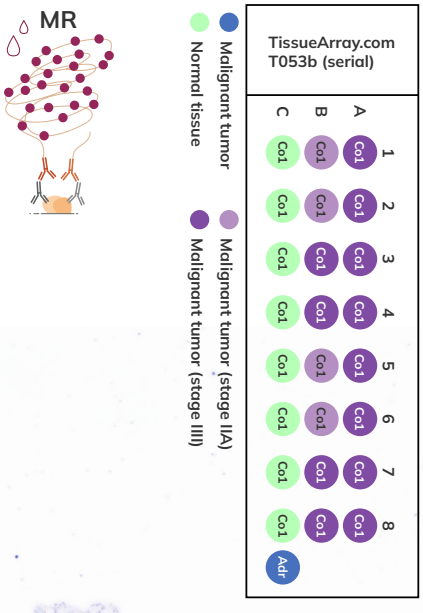


Fig. 4. Performance comparison between manual and BOND RX/RX^m-automated isPLA. A tissue microarray (TMA) containing normal and cancer tissues was stained for the E-cadherin/β-catenin interaction with NaveniBright BOND RX ("Automated") and NaveniBright ("Manual"). Antibody concentrations were matched across experiments. The biological negative controls (individually indicated with the Bio NC icon) were free from staining. For both healthy and cancerous interaction-positive tissues, the intensity and localization of the staining were comparable, demonstrating that the automated isPLA method can consistently and specifically stain tissues and performs as well as the manual counterpart.

