Use of Vector Laboratories ImmPRESS® Polymer IHC Detection Reagents on Open Automated Staining Platforms

Methodology and Comparison with Other Detection Systems

Introduction

Vector Laboratories ImmPRESS Polymer Kits use a proprietary conjugation chemistry that integrates purified secondary antibody with highly reactive enzyme to enable one step immunohistochemistry (IHC) detection. This approach delivers more enzyme at the target site and generates higher sensitivity over other systems, especially when combined with Vector® ImmPACT® substrates. The ImmPRESS reagents are supplied as pre-diluted, ready-to-use solutions thereby streamlining IHC workflows, reducing protocol optimization, and providing unmatched convenience for tissue and cell-based staining applications.

Research labs and core facilities with high IHC work volumes are increasingly evaluating automated staining systems to help augment their processes and reduce labor commitments. Coupled with this move to automate IHC, these facilities are looking for flexibility in what reagents can be applied to the instruments as the demand for multiplex staining grows, and to meet research requirements with different species, xenograft and transgene tissues, and abnormal or tumorigenic specimens.

In response to customer requests we conducted studies to evaluate the suitability of our ImmPRESS HRP polymer reagent, ImmPACT® DAB substrate and associated IHC accessory components on three commercially available automated staining platforms. The studies were performed by a contracted independent third-party laboratory (BioIVT, PHASEZERO Research Services, UK) to eliminate potential bias, and staining assays were run in parallel with the vendor recommended reagents for each instrument.

Presented in this document are the protocols used on each system along with the corresponding staining results obtained. The data provided here should prove helpful for labs looking to apply Vector Laboratories polymer reagents and enzyme substrates on these, or similar, open automated staining platforms.

Materials and Methods

Each of the three automated staining platforms used (see Table 1) was an established instrument in the contracted testing lab and was operated by a trained lab technician, experienced in the use of each machine. The vendor recommended reagents for each instrument (see Table 1) were routinely being used on the corresponding platform prior to this study and were applied according to previously established procedures.

Vector Laboratories detection reagents (see Table 2) were applied to each instrument as direct substitutes for the equivalent vendor recommended reagent at the appropriate step of the IHC workflow. It should be noted that except for the ImmPACT DAB EqV substrate, the other reagents, including the ImmPRESS polymer reagent are supplied as pre-diluted, ready to use solutions. As such, no further dilution or modification of these reagents was required by the technician to apply these to each platform. The ImmPACT DAB EqV requires mixing a 1:1 ratio of the chromogen and diluent supplied in the kit to obtain a stable working solution that was loaded into each instrument.

For consistency and to reduce variables across the platforms, all IHC assays were performed using formalin-fixed, paraffin embedded (FFPE) tissue sections of human skeletal muscle with the same rabbit polyclonal primary antibody against von Willebrand factor (Sigma, F3520). A hematoxylin counterstain was used on all sections to aid in identification of tissue structures and confirm appropriate localization of the target antigen where required.

Table 1.

Automated Platform (Open Systems)	Vendor Recommended Detection Reagents
Agilent/Dako Autostainer Plus	EnVision™ FLEX Visualization System
Leica Bond Rx	Leica Bond™ Polymer Refine Detection System
Ventana Discovery Ultra	Ventana Discovery OmniMap™ Detection System

Table 2.

Vector Laboratories Detection Reagents	Product Catalog Numbers
BLOXALL® (HRP/AP enzyme quencher)	SP-6000
2.5% Normal Horse Serum	MP-7401 Component
ImmPRESS® HRP anti-rabbit IgG polymer	MP-7401
ImmPACT DAB EqV Substrate	SK-4103

Results

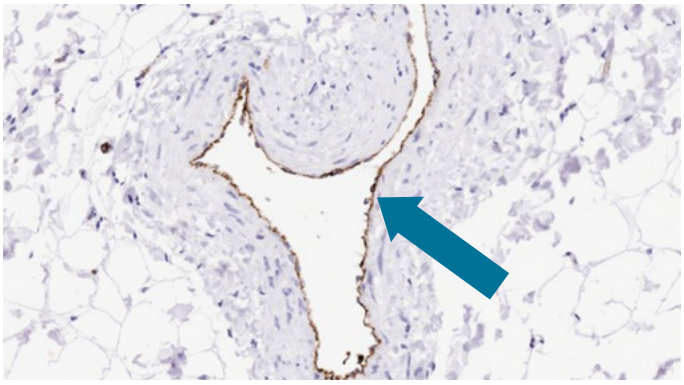
Specific procedures used on each automated platform are featured in Tables 3, 4 and 5. It should be noted that the procedural data for Vector Laboratories reagents shown in Tables 3 and 4 are parameters that were established following four and three rounds of complete staining runs on the Dako® Autostainer Plus and Leica Bond RX instruments respectively. Circumstances did not permit specific optimization of the IHC workflow on the Ventana Discovery Ultra instrument. The parameters for the Vector Laboratories reagents outlined in Table 5 therefore, are instrument program set conditions, and the staining obtained is presented in Figures 6 & 7.

