# Optimization and validation of immunohistochemical assays for the detection of TREM2 and evaluation by image analysis in FFPE human tissue

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## Abstract

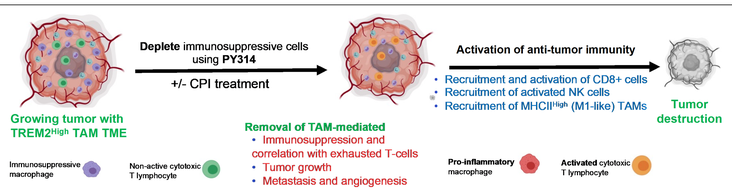
**Introduction:** The tumor microenvironment (TME) often contains high levels of suppressive myeloid cells that may contribute to innate and acquired checkpoint inhibitor (CPI) resistance. Pionyr's Myeloid Tuning™ approach involves altering the composition and/or the function of myeloid cells in the TME. Pionyr has identified the transmembrane protein Triggering receptor expressed on myeloid cells-2 (TREM2) as a highly enriched target on tumor associated macrophages (TAMs) and has developed an anti-TREM2 therapeutic monoclonal antibody, termed PY314, which is currently being tested in a Phase 1 clinical study (NCT04691375). To select patients that would most likely benefit from PY314 therapy, Pionyr has developed a TREM2 immunohistochemical (IHC) assay for the detection of TREM2 in formalin-fixed, paraffin embedded (FFPE) human tissue samples. Moreover, to better understand TREM2 expression, localization within the tumor, and spatial interaction with other immune cells, a multiplex immunofluorescence IHC panel was developed.

**Methods:** The monoplex TREM2 IHC assay was optimized and validated at Mosaic Laboratories, a CLIA-licensed and CAP accredited laboratory, using an anti-TREM2 antibody on the Leica Bond Rx staining platform. Optimization included control selection, pretreatment selection and antibody titration experiments. The assay was validated for sensitivity, specificity, and inter-day precision. Tissues were evaluated by standard manual pathology review and pathologist guided image analysis using HALO (Indica labs). In addition, a multiplex immunofluorescence (IF) assay using Akoya's Opal™ reagents was optimized and validated at Pionyr to include the anti-TREM2 antibody in a 5-plex panel (DAPI, TREM2, CD8, PD-L1, and pan-CK). Tissues were scanned on the Vectra 3 microscope (Akoya) and images were further processed digitally and analyzed using Inform software.

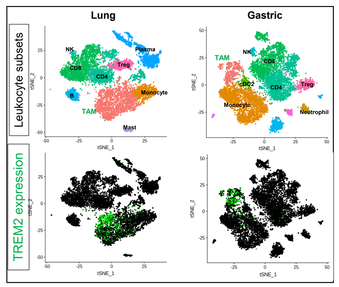
**Results:** The monoplex TREM2 IHC assay was successfully optimized and validated at Mosaic Laboratories and showed excellent specificity, sensitivity, and precision following pathologist guided image analysis. The optimal anti-TREM2 staining concentration was 0.5 µg/mL with a high-pH antigen retrieval. The inter-day precision assay demonstrated that guided image analysis was more robust and reproducible than a standard manual pathologist review for determining the percentage TREM2+ cells in the total tumor area. TREM2 expression and frequency were prevalent across multiple solid tumor indications, while absent from most normal tissues. The anti-TREM2 antibody was successfully integrated and validated into the Akoya's IF multiplex panel.

**Conclusions:** Screening for TREM2 expression using the IHC assay demonstrated that TREM2⁺ TAMs were highly enriched in the TME of the prioritized solid tumor indications currently being pursued in the PY314 Phase 1a clinical trial. The monoplex TREM2 IHC assay is successfully being used on FFPE archival tumor tissues from enrolled patients to determine TREM2 expression. The multiplex IF assay is offering insights into the localization of TREM2⁺ TAMs and their spatial relationship with other immune cells present in the TME to determine what immune composition will be more favorable for patient response to PY314 therapy. This assay may also be used to follow changes in the TME associated with PY314 treatment in pre- and post-tumor biopsies.