Robustness of isPLA automated on BOND RX/RX^m

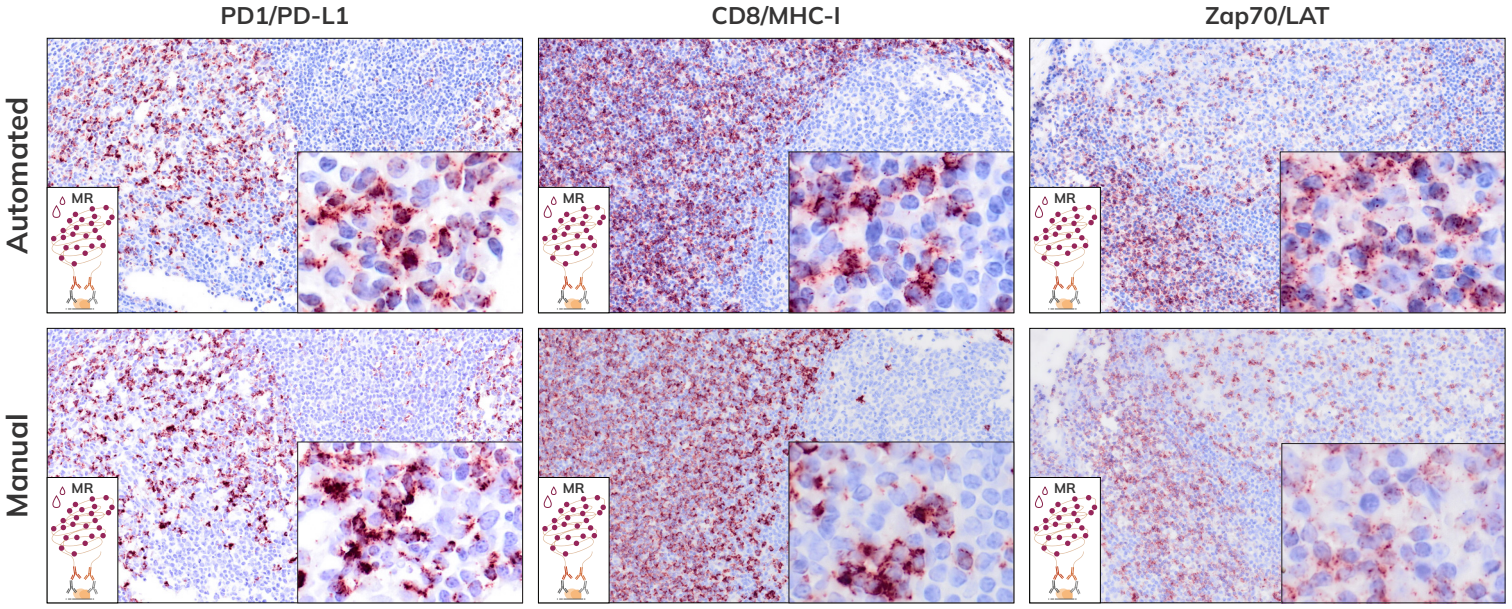


Fig. 5. BOND RX/RX^m-automated vs manual detection of various PPIs with NaveniBright on normal FFPE tonsil tissue. Three different PPIs – the well-known immune checkpoint interaction PD1/PD-L1, the immune interaction between CD8 and MHC-I on T lymphocytes, and the Zap70/LAT interaction downstream of the T cell receptor activation – were detected using both the manual and the BOND RX/RX^m-automated NaveniBright kits in tonsil, which is a positive tissue for all assessed interactions. All staining results were strong and comparable, with minimal variation across assays, demonstrating the ability of the NaveniBright BOND RX kit to detect different interactions with equally reliable outcomes.