Initial first and second round staining results on the Dako Autostainer Plus and Leica Bond Rx platforms indicated that the ImmPRESS Polymer and ImmPACT DAB reagents can easily be applied to these instruments and generate staining results similar to the vendor recommended reagents (Figure 1).

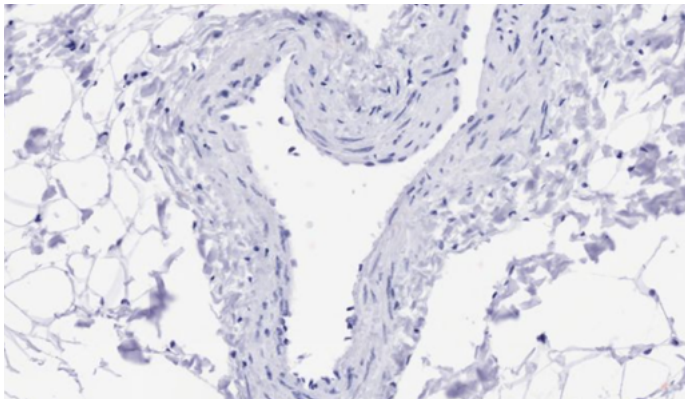
For each instrument, the Vector Laboratories reagents generated appropriate signal intensity, however background (non-specific) toning was higher than the vendor recommended reagents. From this data, it was clear that some modification of the procedure was required to optimize signal to noise ratios when applying Vector Laboratories reagents on each instrument.

Specific procedural modifications were performed on the Dako and Leica instruments to reduce background toning seen with the Vector Laboratories reagents. Incubation time of the ImmPRESS polymer reagent was reduced to 7.5 minutes, and the number of buffer washes immediately following this incubation was increased to five. These two modifications generated significantly improved signal to noise ratios. Evidence of these results is presented in Figures 2 & 3 on the Dako automated platform, and Figures 4 & 5 on the Leica automated platform.

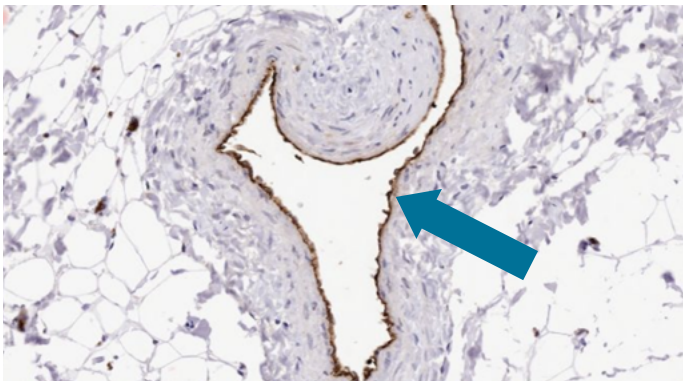
Dako EnVision Flex Detection
1 ug/ml primary antibody concentration



Negative Control – No Primary Antibody



Vector ImmPRESS Polymer/ImmPACT DAB Detection
1 ug/ml primary antibody concentration



Negative Control – No Primary Antibody

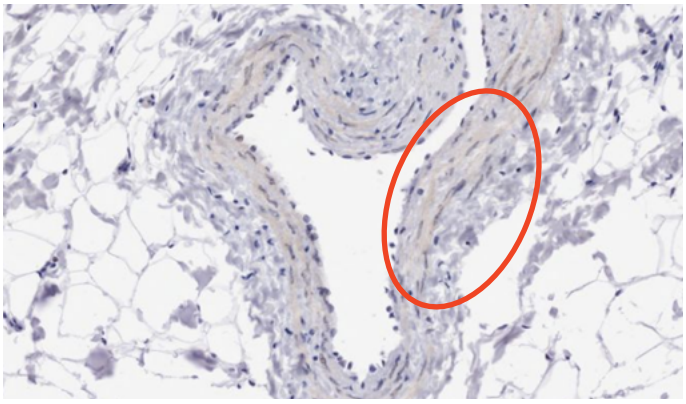


Figure 1: Initial staining results obtained for the Vector Laboratories detection reagents on the Dako Autostainer Plus instrument in comparison with Dako recommended reagents. While specific staining intensity (arrows, brown DAB) was greater using the Vector Laboratories reagents at the same primary antibody concentration, background staining was also higher (note brown toning, red oval).

