Newcastle University

HistoCyte Laboratories

Developing Robust PD-L1 Controls for Cancer Companion Diagnostics

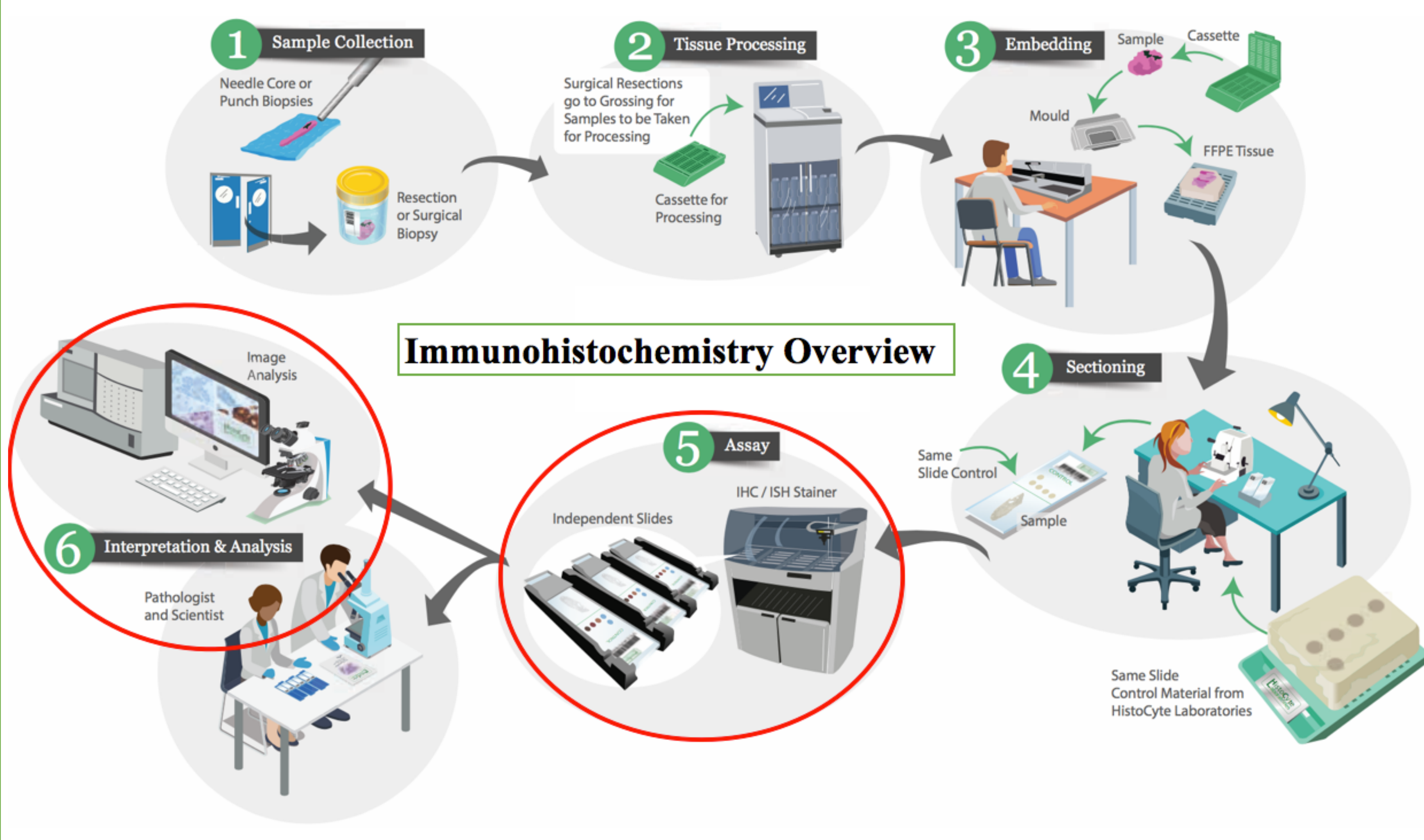
Arunn Sri Ravichelvan 120075436

Introduction

Lung cancer is the most common cancer in men worldwide and the leading cause of cancer death, accounting for almost 1.6 million adult deaths each year. Immunohistochemistry (IHC) is often used to visualise proteins in the cancer cells that can aid physicians in making informed treatment decisions.