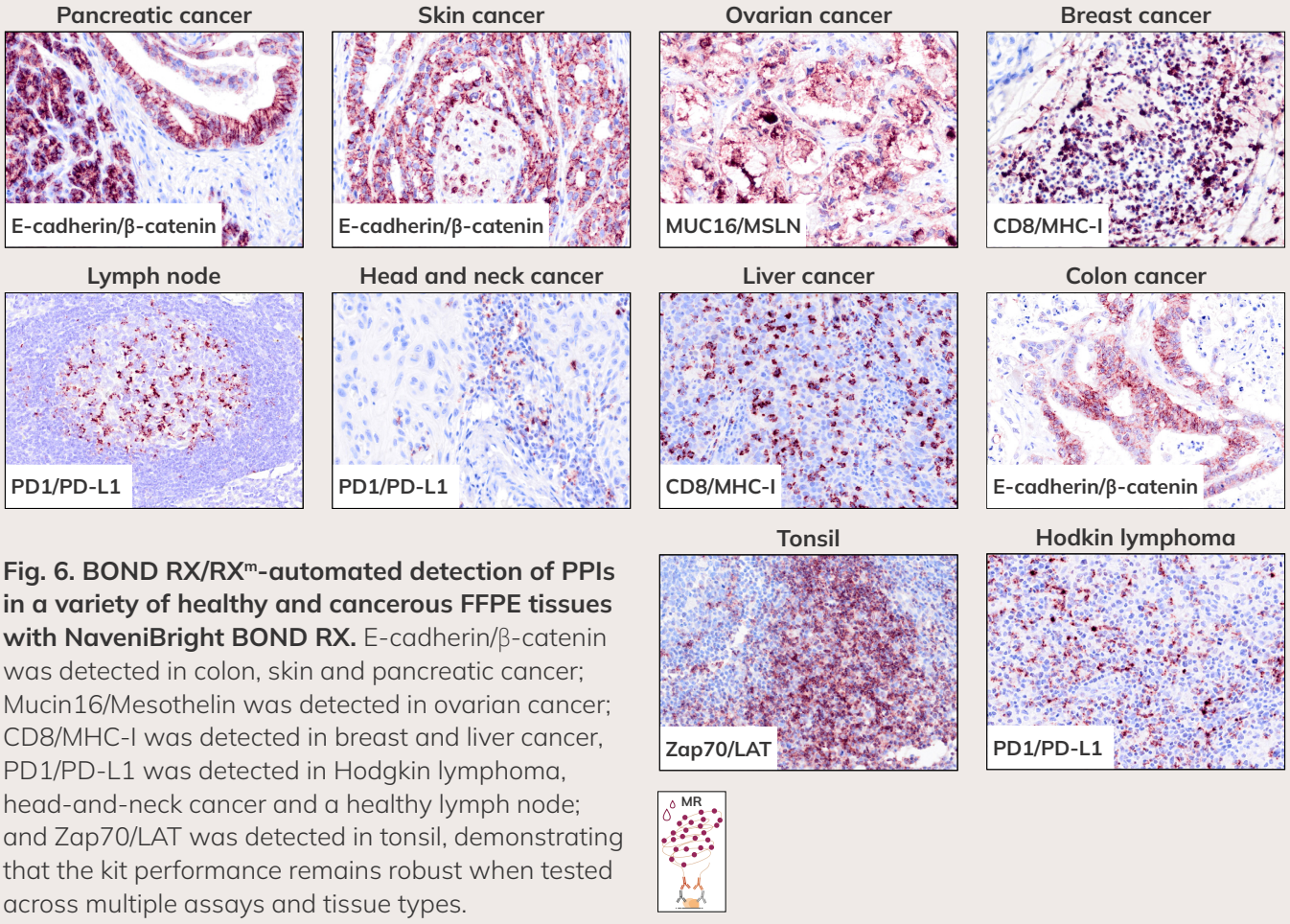


Fig. 6. BOND RX/RX^m-automated detection of PPIs in a variety of healthy and cancerous FFPE tissues with NaveniBright BOND RX. E-cadherin/β-catenin was detected in colon, skin and pancreatic cancer; Mucin16/Mesothelin was detected in ovarian cancer; CD8/MHC-I was detected in breast and liver cancer, PD1/PD-L1 was detected in Hodgkin lymphoma, head-and-neck cancer and a healthy lymph node; and Zap70/LAT was detected in tonsil, demonstrating that the kit performance remains robust when tested across multiple assays and tissue types.

