Analytical validation of a multiplex immunofluorescence 7-Biomarker Immunoprint® assay on the Akoya PhenoImager HT platform for stage I/II Melanoma

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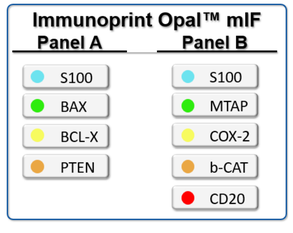
INTRODUCTION

Traditional chromogenic IHC assays are limited to single-marker detection, restricting the ability to resolve spatial relationships and co-expression patterns essential for biomarker-driven clinical decisions. In this study, we transitioned the 7-biomarker Immunoprint® assay requiring 8 serial chromogenic slides into a 2-slide multiplex immunofluorescence (mIF) format, enabling simultaneous quantitative detection within a single tissue section while preserving precious tissue material. The new mIF assay was validated under the CLIA-certified environment, and demonstrates analytical equivalence to the original panel, which robustly stratified early-stage melanoma patients—identifying a high-risk subgroup currently ineligible for adjuvant therapies approved only for later-stage disease, yet exhibiting clinically significant risk of recurrence and mortality.

METHODS

The 2-slide/7-biomarker Opal™ mIF assay was designed as Panel A & Panel B (shown below). mIF panel performance was validated on melanoma control samples, and assessed for accuracy, specificity, sensitivity, precision and reproducibility. Key validation steps included 1) equivalency between "gold-standard" IHC and mIF, 2) evaluation of umbrella effect and spectral crosstalk through drop test, and 3) inter-day/intra-day precision study. Following assay validation, mIF pathologist score guideline was established on training samples.

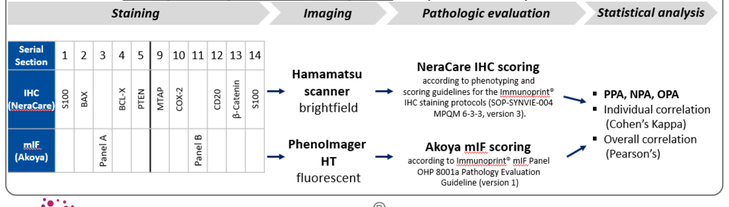
A blinded cohort of 53 stage I/II of cutaneous melanoma pts (6/IA; 18/IB; 15/IIA; 14/IIB, as shown below) were independently assessed with both assay modalities. Risk score was calculated for each modality to stratify patients into High Risk or Low Risk (predefined cut-off 0.135). Study design and workflow are summarized below. To compare the concordance between the two modalities of the Immunoprint® assay, overall/positive/negative percentage agreement (OPA/PPA/NPA) were calculated. Analytical agreement for individual biomarkers and composite risk scores was evaluated using Cohen's kappa and Pearson correlation, respectively.



Blinded Pilot Validation Cohort

AJCC Stage	T-Stage	Number of Samples
IA	T1b	6
IB	T2a	18
IIA	T2b	8
IIA	T3a	7
IIB	T3b	14
	Total	53

Study Design and Workflow - Opal™ mIF panel vs. IHC



RESULTS

Development and validation of 2-slide/7-biomarker Opal™ mIF Assay

The 2-slide/7-biomarker mIF modality of Immunoprint® assay was successfully developed to and evaluated by board-certified pathologists (Fig 1). Regarding assay validation: accuracy was demonstrated by no observation of umbrella effect or spectral crosstalk during drop test (data not shown); sensitivity and specificity was observed through strong equivalency between IHC and mIF assay (Fig 2); precision and reproducibility was evaluated through intra-day/inter-day study (data not shown).

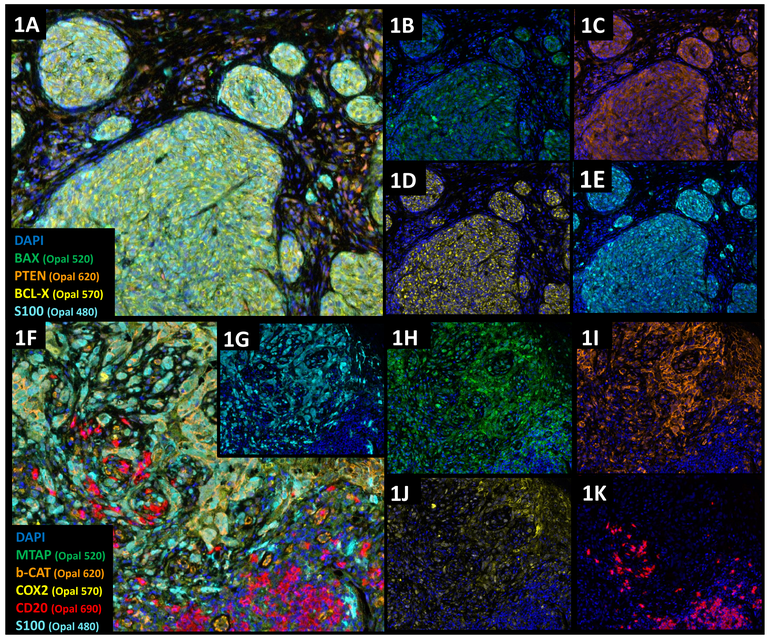


Figure 1. Multispectral image of human, formalin-fixed, paraffin-embedded (FFPE) early-stage melanoma stained with the 2-slide/7-biomarker Opal™ mIF modality of Immunoprint® assay, captured with PhenoImager HT. Figure 1A shows Immunoprint Panel A multiplex 5 color image, while 1B-E presents individually unmixed channels to visualize each marker of interest plus DAPI. Figure 1F shows Immunoprint Panel B multiplex 6 color image; while 1G-K presents individually unmixed channels to visualize each marker of interest plus DAPI. All images are pseudo-colored and brightness increased for visualization purpose only.