Programmed-death ligand 1 (PD-L1) is one of these upregulated proteins, having been found in a number of different cancers including non-small cell lung cancer. It inhibits immune cell activity and prevents the body from attacking the tumour. Detection of PD-L1 with IHC can be used in conjunction with companion drugs to allow the immune system to mount an antitumor response. Variation in PD-L1 testing between laboratories however, remains a problem that can lead to a disparity in treatment selection. Multiple IHC tests, with varying cut-offs for different drugs, creates the opportunity for a situation where a patient can test negative in one lab but positive in another. Standardised controls and analysis could be used to resolve this issue.

One solution is to use external controls to guarantee that an IHC assay is working correctly. HistoCyte Laboratories Ltd. provides standardised external controls. Using image analysis in conjunction with these controls also removes the subjectivity that exists with analysis done by eye; providing quantitative data in place of what was historically only semi-quantitative data.



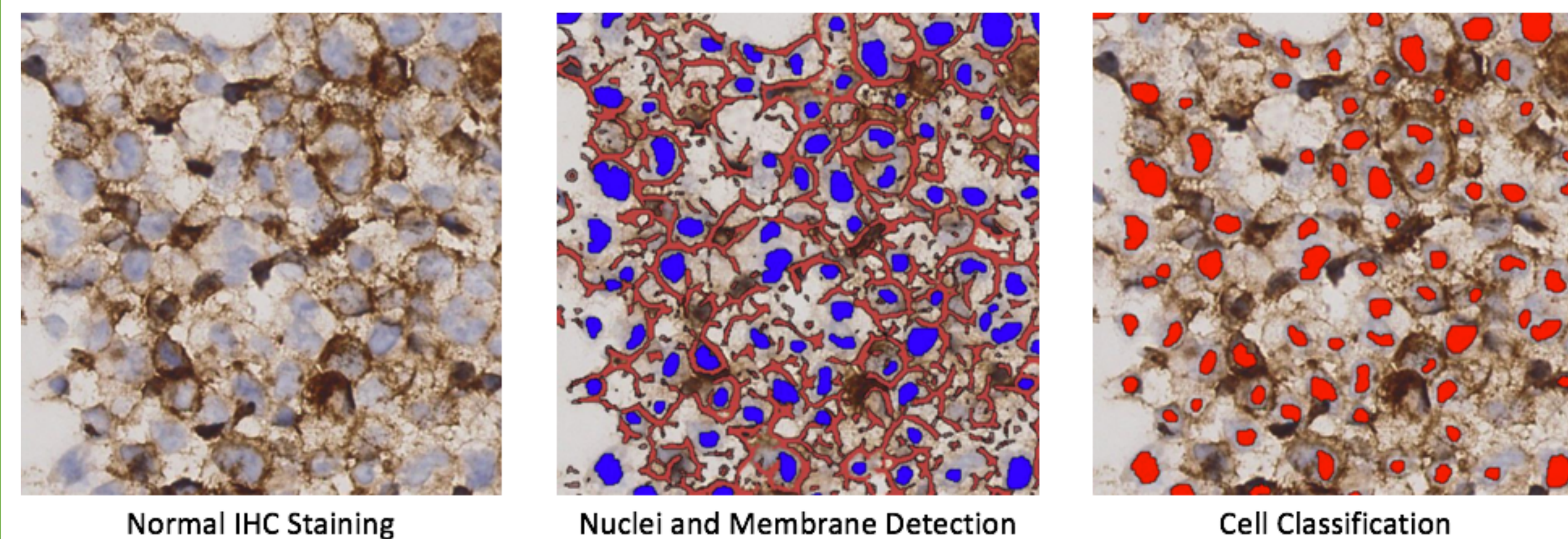
Study Aims

1. Assess consistency of controls on different cell batches
2. Assess controls on a range of section thicknesses
3. Study a range of possible failure parameters
4. Study failure parameters with image analysis software and identify patterns

Methods

HistoCyte's PD-L1 analyte control product was studied through three phases, looking at batch to batch consistency in three batches, variation with different section thicknesses (3, 4, 5 µm) and variation when standard IHC protocols were forced failed in 12 parameters. This resulted in a total of 58 slides providing 174 data points. The data was then analysed using software.