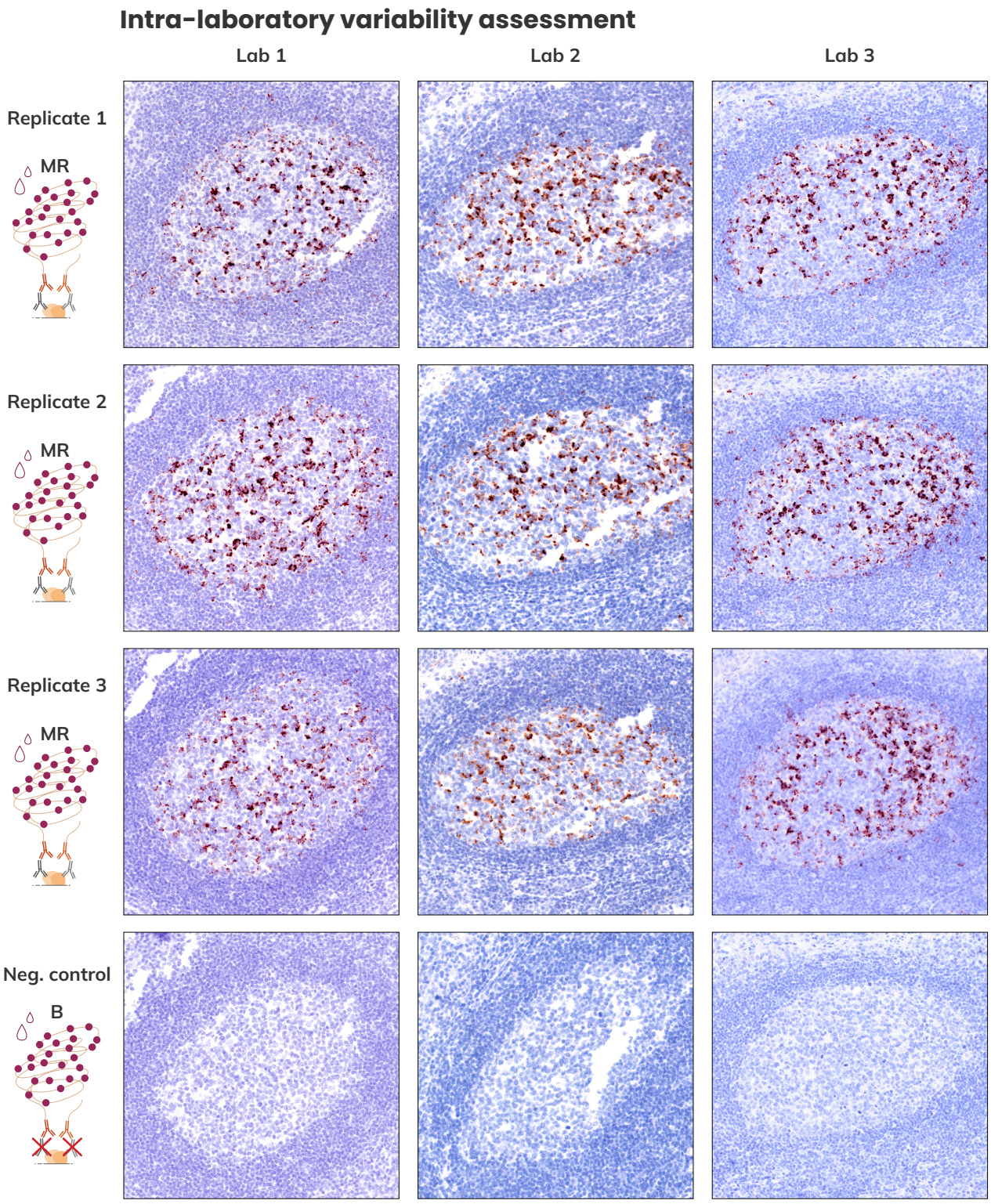


Fig. 7. Assessment of the variability of BOND RX/RX^m-automated isPLA staining between different laboratories and replicates. isPLA experiments were conducted on BOND RX/RX^m in triplicate in three independent facilities, using the same conditions to detect the PD1/PD-L1 interaction in consecutive sections from a human FFPE tonsil tissue block. The kits performed similarly across experiments and laboratories, demonstrating reliable and consistent staining, independent of the user, microscope, or replicate.

Naveni® PD1/PD-L1 BOND RX HRP

Naveni® PD1/PD-L1 BOND RX HRP is a target-specific isPLA kit for the detection of the PD1/PD-L1 immune checkpoint activation. It is intended for use on FFPE tissue or cell sections

and provides an HRP-based chromogenic readout. The workflow is identical to that of the NaveniBright BOND RX (see Fig. 1), with the only difference being that mouse anti-PD1 and rabbit anti-PD-L1 primary antibodies are provided in optimized concentrations in the kit. The kit contains reagents sufficient to stain 30 slides, at a maximum of 4 runs. The Naveni® PD1/PD-L1 BOND RX protocol has been optimized for BOND RX/RX^{m}. The stained slides can be imaged with any brightfield microscope or scanner.