Dako Autostainer Plus Platform Results

Table 3. Methodology using Dako Autostainer Plus

	Dako Autostainer Plus Program	
IHC Automated Workflow	**Modified Reagent Protocol**	**Vendor Recommended Protocol**
Antigen retrieval	PT Link 3 in 1 Module (Dewax/Retrieval)	PT Link 3 in 1 Module (Dewax/Retrieval)
Buffer Wash	Dako Envision Flex wash	Dako Envision Flex wash
Peroxidase Block	**Vector BLOXALL; 10 min**	Dako Envision Flex Block; 10 min
Buffer Wash	Automated washes (2x)	Automated washes (2x)
Protein Block	**Vector 2.5% horse serum, 20 min**	Dako Protein Block; 10 min
Buffer Wash	Air Removal	Air Removal
Primary Antibody Incubation	Rabbit anti-vWF; 30 min (horse serum as diluent)	Rabbit anti-vWF; 30 min (Dako antibody diluent)
Buffer Wash	Automated washes (2x)	Automated washes (2x)
Secondary Detection Reagent	**Vector ImmPRESS HRP anti-rabbit IgG; 7.5 min**	Dako Envision Flex anti-rabbit HRP; 20 min
Buffer Wash	Automated washes (5x)	Automated washes (2x)
Substrate Development	**Vector ImmPACT DAB EqV; 5 min**	Dako Envision Flex DAB; 10 min
Wash	3x dH_20	3x dH_20
Counterstain (Performed manually)	Hematoxylin + H_20 washes	
Dehydration / Clearing (Performed manually)	Sequential steps through 90% EtOH, 99% EtOH and xylene	
Mounting Media (Performed manually)	DePex	

Dako EnVision Flex Detection
1 ug/ml primary antibody concentration

Vector ImmPRESS Polymer/ImmPACT DAB Detection
0.125 ug/ml primary antibody concentration

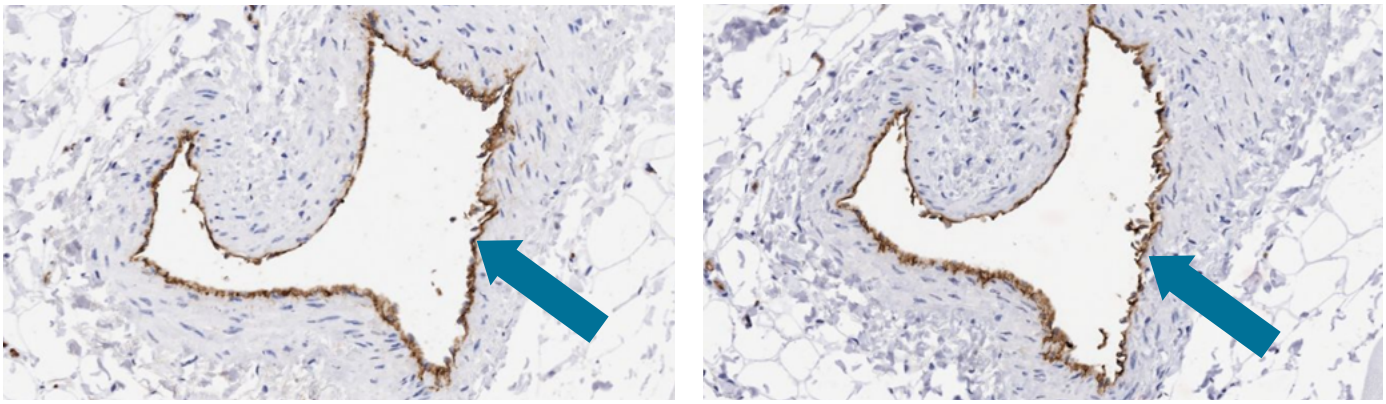


Figure 2. Comparison of specific tissue staining using the detection systems indicated on the Dako Autostainer Plus. Note the significantly lower primary antibody concentration required with the ImmPRESS system to obtain equivalent staining intensity and specificity (arrows, brown DAB) compared with the Dako EnVision Flex reagents.

