POSTER | 3105

Solid tumor analytical validation of a T-regulatory immunohistochemistry multiplex for clinical studies

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BACKGROUND

Accurate characterization of the tumor micro-environment when tissue sample access is limited can be an important challenge in the field of immuno-oncology. The balance and tumor infiltration of T cell subpopulations are of particular interest and its importance has been repeatedly demonstrated in the literature. T cells are important immune effector cells and are therefore preferred targets for immuno-modulation. Conventional T cells can be broadly classified as helper (Th), cytotoxic (Tcyto), memory or regulatory (Treg) cells. Tcyto cells ensure optimal immune responses against invading microbes and tumor antigens. Under homeostatic conditions, Tregs promote peripheral tolerance. However, within tumors, Tregs can supress Tcyto cell functions. The multiplex protocol, Histoprofile®- T-reg light panel, developed at Cerba Research on the Discovery ULTRA (Ventana) platform is designed to stain specific sub-populations of T cells on a single slide, avoiding the need of serial sections from precious patient's FFPE samples in clinical trials while still providing an indepth analysis of the tumor microenvironment.

OBJECTIVE

Analytical validation of a fluorescent multiplex designed to assess cytotoxic T cell ($CD3^+/CD8^+/FoxP3^-$), T helper cell ($CD3^+/CD8^-/FoxP3^-$) and T regulatory cell ($CD3^+/CD8^-/FoxP3^+$) populations and tumor infiltration on solid tumors FFPE samples for use in a clinical trial.

Methods:

Histoprofile®-T-reg light Targets : CD3/CD8/FoxP3

- Cytotoxic T-cells ($CD3^+/CD8^+/FoxP3^-$)
- T helper cells ($CD3^+/CD8^-/FoxP3^-$)
- T regulatory cells ($CD3^+/CD8^-/FoxP3^+$)
- Sequential multiplex protocol with Opal® (Akoya Biosciences) fluorophores on the Discovery ULTRA® (Roche Ventana) slide stainer
- Multispectral images acquired with the VECTRA® PolarisTM (Akoya Biosciences) slide scanner.
- Whole slide image analysis by a pathologist or with HALO® (Indica Labs, v3.3) Highplex module on unmixed images
- Panel statistics were analyzed with JMP statistical analysis software (SAS, V16.1.0)

The multiplex protocol was optimized and pre-validated on a non-small cell lung cancer FFPE sample. The analytical validation included specificity, sensitivity, precision and antigen stability tests on Healthy tonsil (control) and pathological Breast, Lung, Head & Neck and Colon FFPE samples sourced from the Cerba Research Montpellier Biobank.

HISTOPROFILE®- T-REG LIGHT VALIDATION

Analytical Specificity

To assess Histoprofile®-T-reg light protocol specificity, the multiplex protocol was applied on healthy human tonsil samples (negative/positive controls), and a healthy multi-tissue FDA approved TMA containing 33 different tissues from 3 donors. A pathologist confirmed the specificity of the three biomarkers on all tested tissues.

Figure 2.

Specificity assessment on Healthy Tonsil FFPE samples. Representative images of two tonsil samples stained with the Histoprofile®-Tregs light panel. CD3 (Red), CD8 (Orange) and FoxP3 (Green). Scale bar: 50µm

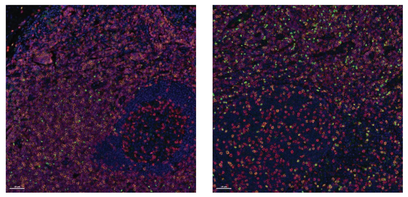
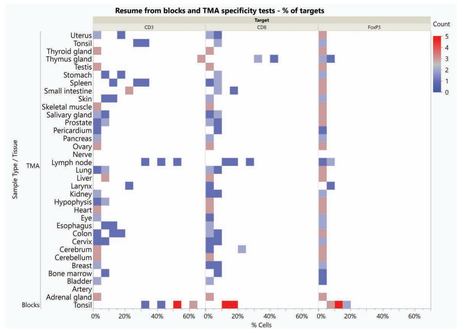


Figure 3.

Heat Map of specificity results obtained on Healthy Tonsil Blocks and Healthy Multi-organ TMA. Count represents the number of stained samples for each tissue and each target in each percentage of positive cells range.