IHC staining was performed on the Ventana BenchMark ULTRA autostainer, using the SP263 assay. Images were scanned on the Leica SCN400 slide scanner and image analysis was done by Visiopharm with the VIS APP.

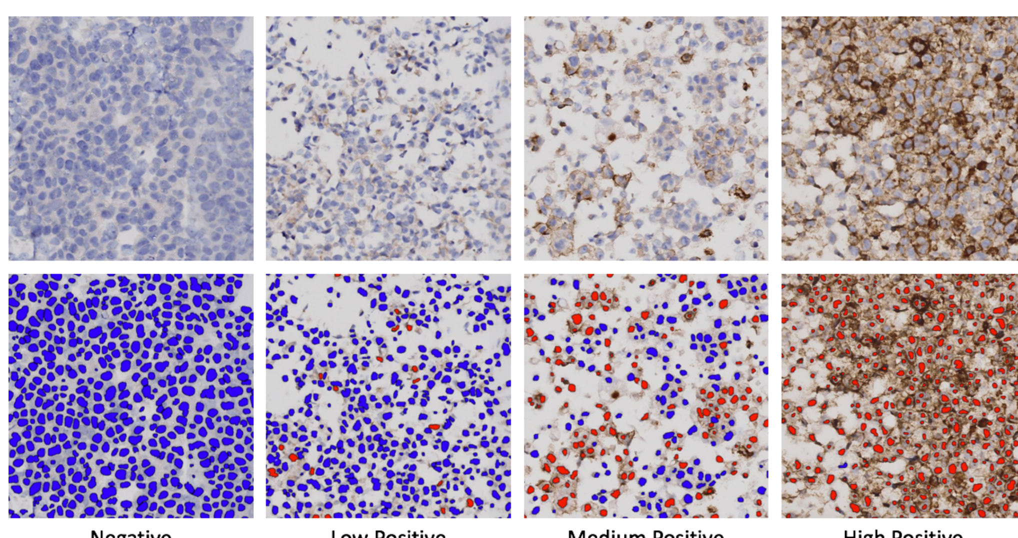


Normal IHC Staining | Nuclei and Membrane Detection | Cell Classification

The image above illustrates how the VIS software analysed scanned images, differentiating cell staining (blue) from membrane staining (brown), and filtering to show only positive cell staining (red) in the final results.

Image Analysis

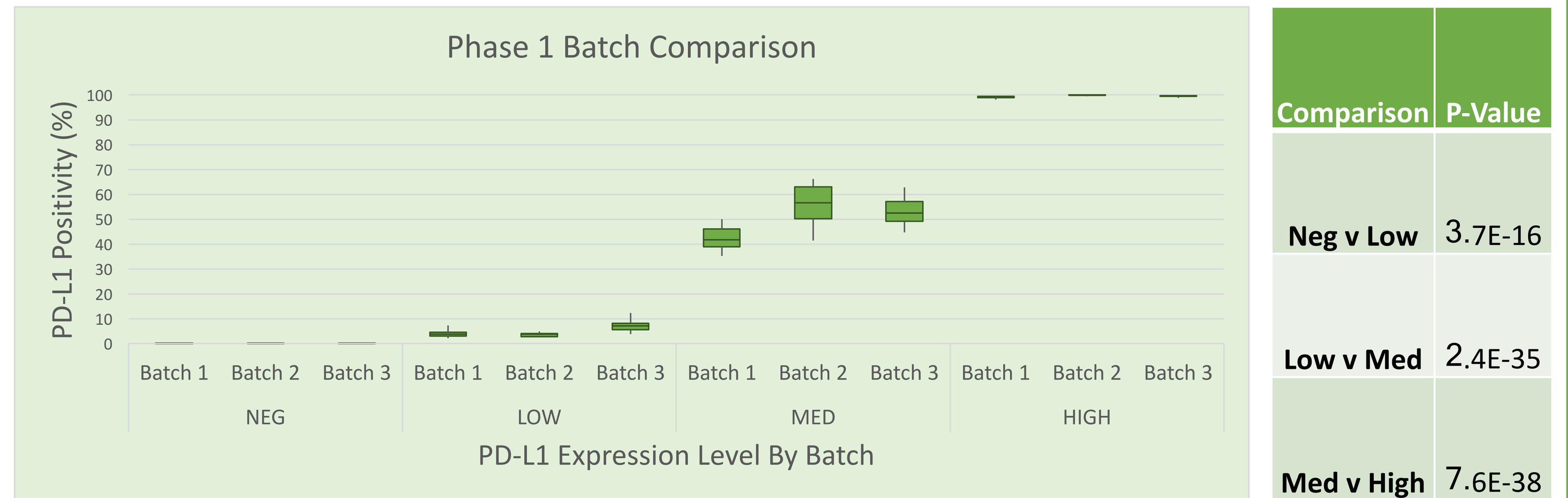
Image analysis provided quantitative data in the form of PD-L1 percentage positivity, number of positive and negative cells, and total cell core area. As expected, image analysis detected no positivity in negative controls, high positivity in high controls, and a mixture in low and medium controls.