Negative Control – No Primary Antibody

Negative Control – No Primary Antibody

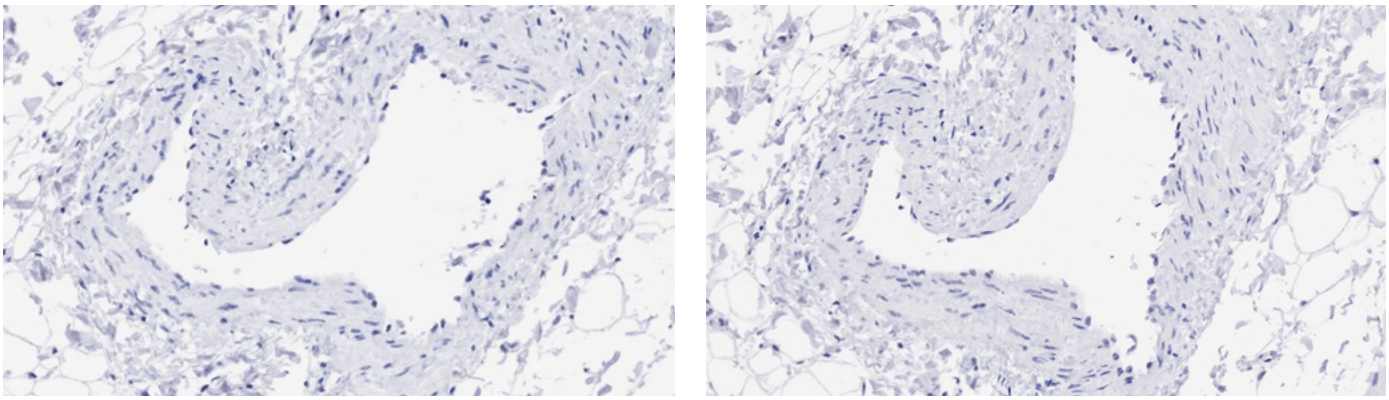


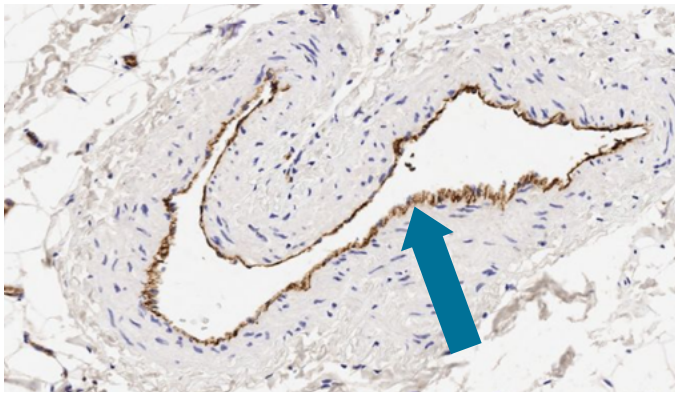
Figure 3. Primary antibody omission negative controls to determine presence of background staining due to the secondary detection reagents on the Dako Autostainer Plus. Note comparable absence of staining for both detection systems.

Leica Bond Rx Platform Results

Table 4. Methodology using Leica Bond Rx

	Leica Bond Rx Program	
IHC Automated Workflow	**Modified Reagent Protocol**	**Vendor Recommended Protocol**
Dewax/Antigen retrieval	Leica ER1 solution; 20 min, 95 °C	Leica ER1 solution; 20 min, 95 °C
Buffer Wash	Leica/Bond wash	Leica/Bond wash
Peroxidase Block	**Vector BLOXALL; 10 min**	Leica Refine Block; 8 min
Buffer Wash	Leica/Bond washes (2x 2 min + 1x brief rinse)	Leica/Bond washes (2x 2 min + 1x brief rinse)
Protein Block	**Vector 2.5% horse serum, 20 min**	Dako Protein Block; 20 min
Buffer Wash	None	None
Primary Antibody Incubation	Rabbit anti-vWF; 30 min (horse serum as diluent)	Rabbit anti-vWF; 30 min (Leica antibody diluent)
Buffer Wash	Leica/Bond washes (2x 2 min + 1x brief rinse)	Leica/Bond washes (2x 2 min + 1x brief rinse)
Secondary Detection Reagent	**Vector ImmPRESS HRP anti-rabbit IgG; 7.5 min**	Leica Polymer Refine anti-rabbit HRP; 20 min
Buffer Wash	Leica/Bond washes (5x 2 min + 1 rinse + 1x dH_20)	Leica/Bond washes (5x 2 min + 1 rinse + 1x dH_20)
Substrate Development	**Vector ImmPACT DAB EqV; 5 min**	Leica Polymer Refine DAB; 10 min
Wash	3x dH_20	4x dH_20 + 1 Bond wash
Counterstain	Leica Polymer Refine Hematoxylin + H_20 washes (instrument stain)	
Dehydration / Clearing (Performed manually)	Sequential steps through 90% EtOH, 99% EtOH and xylene	
Mounting Media (Performed manually)	DePex	

Leica Bond Polymer Refine Detection
1 ug/ml primary antibody concentration



Vector ImmPRESS Polymer/ImmPACT DAB Detection
1 ug/ml primary antibody concentration

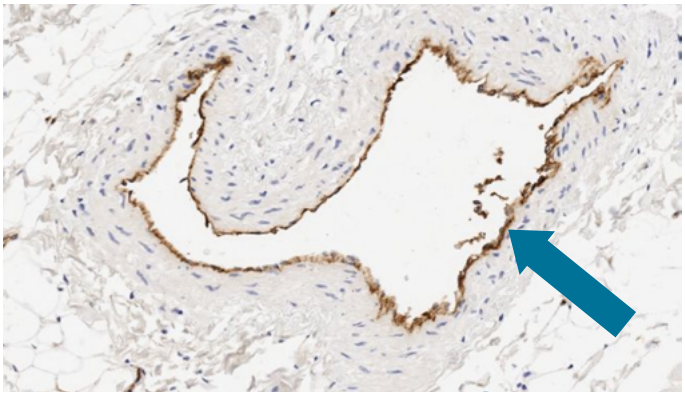


Figure 4. Comparison of specific tissue staining using the detection systems indicated on the Leica Bond Rx. Staining intensity and specificity (arrows, brown DAB) appear equivalent between the two systems.