Analytical precison

To assess the analytical precision of the Histoprofile®-T-reg light protocol, both intra-run and inter-run reproducibility were performed on two samples of breast, lung, colorectal, and head & neck cancers **(Figure 5)**. The slides were analyzed with Halo for cell density **(Figure 6)**. The mean CV of all samples was calculated for each tissue type and for solid tumors. Repeatability results: When using positive cell densities for CD3, CD8 and FoxP3 as the readout for the protocol, the acceptance criteria (CV<20%) is met for solid tumors. Reproducibility results: When using positive cell densities for CD8 and FoxP3 as the readout for the protocol, the acceptance criteria (CV<20%) is met for solid tumors. For CD3, the CV is 21% but Cerba Research considers this acceptable due to a low positive cell density (<5% of total cells).

Figure 5.

Representative images from precision assessment of the Histoprofile®-T-reg light protocol on NSCLC, TNBC, HNSCC and CRC. CD3 (Red), CD8 (Orange) and FoxP3 (Green). Scale bar = 100µm.

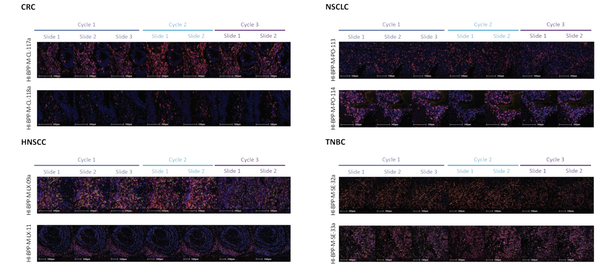
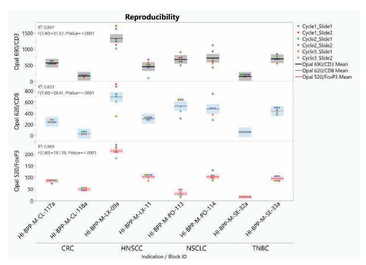


Figure 6.

Distribution Graph of reproducibility test slides. CD3 (top row), CD8 (middle row) and FoxP3 (lower row) cell densities in function of sample and indications



Antigen stability

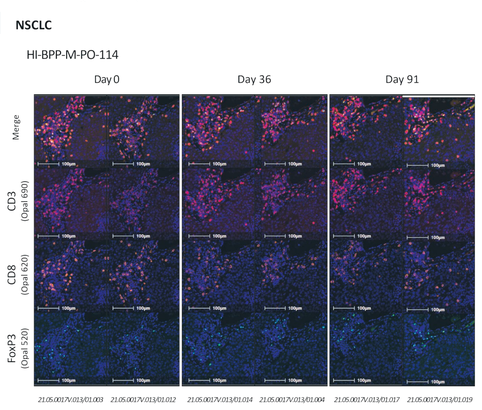
To test the stability of the targets in the Histoprofile®-T-reg light protocol, a kinetics experiment was performed, analyzing the same sample with slides aged over a range of time (fresh up to 3 months). This test was done on two different samples per indication, with varying ranges of signal expression. Example images of a NSCLC sample over time can be appreciated in Figure 7. Images were assessed with Halo image analysis software.

The slides included in the stability test were analyzed with Halo software to determine the cell density of each target of the multiplex.

Antigen Stability: When using the cell density of CD3, CD8 and FoxP3 as the readout for the protocol, the acceptance criteria (variation<20% from day 0) is met up to 90 days for all targets and therefore sets the limits for CD3, CD8 and FoxP3 antigen stability on FFPE slides.

Figure 7.

CD3/CD8/FoxP3 stability assessment on NSCLC. From top to bottom: merge, CD3 (red), CD8 (orange), FoxP3 (green). Scale bar = 100µm.



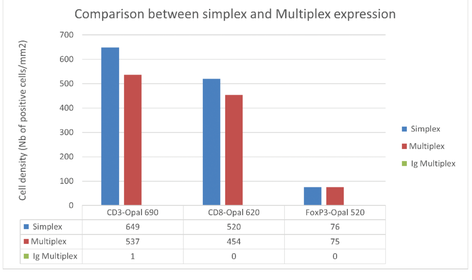
HISTOPROFILE®- T-REG LIGHT PREVALIDATION

Simplex slides were stained for each individual biomarker in a simplex protocol and compared to a serial section stained with the HISTOPROFILE®-T-reg light multiplex panel. Staining concordance between the multiplex slide and simplex slides was determined by analysis with HALO® without multispectral deconvolution.

The cell densities from the simplex (blue bars) and multiplex panel (red bars) slides showed comparable staining profiles between the slides.

Figure 1.