Negative | Low Positive | Medium Positive | High Positive

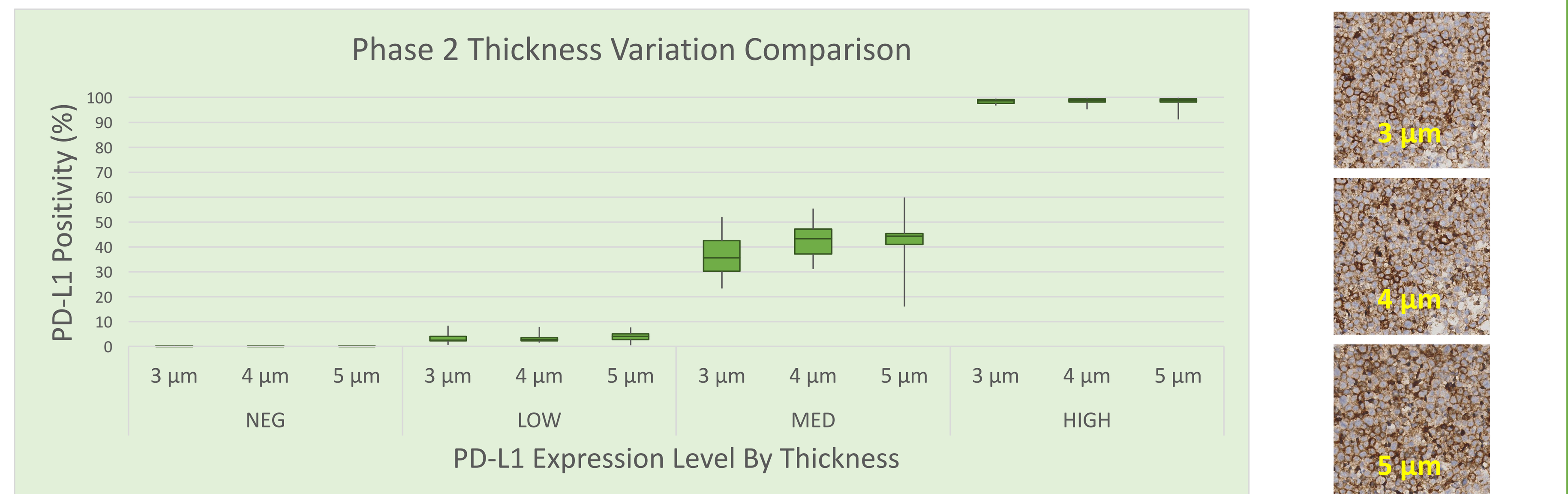
Results

The PD-L1 product was consistent from batch to batch for each level of expression and allowed for a baseline to be established. Greater variation in the low positive and medium positive levels showcased the sensitivity of this control to better detect fluctuating mid-range positivity.