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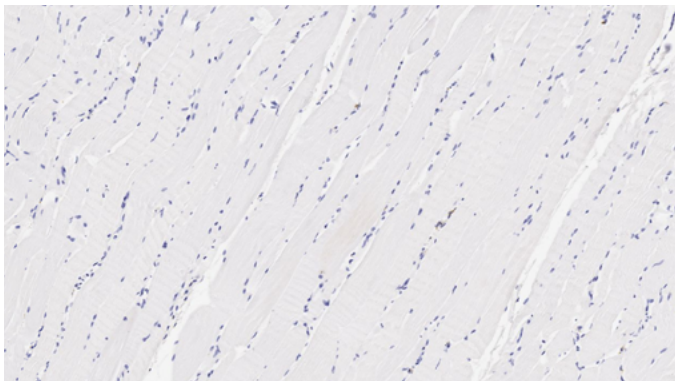


Negative Control – No Primary Antibody

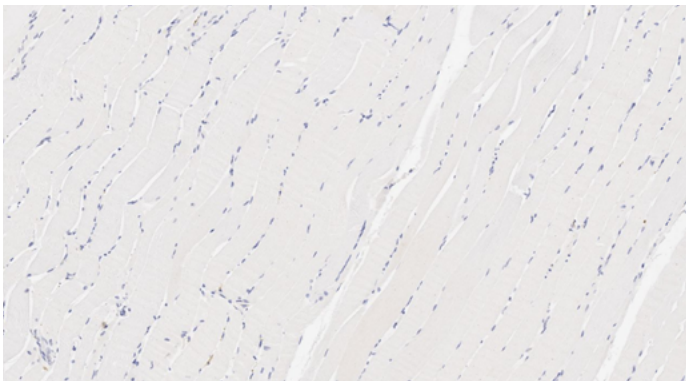


Figure 5. Primary antibody omission negative controls to determine presence of background staining due to the secondary detection reagents on the Leica Bond Rx. Note comparable absence of staining for both detection systems.

Ventana Discovery Ultra Platform Results

Table 5. Methodology using Ventana Discovery Ultra

	Ventana Discovery RUO Universal Automated Program	
IHC Automated Workflow	**Modified Reagent Protocol**	**Vendor Recommended Protocol**
Antigen retrieval	Ventana Cell Conditioning Solution (CC2); 32 min	Ventana Cell Conditioning Solution (CC2); 32 min
Buffer Wash	Ventana Buffer	Ventana Buffer
Peroxidase Block	**Vector BLOXALL; 8 min**	Ventana Inhibitor CM; 8 min
Buffer Wash	Program specific	Program specific
Protein Block	**Vector 2.5% horse serum, 20 min**	None
Buffer Wash	Program specific	None
Primary Antibody Incubation	Rabbit anti-vWF; 20 min (horse serum as diluent)	Rabbit anti-vWF; 20 min (Dako antibody diluent)
Buffer Wash	Program specific	Program specific
Secondary Detection Reagent	**Vector ImmPRESS HRP anti-rabbit IgG; 32 min**	Ventana OmniMap anti-rabbit HRP; 16 min
Buffer Wash	Program specific	Program specific
Substrate Development	**Vector ImmPACT DAB EqV; 4 min**	ChromoMap DAB; 4 min
Wash	Program specific	Program specific
Counterstain	Ventana Hematoxylin II (instrument stain)	
Dehydration / Clearing (Performed manually)	Sequential steps through 90% EtOH, 99% EtOH and xylene	
Mounting Media (Performed manually)	DePex	

Ventana Discovery OmniMap Detection
0.5 ug/ml primary antibody concentration

Vector ImmPRESS Polymer/ImmPACT DAB Detection
0.5 ug/ml primary antibody concentration

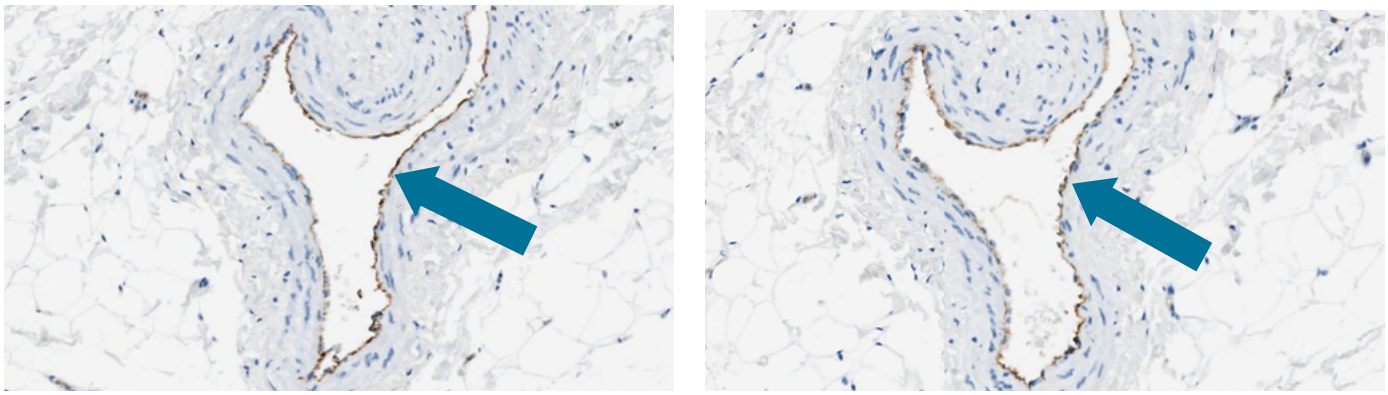


Figure 6. Comparison of specific tissue staining using the detection systems indicated on the Ventana Discovery Ultra. Staining intensity and specificity (arrows, brown DAB) appear equivalent between the two systems.