Comparison of cell density between simplex and multiplex slides



	CD3-Opal 690	CD8-Opal 620	FoxP3-Opal 520
Simplex	649	520	76
Multiplex	537	454	75
Ig Multiplex	1	0	0

Analytical Sensitivity

The Histoprofile®-Tregs light protocol was tested on a range of solid tumor FFPE samples including Triple Negative Breast Cancer (TNBC), Non-Small Cell Lung Cancer (NSCLC), Head & Neck Squamous Cell Carcinoma (HNSCC) and Colorectal Cancer (CRC). Tissue blocks were complemented by a Multi-organ Cancer TMA to evaluate a sufficient number of samples for each indication. Example images can be seen in **Figure 5**. Slides were scored by a pathologist to provide semi-quantative analysis of the targets and confirm specificity.

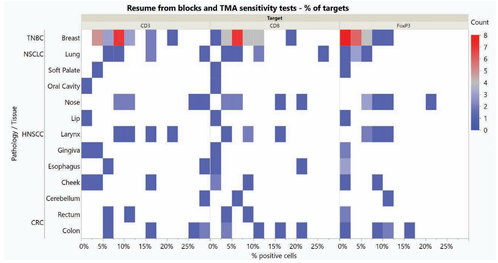


Figure 4.

Heat Map of sensitivity results obtained on Blocks and Multi-organ TMA. Count represents the number of stained samples for each tissue and each target in each percentage of positive cells range.

HISTOPROFILE®- T-REG LIGHT SPATIAL ANALYSIS

T cell infiltration into the tumor has become an important biomarker to estimate response to checkpoint immune therapies. Five colorectal cancer (CRC) samples stained with the Histoprofile®-T-reg light panel (representative images in **Figure 8**) were analyzed with Halo to determine the densities of CD8 T cells and Treg cells in the tumor and stroma compartments **(Figure 9)** and the densities of the cell populations at the tumor/stroma border **(Figure 10)**. There are significantly more Tregs in stroma than the tumor ($p = 0.026$). Similarly, more T cells are in the stromal compartment than the tumor but not to a significant level ($p = 0.096$). A particular accumulation of the T cells and Tregs cells on the stromal side of the interface is evident. The variability in the CD8 T cells and Treg profiles at the tumor/stroma interface for the individual samples can be greatly appreciated in **Figure 10**.

Figure 8.

Representative images from spatial analysis using HaLo (Green line represents the Tumor/Stroma interface. Left image: Histoprofile®-Tregs light panel CD3 (red), CD8 (orange), FoxP3 (green). Middle image: Halo masks for T cells (top) and Tregs (bottom). Right image: Halo representation of the areas evaluated during the interface analysis. Each color represents a distance of 10 µm. Scale bar = 100µm.

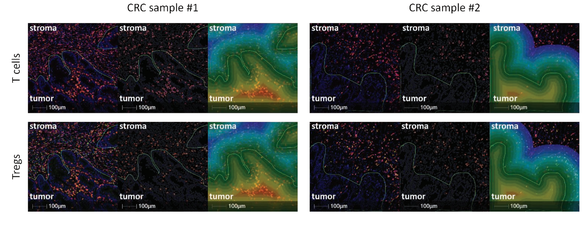


Figure 9.

Box Plot for Tregs (top) and T cells (bottom) density in the Stroma (blue) and Tumor (red) for five CRC samples.

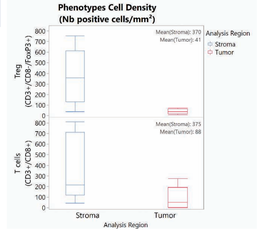
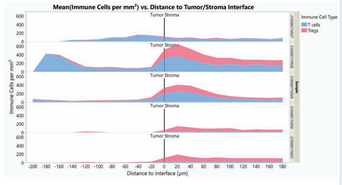


Figure 10.

Area Plot showing Tregs (red) and T cells (blue) infiltration in the Tumor (left) from the Stroma (right) for five CRC samples.



CONCLUSION

After having shown specificity and sensitivity analytical performance of the Histoprofile®-T-reg light protocol using anti-CD8, anti-CD3 and anti-FoxP3 to detect Tcyto and Treg cells in a range of solid tumors including but not limited to Lung, Head & Neck, Breast and Colorectal Cancer FFPE tissues, the protocol meet the acceptance criteria regarding repeatability and inter-assay reproducibility. Antigen stability showed that the antigens can be detected at a level similar to fresh sections after three months. Therefore, Cerba Research confirms that the Histoprofile®-T-reg light protocol is validated on solid tumors to an exploratory endpoint level. Spatial analysis of this protocol offers a more in depth analysis of the tumor microenvironment, allowing appreciation of immune cell infiltration into the tumor.

These results demonstrate the Histoprofile®- T-reg light panel as a useful and robust tool to investigate T cell populations in various human solid tumors in clinical trials.

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