AUTOMATING 6-PLEX CHROMOGENIC MULTIPLEXING WITH BOND RX

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INTRODUCTION

There is an ever-increasing demand to derive more information from limited biopsy samples. Multiplex staining provides a means of visualizing many targets in a single tissue section, with the ability to accurately resolve spatial relationships between targets. It is a vital tool in establishing expression profiles of immune checkpoint markers in the tumor microenvironment. Multiplex staining paired with digital imaging provides a permanent image that can support deeper analysis.

Multiplex staining has historically been a long process, and when done manually is generally performed over two or more days, depending on the number of marker and chromogen combinations used. Automated chromogenic multiplexing applications have largely been limited to 2- or 3-plex stains and to conduct more than 3-plex had required a combination of manual steps (e.g. pre-mixing chromogens) incorporated with several processing runs on the instrument. As a result, these workflow inefficiencies often hindered optimal assay design and staining quality.

Presented herein is the new fully-automated 6-plex staining functionality on the Leica Biosystems research platform (BOND RX and BOND RX^{m}.)*

** BOND RX is for research use only. Not for use in diagnostic procedures.*

METHODS

Sample Preparation:

Formalin-Fixed Paraffin-Embedded (FFPE) tissue sections of 3µm thickness were mounted onto charged slides. Slides were baked at 60°C for 30 minutes. Slides were deparaffinized, epitope retrieved and stained on the BOND RX instrument.

IHC Multiplex Staining

Slides were deparaffinized (BOND Dewax protocol), heat induced epitope retrieved (BOND Epitope Retrieval Solution 2 for 20 mins protocol) and stained on the BOND RX stainer. All primary antibodies were BOND Ready-To-Use reagents and of monoclonal mouse or rabbit origin. Chromogens were from Leica Biosystems, Abcam, Vector or Diagnostic BioSystems. All chromogens, with the exception of the purple, were mixed on-board the BOND RX research stainer as part of the automated staining protocols and used enzymatic conversion with BOND Polymer Refine Detection (HRP) and BOND Polymer Refine Red Detection (AP).

Following deparaffinization and heat induced epitope retrieval, multiple cycles of sequential staining were conducted. Marker, enzyme, chromogen, followed by heat-mediated antibody stripping/elution (BOND Epitope Retrieval Solution 2 [ER2] at 80°C for 12 minutes or 20 minutes or 100 °C for 10 minutes) and then repeated with the next round of staining with marker, enzyme, and chromogen. Only the final staining protocol included a counterstain dispense of hematoxylin or Nuclear Fast Red (Figure 1).

Mounting and Digital Pathology

Sections were air dried for a minimum of 30 minutes at room temperature or in a 60°C oven, then mounted with Leica CV Ultra Mounting Medium and scanned on either the Aperio GT 450 or Aperio AT2 digital slide scanners.

WORKFLOW

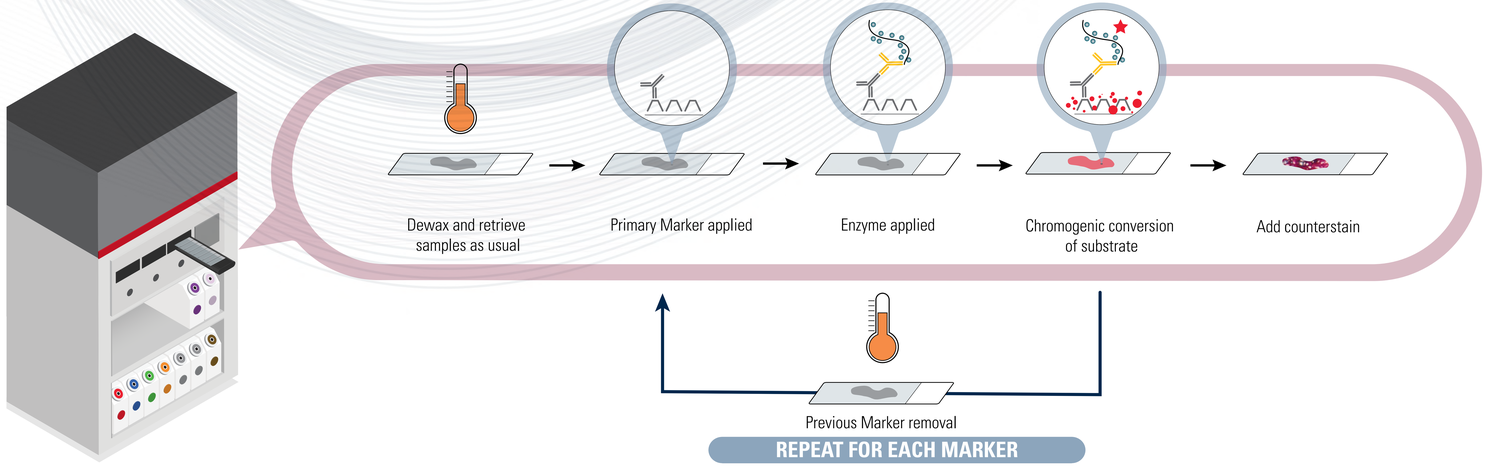
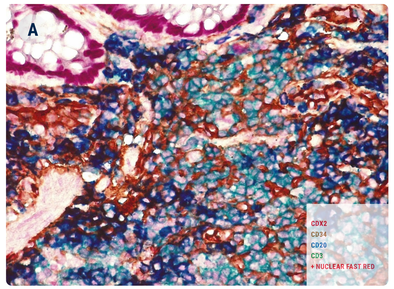