Negative Control – No Primary Antibody

Negative Control – No Primary Antibody

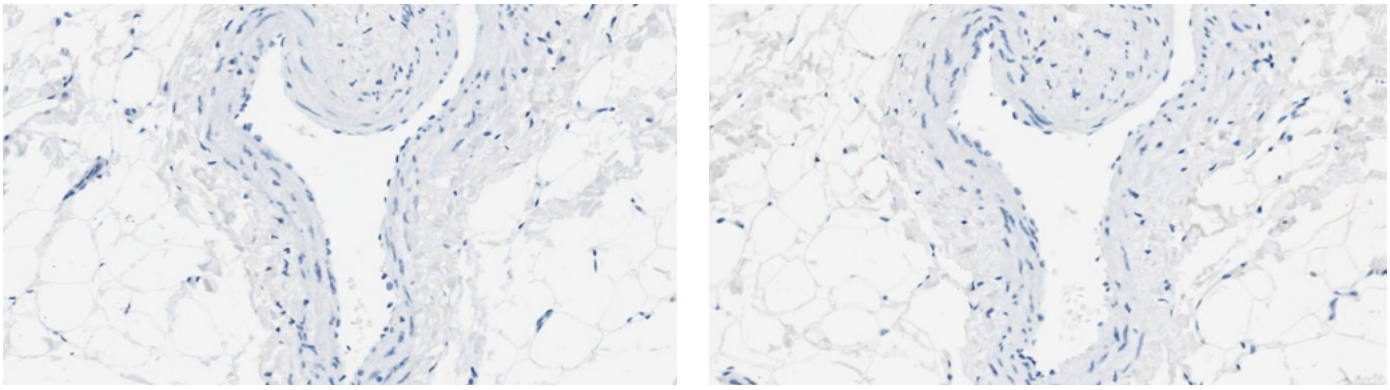


Figure 7. Primary antibody omission negative controls to determine presence of background staining due to the secondary detection reagents on the Ventana Discovery Ultra. Note comparable absence of staining for both detection systems.

Discussion

Vector Laboratories first introduced the ImmPRESS polymer detection system in 2005 launching three peroxidase-based kits, each in a 50 ml volume format. Since that time the ImmPRESS polymer product portfolio has grown to over 30 products featuring choices of detection enzyme, species cross-adsorbed formats, single step and two step detection, double labeling kits, different volume formats and a Mouse on Mouse ImmPRESS kit.

These ImmPRESS polymer kits are widely used throughout the life science research market and are found in academic labs, biotech and biopharma companies, and private and public research institutes. Current use of the ImmPRESS reagents in these facilities is primarily by manual application, dispensed by hand, onto cell and tissue section specimens. Indeed, this method of application is the intended use of the products and how each lot is largely evaluated on tissue sections for quality testing at Vector Laboratories prior to release for sale.

As previously mentioned, considerable interest has been expressed by Vector Laboratories customers regarding the use of the ImmPRESS polymer reagents on open automated staining platforms. To address these customer inquiries, we contracted BioIVT, a specialized CRO, experienced with open automated IHC staining systems, to conduct studies on the suitability of a one-step ImmPRESS HRP Polymer anti-rabbit IgG kit, ImmPACT DAB EqV substrate, and accessory reagents on three commercial platforms.

Overall, the results presented here, clearly show Vector Laboratories polymer detection reagents are well-suited for IHC detection on each of the three platforms evaluated.

Dako Autostainer Plus System

One surprising finding was how well the Vector Laboratories detection reagents worked on the Dako platform. From the initial rounds of optimization (Figure 1), it was evident that the ImmPRESS polymer and ImmPACT DAB EqV generate a robust signal on the autostainer in direct comparison with the vendor recommended detection reagents. Subsequent minor modifications to the procedure, that consisted of a shorter incubation time with the ImmPRESS anti-rabbit IgG polymer and an increase in the number of buffer washes, yielded significant improvement in signal to noise ratio. These combined procedural modifications when applying the ImmPRESS polymer reagents reduced background levels comparable to that of the Dako EnVision reagents (Figure 3).

Using the parameters outlined in Table 3, the ImmPRESS polymer and ImmPACT DAB EqV generated superior specific staining intensity over the EnVision reagents. This increased sensitivity of the ImmPRESS/ImmPACT reagents allowed for further dilution of the primary antibody up to 8x compared to the EnVision reagents to yield the same staining intensity (Figure 2). This result has obvious implications for investigators looking for alternative reagents to apply to this automated platform.