Validation of Naveni® PD1/PD-L1 BOND RX on BOND RX/RX^{m}

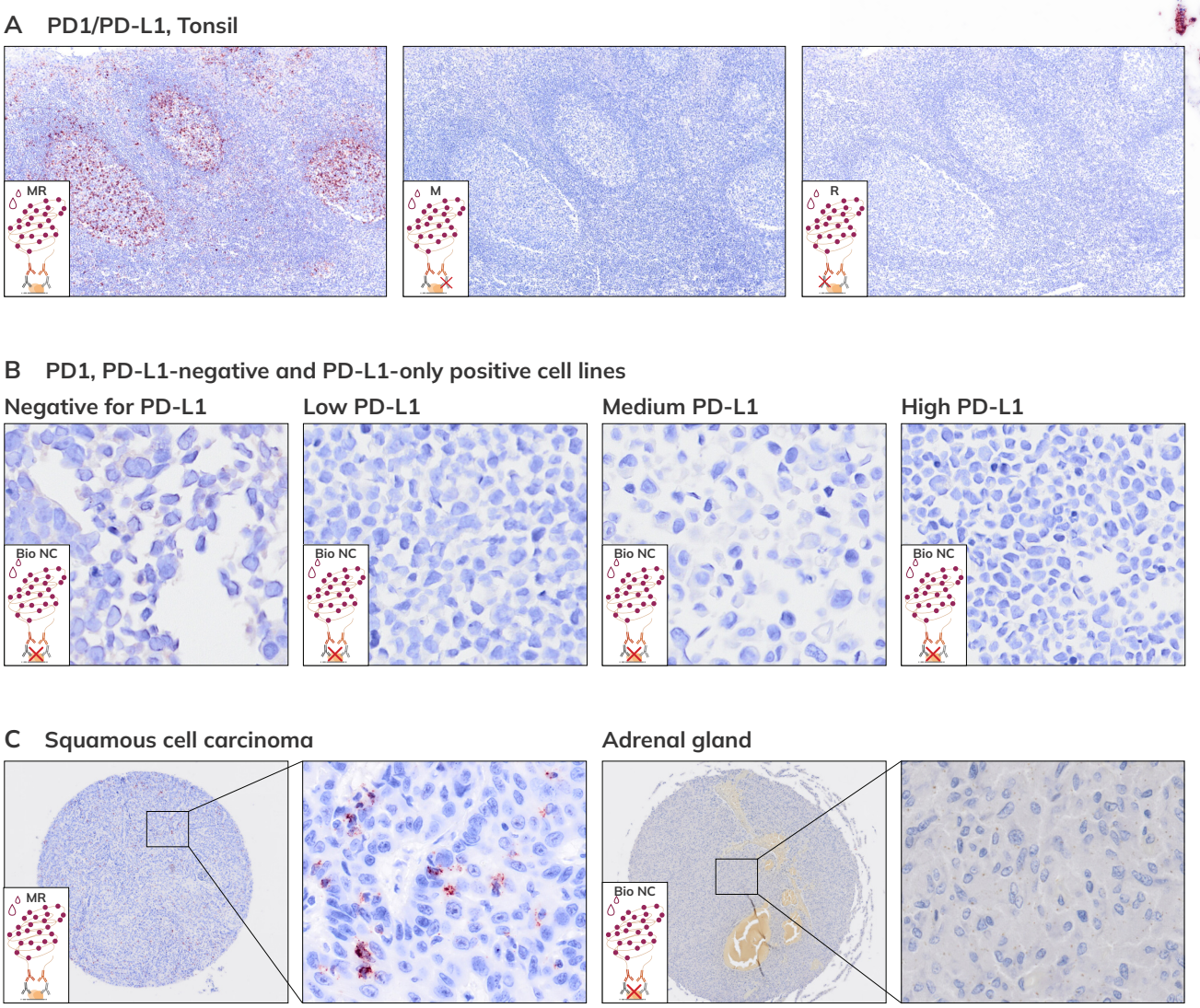


Fig. 8. Automated PD1/PD-L1 staining of interaction-positive tissues and negative controls on BOND RX/RX^{m}. (A) Naveni® PD1/PD-L1 BOND RX was used to detect PD1/PD-L1 in human tonsil FFPE tissue, a positive control for the interaction. Specificity and background were assessed by removing one of the primary antibodies (MR vs technical negative controls M and R). Strong and specific staining was observed in the MR reaction, whereas the negative controls were blank. (B) Naveni® PD1/PD-L1 BOND RX was used to detect PD1/PD-L1 in four negative control cell lines which either did not express PD1 and PD-L1, or only expressed PD-L1 to varying degrees. No interaction was detected in any of the cell lines, confirming the specificity of the assay. (C) An interaction-positive (head-and-neck cancer, squamous cell carcinoma) and an interaction-negative (adrenal gland) FFPE tissue were compared after staining with the Naveni® PD1/PD-L1 BOND RX. Clear positive staining was observed in the cancer tissue, whereas in the biological negative control no PD1/PD-L1 interaction was detected.