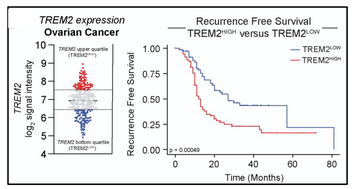
## Introduction




- TREM2 is an immunomodulatory receptor expressed on TAMs.
- PY314 is a humanized mAb that specifically binds human TREM2 and "re-tunes" the TME via targeted depletion of TREM2⁺ TAMs via antibody-dependent cell-mediated cytotoxicity and/or cellular phagocytosis.
- PY314 is currently in Phase 1a clinical trials in advanced solid tumors relapsed or refractory to standard of care (NCT04691375)




TREM2 is predominantly expressed on human TAMs as show here by single cell RNA sequencing in examples of lung and gastric tumors.




TREM2 expression is inversely correlated with survival in ovarian cancer and is also seen in:

- Gastric cancer
- Hepatocellular carcinoma
- Renal cell carcinoma

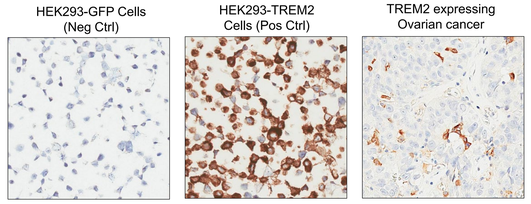
Binneweis M. et al, *Cell Reports.* Accepted for Publication, October 2021. **Targeting TREM2 on Tumor Associated Macrophages Enhances Efficacious Immunotherapy.**

## Objectives

1. To optimize and validate TREM2 DAB IHC assay that could aid in the selection of patients that may benefit of PY314 therapy.
2. To optimize and validate a multiplex IHC assay to better understand the TREM2 expression, localization within the TME, and spatial interaction with other immune cells.

## Optimization of the Monoplex TREM2 IHC Assay

The TREM2 IHC assay was optimized using the anti-TREM2 human antibody. FFPE HEK-293T cell lines were used as positive (TREM2 expressing) and negative (GFP expressing) controls, and an ovarian tumor block was selected to use as control tissue. Titration experiments determined the anti-TREM2 antibody at a concentration of 0.5 µg/mL stained on the Leica Bond Rx to be optimal using high pH antigen retrieval.




## Validation of the TREM2 IHC Assay : Inter-day precision, Sensitivity, and Specificity

### Inter-day precision and manual versus guided image analysis scoring

A

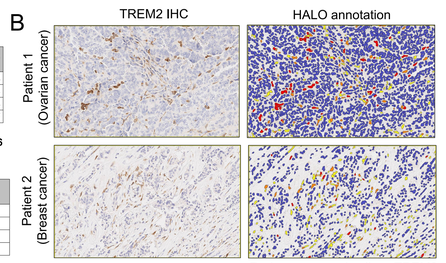
Manual Scoring (over total cells)

| Patient ID | Tissue Type | %Average TREM2+ | SEM | Standard Deviation | %CV |
|---|---|---|---|---|---|
| Patient 1 | Ovarian Carcinoma | 14.00 | 1.87 | 4.18 | 29.88 |
| Patient 2 | Breast Carcinoma | 5.80 | 1.24 | 2.77 | 47.84 |
| Patient 3 | Breast Carcinoma | 19.00 | 2.45 | 5.48 | 28.83 |
| Patient 4 | Ovarian Carcinoma | 36.00 | 5.10 | 11.40 | 31.67 |
| | | | | Average %CV | 34.56 |

Digital Scoring (over total cells)

| Patient ID | Tissue Type | % Average TREM2+ | SEM | Standard Deviation | %CV |
|---|---|---|---|---|---|
| Patient 1 | Ovarian Carcinoma | 18.59 | 2.46 | 5.49 | 29.54 |
| Patient 2 | Breast Carcinoma | 8.75 | 0.89 | 2.00 | 22.86 |
| Patient 3 | Breast Carcinoma | 14.32 | 1.49 | 3.32 | 23.22 |
| Patient 4 | Ovarian Carcinoma | 22.66 | 1.26 | 2.82 | 12.42 |
| | | | | Average %CV | 22.01 |