Further studies would be required to determine if this same degree of sensitivity is common with other primary antibodies and tissue specimens when using the ImmPRESS polymer reagents on the Dako Autostainer Plus instrument. However, the ease with which the Vector reagents were substituted on the platform, suggests that at least equivalent specific staining intensity and low background can be achieved for other antigens compared with the instrument manufacturer's recommended detection reagents.

Leica Bond Rx System

Similar to the initial staining performed on the Dako instrument, it was quickly established that the ImmPRESS polymer reagent and ImmPACT DAB EqV substrate could easily be applied to the Leica Bond Rx platform and generate staining for the specific target antigen. However, also as seen on the Dako instrument, these initial rounds of staining on the Leica instrument did not produce the most optimal signal to noise staining ratio (staining results not shown). To address this, the same procedural modifications used on the Dako instrument (i.e. shortened ImmPRESS polymer incubation time and increased number of buffer washes) were applied to the Leica Bond Rx. Combined, these modifications significantly lowered background tone to levels equivalent to the Leica Polymer Refine detection reagents (Figure 5).

Once the minor procedural modifications were implemented (Table 4), specific staining intensity and specificity were equivalent to that of the Leica Polymer Refine detection reagents on the Lecia Bond Rx platform using the same primary antibody concentration (Figure 4). These results support the use of the ImmPRESS Polymer reagent and ImmPACT DAB EqV on this automated instrument. Furthermore, in some labs and facilities, the shorter incubations, and thus potential time savings when using these Vector reagents compared with the instrument manufacturer's recommended detection reagents, would be considered advantageous.

Ventana Discovery Ultra System

As mentioned, no optimization of the staining procedure was performed on the Ventana Discovery Ultra with substitution of the Vector Laboratories detection reagents. Optimization may have generated more intense staining or shorter reagent incubation times than the results indicated. Regardless, with the parameters used, staining intensity and specificity of the ImmPRESS/ImmPACT reagents were equivalent to the Ventana OmniMap detection reagents (Figure 6). Negative controls (Figure 7) show little to no difference in background tone between the two detection systems.

These results reinforce the data from the Dako and Leica instruments regarding the suitability of Vector detection reagent use on open automated staining systems, and underscore some of the inherent differences between automated platforms.

Published Data

During the course of this study, a literature search of recent scientific publications revealed that the ImmPRESS polymer detection reagents and enzyme substrates are currently being applied to these same open automated staining instruments (see References 1-3). The data from these peer-reviewed published references compliment the findings of this study and broaden the scope of the results reported here by describing the use of different one-step ImmPRESS Polymer reagents and enzyme substrates. Furthermore, as highlighted in Figure 8, one study goes beyond our single stain approach and successfully uses Vector reagents in a triple label on the same tissue section, on an automated staining instrument.

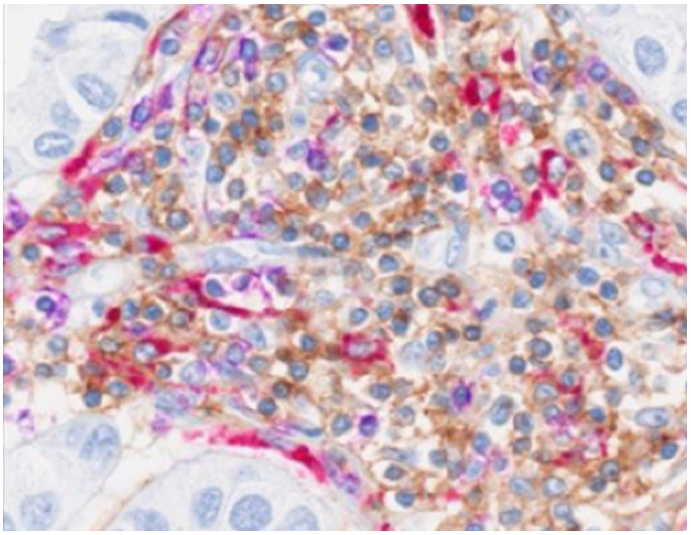


Figure 8. Extracted image from: Ma, Z., et al. (2017) Diagnostic Pathology 12:69 Human Breast Tumor: Tissue section triple stained for CD4 (brown), CD8 (purple) and CD 68 (red) with a hematoxylin (blue) nuclear counterstain. Staining performed on Ventana Discovery Ultra automated stainer. ImmPRESS AP anti-mouse IgG (MP-5402) in combination with Vector Red AP substrate (SK-5100) were used for the localization of CD68 (red).

Emerging Automated IHC Technology

A recent publication (see Reference 4) has described the successful use of the ImmPRESS HRP polymer reagents and ImmPACT DAB substrate on a microfluidic automated IHC device. While this specific device and technology for IHC is yet to be made commercially available, the underlying premise that these reagents can be used on this novel automated system further support the overall findings of our study. Each of the systems described in this document differ in the delivery method of the reagents to the specimen, and the microfluidics technology is certainly a further variation.