Comparison of Naveni® PD1/PD-L1 BOND RX with Leica BOND Polymer Refine IHC

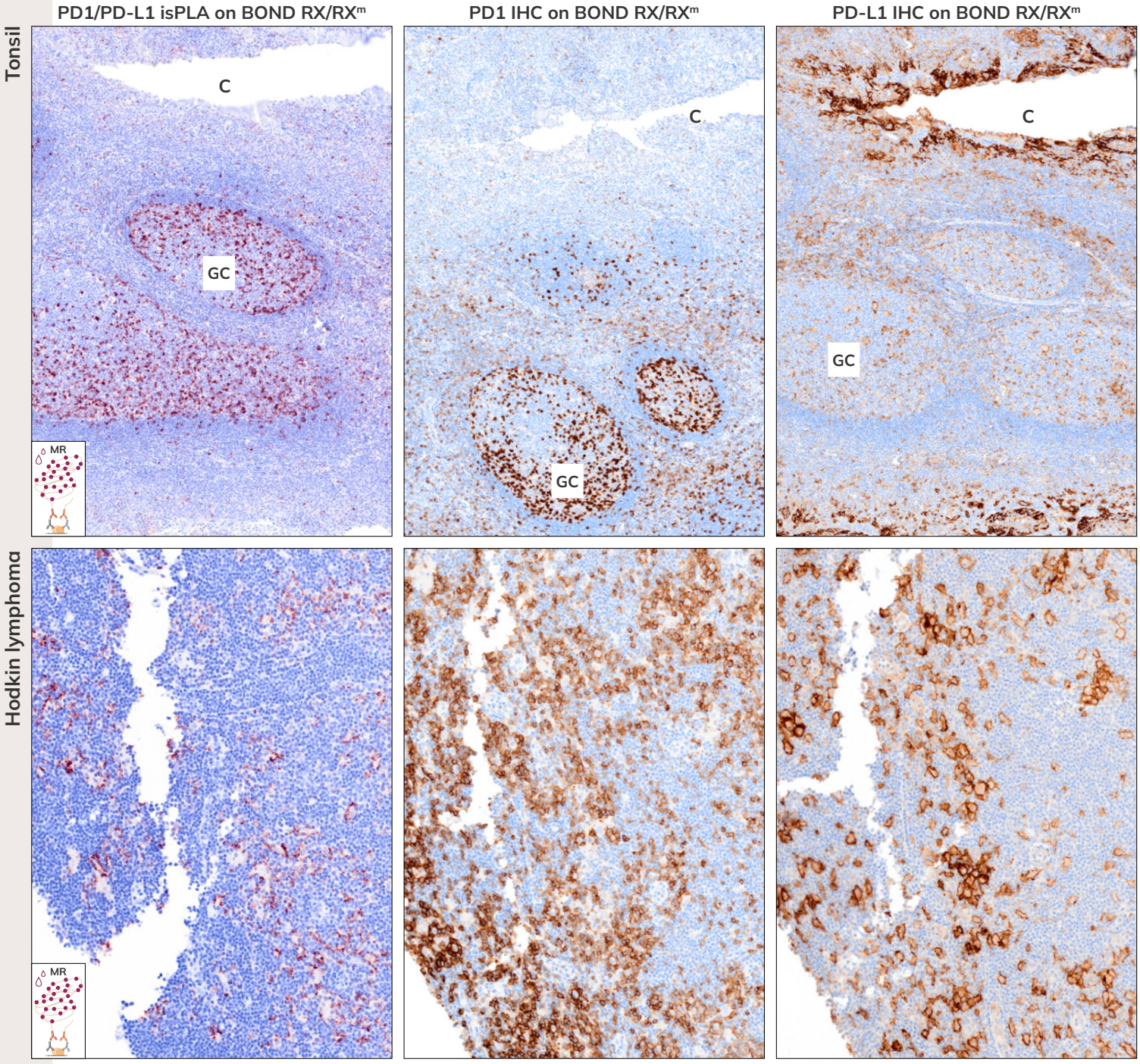


Fig. 9. Performance comparison of isPLA with IHC on BOND RX/RXm. Naveni® PD1/PD-L1 BOND RX staining on tonsil and Hodgkin lymphoma FFPE tissues was compared to IHC (BOND Polymer Refine) using the same primary antibodies, all on BOND RX/RXm. Antibody concentrations and exposure times were matched across experiments and samples. GC = germinal center, C = crypt. In Hodgkin lymphoma, PD1/PD-L1 interactions were observed around the same regions where the proteins were expressed individually as detected by IHC. In tonsil, PD1/PD-L1 was mostly detected in the germinal centers. This was consistent with