

# ROBOTIC AND SEMI-AUTOMATED MICROTOMY CAN DECREASE VARIABILITY IN HER2 STAINING INTENSITY

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## INTRODUCTION

**BACKGROUND:** Advancements in targeted therapeutics demand increasingly quantitative evaluation of biomarker expression in prognostic and predictive assays. Calibration of such assays is critical for accurate results. Pre-analytic variables, such as section thickness, are known to influence IHC staining intensity. We hypothesized that the variability of HER2 IHC staining intensity would correlate inversely with the degree of automation of microtomy instrumentation.

**DESIGN:** We investigated the influence of automated microtomy methods on the intensity of IHC staining using genetically defined HER2 cell line reference standards. We compared four sectioning methods at several micron settings: manual (Leica, RM2145), semi-automated (Microm, HM355S with Cool-Cut attachment), automated (Tissue-Tek AutoSection<sup>®</sup>, Sakura Finetek), and fully robotic (Tissue-Tek SmartSection<sup>®</sup>, Sakura Finetek). Section thickness selections were intended to span the manufacturers recommended ranges: DAKO HercepTest<sup>™</sup> (4-5µm), Roche PATHWAY<sup>™</sup> 4B5 (3-5µm) and Leica Bond Oracle<sup>™</sup>SP3 (3-5µm).

## MATERIALS AND METHODS

**CELL LINES AND SLIDES:** Two cell culture microarray blocks, from Horizon Discovery, with cell lines expressing HER2 protein at controlled levels were utilized. Each micro-array block contained four cell lines with HER2 protein expression ranging from 0 to 3+ (Figure 1). The two blocks were constructed identically except for core A02 (HER2 1+). All cell lines selected were formalin fixed, routinely processed, and paraffin embedded. One block was used for the manual and semi-automated sectioning methods while the other block was used for the automated and robotic sectioning methods. Slides were sectioned at 3µm, 4µm, 5µm and 8µm, air dried and baked at 60°C prior to IHC staining.

**MICROTOMY:** Four sectioning methods were used: manual (Leica, RM2145), semi-automated (Microm, HM355S with Cool-Cut attachment), automated (Tissue-Tek AutoSection<sup>®</sup>, Sakura Finetek), and fully robotic (Tissue-Tek SmartSection<sup>®</sup>, Sakura Finetek). High profile HP35 Ultra microtome blades from Thermo Scientific were used with the Leica RM2145 microtome while low profile Accu-Edge<sup>®</sup> microtome blades from Sakura Finetek were used on all other microtomes. At least 6 slides were collected at each of the 4 different micron settings on each of the different microtomes.

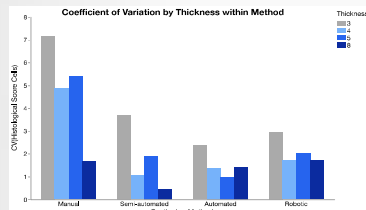
**IMMUNOHISTOCHEMICAL (IHC) STAINING:** Six slides of each section thickness from the four microtomy methods were prepared and divided into two staining runs (48 slides per run, for a total of 96 slides). Staining was performed using the Dako HercepTest<sup>™</sup> for Automated Link Platforms kit (SK001). HIER was performed to kit specifications on a Dako PT Link. Preprogrammed staining protocols were used on a Dako AutostainerPlusLink IHC stainer.

**SLIDE SCANNING AND IMAGE ANALYSIS:** Stained slides were scanned at 20X using the Aperio ScanScope XT imaging system, all slides were scanned in the same session. H-scores were determined by a customized algorithm generated in Definiens Tissue Studio (TMA) by utilizing the Breast Her-2 Score solution modified with a cell simulation action to better detect membrane staining. A total of 384 cell line cores were analyzed.