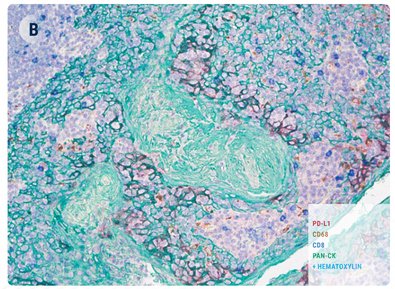
Figure 1. *Visual description of the fully-automated multi-cycle sequential multiplex staining on the BOND RX System.*

RESULTS

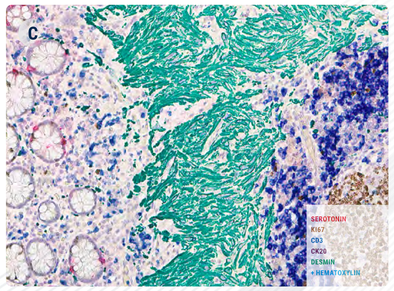
Full automation of 4- to 6-plex chromogenic multiplexing on the BOND RX stainer was achieved. Using a sequential staining and antibody stripping method, the 4-plex stain was achieved in 8 hours and 20 mins, within a workday, and the 6-plex stain was achieved in 10.5 hours, equivalent to an overnight run on the instrument.



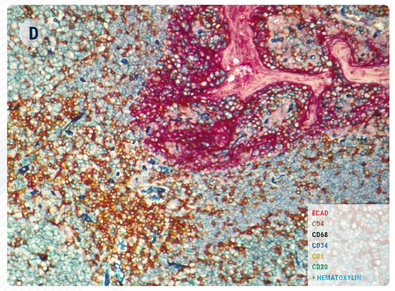
4-PLEX – BOWEL



4-PLEX – SKIN MELANOMA



5-PLEX – BOWEL



6-PLEX – TONSIL

Figure 2. *Fully-automated chromogenic multiplexing on 3µm FFPE sections on the BOND RX stainer.* ***A.*** *4-Plex on normal bowel tissue, CDX2 LBS Red, CD34 LBS DAB, CD20 LBS Blue, CD3 Abcam Green, Nuclear Fast Red counterstain. Magnification 40x.* ***B.*** *4-plex on skin Melanoma tissue; PD-L1 LBS Red, CD68 LBS DAB, CD8 LBS Blue, Pan-CK LBS Green, Hematoxylin counterstain.* ***C.*** *5-plex on normal bowel tissue; Serotonin LBS Red, Ki67 LBS DAB, CD3 LBS Blue, CK20 Vector Purple, Desmin LBS Green, Hematoxylin counterstain.* ***D.*** *6-Plex on normal tonsil tissue; ECAD LBS Red, CD4 LBS DAB, CD68 Abcam Black, CD34 LBS Blue, CD8 Abcam Yellow, CD20 LBS Green, Hematoxylin counterstain.* ***B-D*** *Magnification 20x.*

DISCUSSION

Chromogenic multiplexing requires optimization of the marker and chromogen set. The order of the markers and chromogens needs to be considered. For instance, some epitopes can be more susceptible to impact from multiple rounds of staining (e.g. repeated epitope retrieval), while others may become further retrieved and the signal may in fact improve. Therefore, marker order is key. Some chromogens are less stable or may block the formation of subsequent chromogens. The green chromogen for instance is shown to perform best when used late in the staining sequence.

Successful sequential chromogenic multiplexing was achieved using multiple elution rounds. Elution in this context refers to the use of ER2 and heat as part of the process of removing previously bound primary antibodies and chromogen enzymes from the tissue and washing them away. The purpose of this is to denature bound antibodies yet leave the deposited chromogen unaffected. This allows markers which are raised in the same species to be used as well as reusing the same tagged enzymes without cross-reactivity between detection systems. Setting the temperature of the elution step to 80°C allowed the elution to occur without over retrieving the target epitopes.

Optimizing with individual markers versus the multiplex protocol is a key part of multiplex validation to ensure the right staining profile is achieved.

CONCLUSION

Fully-automated 4- to 6-plex chromogenic multiplexing is both efficient and effective on the Leica Biosystems BOND research automated stainers. The flexibility of the system allows different bespoke protocols to be designed to support different elution needs and chromogen types. The total automation of the stains from deparaffinization to counterstain relieves significant time burden from the user.

The quality of the images and color separation indicates that full automation of chromogenic multiplexing above the conventional 2- and 3-plex stains has great potential. It avoids commonly known issues associated with fluorescent multiplexing and there is no risk of signal quenching associated with fluorophore excitation or autofluorescence inherent in some tissue structures. It also negates the requirement for more specialized imaging equipment making it generally more accessible to a wider research base.

Leica BIOSYSTEMS

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