Comparison	P-Value
Neg v Low	3.7E-16
Low v Med	2.4E-35
Med v High	7.6E-38

Variation in thickness caused the product to perform outside of baseline values in no discernible pattern. 5 µm sections appeared to have stronger staining and greater variation over 3 and 4 µm.



Of the 12 parameters that were tested in forced parameter fails, three showed significant variation in control performance:

- No Deparaffinization – controls stained stronger, PD-L1+ was greater than baseline values
- No Cell Conditioning – controls stained weaker, PD-L1+ was less than baseline values
- CC2 Cell Conditioning – controls did not stain at all, image analysis had few/no results

All other parameters showed minor variation, often just outside baseline values.

Phase 3 Results

Parameter	Function	Result
No Deparaffinization	Wax is removed from specimens.	Stronger
No Cell Conditioning	Prepares antigens for antibody binding.	Weaker
CC2 Cell Conditioning	Antigen preparation in a pH of 6 (normal is pH 9).	No Staining
Cell Conditioning Incubation	Allows time for antigen preparation to occur.	Minor Variation
No Peroxidase Inhibitor	Prevents background staining from endogenous peroxidase.	No Change
Antibody Incubation	Allows time for antibodies to bind to antigens.	Minor Variation
HQ Linker Incubation	Linkers bind to primary antibody.	Minor Variation
HRP Multimer Incubation	Multimers bind to Linkers, which will bind to dyes.	Minor Variation
Hematoxylin II Incubation	Counterstaining for better stain visibility.	Minor Variation

Discussion

Random variation in phase 2 results demonstrated that even with a calibrated microtome and trained histotechnician, thickness cannot be guaranteed at such small measurements. This highlights known issues that manual input plays in a process that dictates treatment decisions.[1]

Phase 3 results demonstrated that the most important protocols in IHC staining (and the ones that are most likely to significantly affect results if incorrect) are deparaffinization and cell conditioning. The likely explanations for the three notable failures are that wax residue affected staining analysis, no cell conditioning prevented sufficient exposure of the epitope for effective antibody-antigen interactions, and a more acidic pH was generally detrimental to the sample and protocol.

Image analysis was useful in quantifying the results. In this study, the software utilised one algorithm for PD-L1 positivity and applied it to all phases and parameters. Future versions could be tailored to not only detect improper use of an assay, but also the specific failure that has occurred, allowing for adjustments to readings. One example is that detection of improper dewaxing could prompt the software to use an algorithm that ignores wax residue in calculations.

Laboratory failure rates for IHC are about 2% with the majority manifesting as individual slides, avoiding batch control detection.[2] Standardised PD-L1 assays are also expensive, forcing laboratories to create their own assays using generic antibodies in varying concentrations. This creates greater variation in a process that already warrants more standardisation. Quality control products like same-slide PD-L1 analyte controls, allow laboratories to inexpensively ensure that at the least, their IHC assay is working as intended. This allows patients and physicians testing for PD-L1 positivity to be confident in the laboratory results that will drive their treatment decisions.

Conclusions

1. The PD-L1 controls work as reputed, allowing for a tight baseline consistent between batches
2. Variation from section thickness differences must be considered when performing IHC assays
3. Standardised IHC assays are robust, however dewaxing and cell conditioning are critical steps
4. Image analysis software can aid in detecting and accounting for certain laboratory errors

Acknowledgements

This project was done in collaboration with HistoCyte Laboratories Ltd. and Visiopharm. Special thanks to Colin Tristram, Director of HistoCyte Laboratories Ltd, and Dr Richy Hetherington of Newcastle University, without whose help this study would not have been possible.

1. Barker C, Ibrahim M, Miller K, Reid V. Nondestructive Quality Control of HER2 Control Cell Line Sections. Applied Immunohistochemistry & Molecular Morphology. 2009;17(6):536-542.
2. Cheung C, Taylor C, Torlakovic E. An Audit of Failed Immunohistochemical Slides in a Clinical Laboratory. Applied Immunohistochemistry & Molecular Morphology. 2017;25(5):308-312.