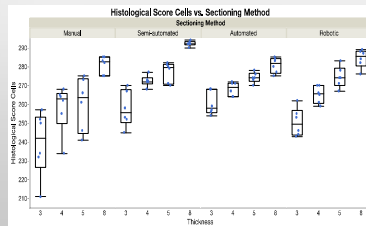
**STATISTICAL ANALYSIS:** Nested variance component models were run on JMP statistical software (SAS Institute, Cary, NC) and used to investigate the variability among the different sectioning methods and section thicknesses on the H scores. The variance components are expressed as %CVs (standard deviation/mean) and summarized across the sample types.



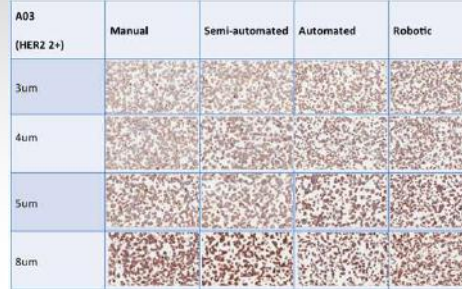
**Figure 1:** Cell culture microarray cores from an example Horizon Discovery HER2 Reference Standard - Labeled A01 through A04, representative of 0 to 3+ scoring from left to right.



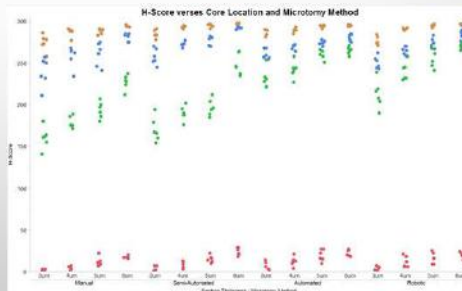
**Figure 2:** Coefficient of variation in HER2 staining intensity, within core A03, by section thickness (in microns) and microtomy method, demonstrating a decrease in staining intensity variation with increasing automation and increasing section thickness. Average %CV's for each sectioning method from manual to robotic 4.8, 1.8, 1.55 and 2.1.



**Figure 3:** Histogramical H-scores versus sectioning methods for core A03 to include box plots demonstrating the decreases in staining intensity variation with increased automation



**Figure 4:** Example photomicrographs of HER2 IHC staining in core A03 (2+). Images are representative of section thickness and sectioning method. 20X magnification.



**Figure 5:** H-score versus core location (A01 red, A02 green, A03 blue and A04 orange). Each dot represents one cell line core, there are 6 dots for each cell line per micron setting. The more closely clustered dots of the same color indicate less variable staining intensity. (Of note, the cell line in the A02 (1+) position, was different in the block used for the automated and robotic microtomy from that used in the manual and semi-automated methods. All other cell lines were the identical.)

## RESULTS

- HER2 staining intensity, as measured by an image analysis system, increases with section thickness across all microtomy methods tested.
- The variation in staining intensity at a given section thickness tends to decrease with greater levels of sectioning automation.
- The coefficient of variation for staining intensity decreases with increasing section thickness across all methods.

## CONCLUSIONS

- Cell line reference standards and digital image analysis can be used to assess the reproducibility of IHC assays.
- Increases in section thickness yield increased staining intensity for the membrane marker HER2.
- Greater staining variability is seen among thinner sections. Whether these variations may be clinically significant in borderline cases for HER2 and other assays, warrants further investigation.
- Decreased variability among thicker sections may represent saturation of the assay system.
- Automation in microtomy may provide a means to decrease variability in immunohistochemical assay results.

## STUDY LIMITATIONS

- For the purposes of this study we examined different sectioning methods. However, other factors can influence section thickness, such as waterbath temperature, blade sharpness, etc., and these variables were not strictly controlled.
- Cell lines may not function precisely like tissue-based IHC controls for an image analysis solution.
- In this study, the cell line for the A02 (1+) core, used for the manual and semi-automated methods, was different from the A02 (1+) core in the block used in the automated and robotic methods.

## FUTURE DIRECTIONS

- Examine whether other critical predictive assays, such as PD-1, show similar variations in staining intensity at different section thicknesses and different microtomy methods.
- Expand on the analysis to include nuclear and cytoplasmic markers, such as Ki-67 and ALK.
- Define whether variations in staining intensity may be clinically significant in borderline cases.