PD1 expression as detected by IHC, whereas PD-L1 IHC staining was strongest around the crypt. These observations suggest that individual protein expression does not always have biological relevance, and that PD-L1 expression is not synonymous with the activation of the immune checkpoint. Furthermore, individual proteins may be expressed to different degrees and without complete overlap in subcellular/tissue localization, whereas interaction between them is limited to specific areas and is always less abundant than the individual protein partners.

Automated Naveni® PD1/PD-L1 BOND RX performance is comparable to the manual method

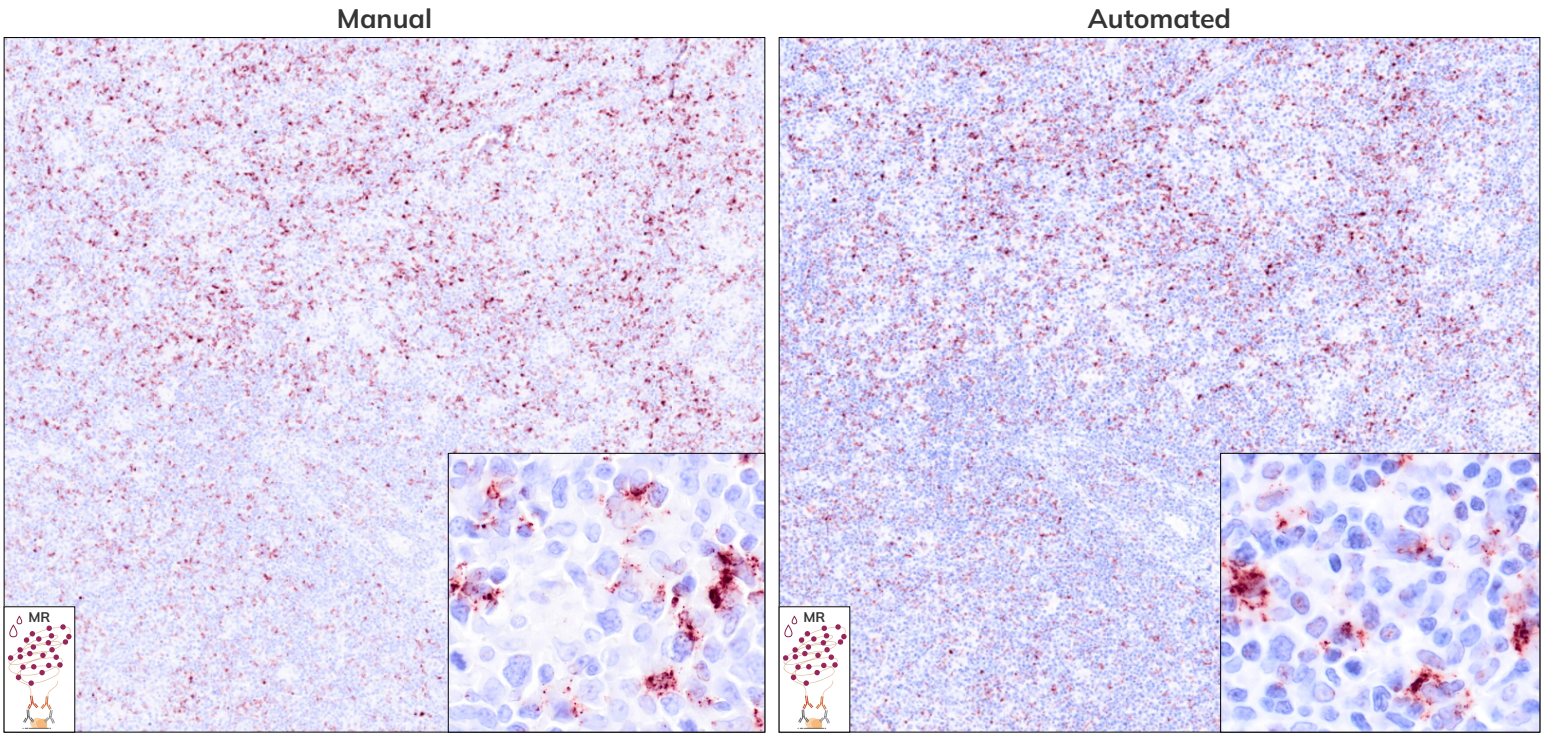
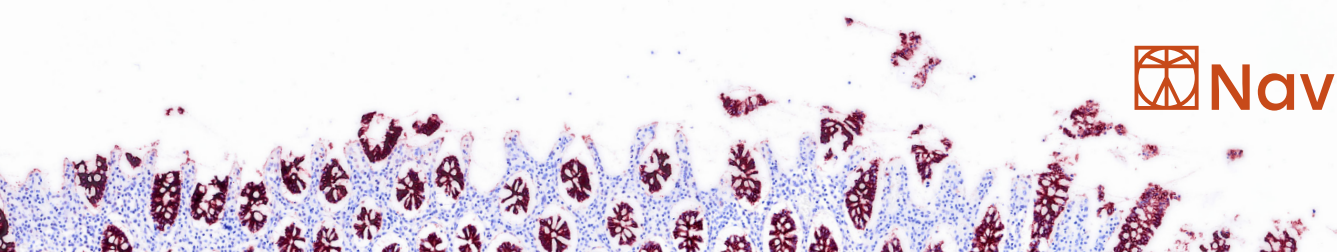