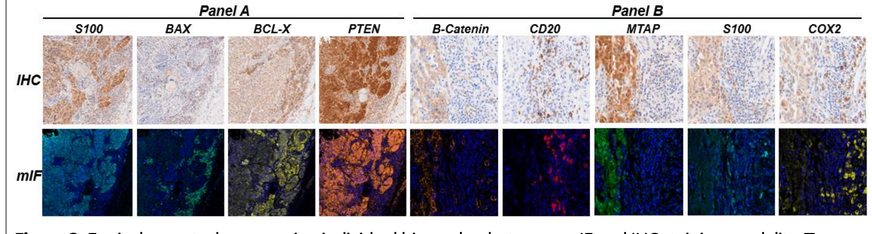


Figure 2. Equivalency study comparing individual biomarker between mIF and IHC staining modality. Top panel shows the "gold-standard" IHC staining pattern. Bottom panel shows the Immunoprint® Panel A and B individually unmixed channels to visualize each biomarker.

RESULTS

Analytical Validation of Opal™ mIF assay compared to the original 7-P-IHC assay on a blinded pilot cohort

The mIF assay bridging and analytical validation was successful with OPA = 96.2% (95 % CI 87.0–99.0), PPA = 100 % (86.7–100) and NPA = 88.9 % (65.3–98.6) (Table 1). Strong analytical agreement was observed between all seven individual biomarker scores (Cohen's κ 0.61–0.81) (Figure 3), and for the composite risk score (Pearson r = 0.84) (Figure 4).

Table 1. Comparison of risk categories of two Immunoprint® assay modalities: mIF assay and 7-P-IHC assay with overall/positive/negative percentage agreement (OPA/PPA/NPA), with 95% CI provided (N = 53).

AEC	mIF		Total	Agreement	
	High Risk	Low Risk		Parameter(1)	Estimate (95% CI)(2)
High Risk	35	0	35	PPA	100 (90.1, 100)
Low Risk	2	16	18	NPA	88.9 (67.2, 96.9)
Total	**37**	**16**	**53**	**OPA**	**96.2 (87.2, 99.0)**

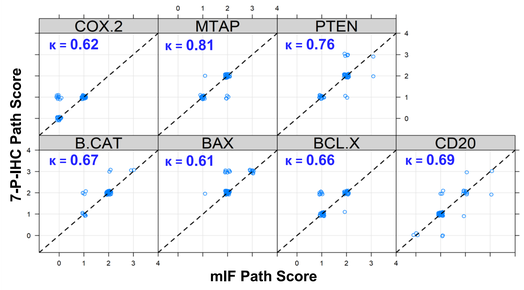
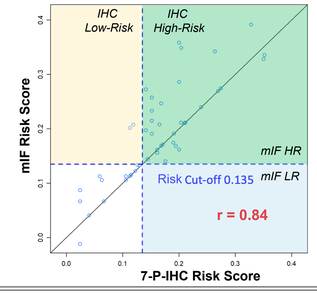


Figure 4 (right). Agreement of risk scores calculated from two modalities of Immunoprint® assay: mIF and 7-P-IHC, evaluated by Pearson correlation coefficient (r = 0.84) (2). Blue line indicates the cut-off value for risk score stratifying high risk (HR) versus low risk (LR).

(2) Pearson correlation coefficient (r), strength of relationship: r ≤0, no; 0.1 ≤ r < 0.4, weak; 0.4 ≤ r < 0.7 moderate; 0.7 ≤ r < 1, strong; r = 1, perfect

Figure 3 (left). Agreement of individual biomarker score between mIF and 7-P-IHC modalities of Immunoprint® assay , evaluated by Cohen's kappa (1).

(1) Cohen's Kappa (κ), strength of agreement: κ≤0, poor; 0<κ≤0.20 slight; 0.21≤κ≤0.40, fair; 0.41≤κ≤0.60, moderate; 0.61≤κ≤0.80, substantial; 0.81≤κ≤1.00, almost perfect



CONCLUSION

- The 2-slide/7-biomarker mIF Immunoprint® assay was successfully developed and validated by board-certified pathologists, addressing the limitation of traditional IHC by enabling simultaneous detection of 7 biomarkers with just 2 slides.
- The new mIF assay retained the clinical relevance of the original design in stratifying high-risk early-stage melanoma patients. Analytical equivalence between the mIF Immunoprint® assay to the original 7-P-IHC assay was demonstrated in this analytical-bridging pilot. The data satisfy CLSI EP09-A2 guidance for platform transfer.
- By leveraging Akoya's mIF capabilities and PhenoImager HT platform, the Immunoprint® assay achieved significant improvement including reduced the tissue usage, high throughput of 30-60 slides per day, automated workflow, robust validation in the CLIA environment, and access to a mature commercialization platform.