Supplemental Information

BioCare intelliPATH FLX Automated IHC Staining Platform

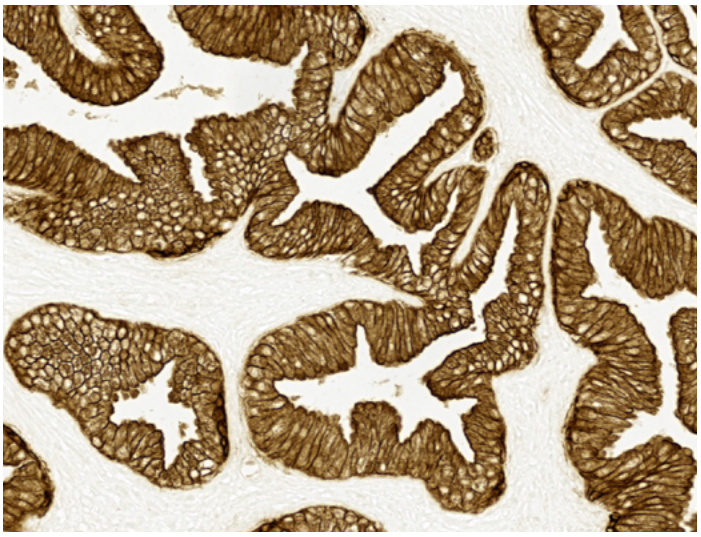
Vector Laboratories has on-site a BioCare intelliPATH FLX automated IHC staining platform that is used on a routine basis. This platform is described by the manufacturer as a flexible, fully open system that allows for the use of reagents from any source. Most of the work performed on this instrument at Vector Laboratories serves primarily a commercial purpose for product development rather than for scientific research. It should be noted though that use of the Vector Laboratories reagents on this or other vendors open automated instruments, is the same regardless of the intended purpose of the IHC staining application.

Supplemental Table 1 provides the standard methodology that is used in-house by Vector Laboratories when applying ImmPRESS polymer systems and accessory detection reagents on the Biocare intelliPATH FLX automated IHC staining platform. While this data was not part of the contracted study conducted by BioIVT, this data is pertinent to the use of Vector Laboratories products on automated platforms and hence, is presented here as supplemental information.

The procedure and parameters outlined in Supplemental Table 1 were developed by Vector Laboratories technicians to closely match those when the reagents are applied to tissue sections manually. The key variables that were optimized on this instrument were the volume and length of the buffer washes. From our experience with this automated platform, the methodology indicated in Supplemental Table 1 generates equivalent specific staining intensity and specificity, with comparable negligible background on tissue sections, when using these same reagents in parallel by manual application. Supplemental Figure 1 is a representative image of tissue section staining obtained when using Vector Laboratories reagents on the BioCare intelliPATH instrument.

Supplemental Table 1

	Protocol for applying Vector Laboratories reagents on BioCare intelliPATH instrument
IHC Automated Workflow	**Current in-house Protocol for ImmPRESS Staining**
Preparation / Antigen retrieval	Offline Pressure Cooker using H-3300, 1 min
Buffer Wash	Automated washes (1x), 5 min (buffer = PBS + 0.05% Tween 20, pH 7.4)
Peroxidase Block	**Vector BLOXALL; 10 min**
Buffer Wash	Automated washes (1x), 5 min
Protein Block	**Vector 2.5% horse serum, 20 min**
Buffer wash	Blow
Primary Antibody Incubation	30 minutes
Buffer wash	Automated washes (1x), 5 min
Secondary Detection Reagent	**Vector ImmPRESS HRP anti-rabbit IgG, 30 min**
Buffer wash	Automated washes (2x), 5 min ea
Substrate Development	**Vector ImmPACT DAB, 8 min**
Wash	Automated washes (1x), 5 min
Counterstain	Not done / Optional
Dehydration / Clearing (Performed manually)	Sequential steps through 95% EtOH, 100% EtOH and xylene
Mounting Media (Performed manually)	Vectamount



Supplemental Figure 1. Human prostate tissue section stained with anti-cytokeratin antibody (AE1/AE3) and detected using ImmPRESS HRP horse anti-mouse IgG polymer (MP-7402) and ImmPACT DAB (SK-4105). No counterstain.

Conclusions

Combined, the results of our contracted study, published literature and our own firsthand experience are unequivocal. The ImmPRESS Polymer detection kits, enzyme substrates and accessory reagents can be applied to open automated staining systems, and at minimum generate equivalent IHC staining results compared with the instrument manufacturers recommended reagents. Depending on the automated staining platform being used, minor modifications to the procedure may be required to achieve optimal signal to noise ratio.

The relative ease with which the Vector Laboratories reagents can be applied to automated IHC platforms for single and multiple antigen labeling indicates a much wider adoption of these products on open autostainer systems in the near future.

Published References

1. Hercher, C., *et al* (2014) *J Psychiatry Neurosci.* 39(6):376–85.
2. Ma, Z., *et al.* (2017) *Diagnostic Pathology* 12:69
3. Habiel, D. M., *et al* (2017) *Scientific Reports* 7: 15444
4. Brajkovic, S., *et al* (2017) *Lab Invest.* 97(8):983-991