Fig. 10. Comparison of the performance of manual vs automated isPLA. Consecutive FFPE sections from an interaction-positive Hodgkin lymphoma tissue block were stained for the PD1/PD-L1 interaction with Naveni® PD1/PD-L1 BOND RX (“Automated”) and Naveni® PD1/PD-L1 (“Manual”). Antibody concentrations were matched across experiments. The intensity and localization of the staining were comparable, demonstrating that the automated isPLA method performs as well as the manual one.

Conclusion

Both the NaveniBright™ kit and the Naveni® PD1/PD-L1 kit demonstrated stable and comparable performance when used manually and when automated on the BOND RX/RX^{m} Fully Automated Research Stainer. The kits were successfully implemented at different laboratories, showing user-friendliness with consistent results and minimal optimization needed. The NaveniBright BOND RX kit can be used to detect an assortment of targets, with possible applications in basic research, drug development, cancer biology, neuroscience and many others. Naveni® PD1/PD-L1 BOND RX is a specialized version of NaveniBright BOND RX, which specifically detects the PD1/PD-L1 interaction on the BOND RX/RX^{m} Fully Automated Research Stainers. It is suitable for studying clinical samples and comparing standard patient stratification methods to the novel interactomics approach. It is a tool for both studies on autoimmunity and for immunooncology research, providing a better understanding of the TME and the activation/silencing of immune responses to tumors. The possibility to study PPIs in their native environment is now expedited by streamlining the protocol and allowing for the simultaneous handling of multiple slides with no manual input on the BOND RX/RX^{m} Fully Automated Research Stainers.

For research use only. Not for use in diagnostic procedures.



Navinci