B




Brown: TREM2-DAB

Red, orange, and yellow: TREM2⁺

The IHC inter-day precision analysis was performed in 2 ovarian and 2 breast cancer tissues stained on 5 separate days, which were evaluated for % TREM2 positive cells and scored manually and digitally by pathologist guided image analysis. **(A)** The assay showed robust and similar staining across different days with an average %CV (coefficient of variation) of 34.56% for manual scoring and 22.01% for digital scoring suggesting that pathologist guided image analysis is a robust and reproducible method for TREM2 scoring. **(B)** Digital scoring using the HALO image analysis tool shows accurate detection of TREM2 DAB staining.

### Sensitivity analysis

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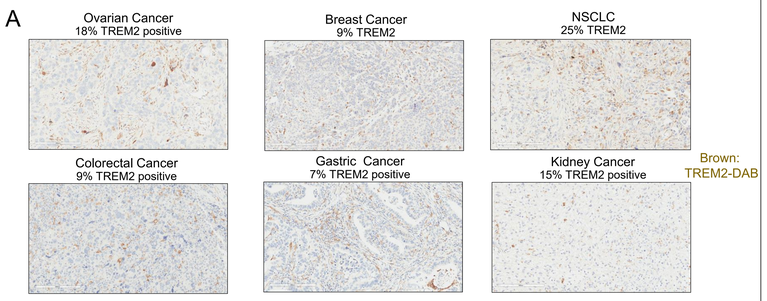


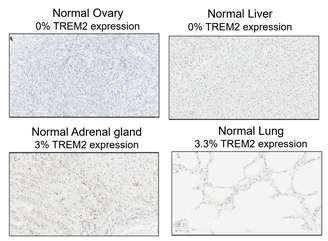

The sensitivity of the TREM2 DAB IHC assay was tested on 127 preclinical archival tumor blocks from Mosaic Labs and evaluated by pathology guided image analysis. **(A)** Representative IHC images (20x) of high and low TREM2 expression on FFPE tissues from six solid tumor indications stained with the anti-TREM2 antibody. *Note: Due to tissue size and heterogeneity of staining, images may not be truly representative of the analysis.*

### Specificity analysis

The IHC specificity of the TREM2 DAB assay was tested on a tissue microarray containing 30 unique tissues cores from 30 different normal human tissues. All tissues besides lung and adrenal gland tissues had TREM2+ percentages ≤1 or were completely negative. Only ~3% of cells in the lung and and adrenal gland were positive for TREM2 in the normal tissue cores tested.

**(A)** Representative IHC images (20x) of TREM2 expression on FFPE human normal tissues stained with the anti-TREM2 antibody. *Note: Due to tissue size and heterogeneity of staining, images may not be truly representative of the analysis.*

A



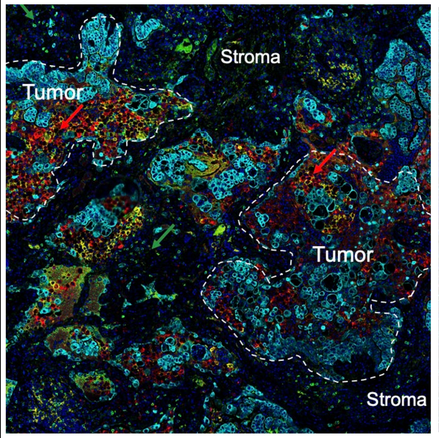

Brown: TREM2-DAB

## Optimization of a Multiplex IHC Assay (5-plex)

A multiplex immunofluorescence (IF) assay using Akoya's Opal™ reagents was optimized and validated at Pionyr to include the anti-TREM2 antibody in a 5-plex panel. Tissues were scanned on the Vectra 3 microscope (Akoya) and images were further processed digitally and analyzed using Inform software.

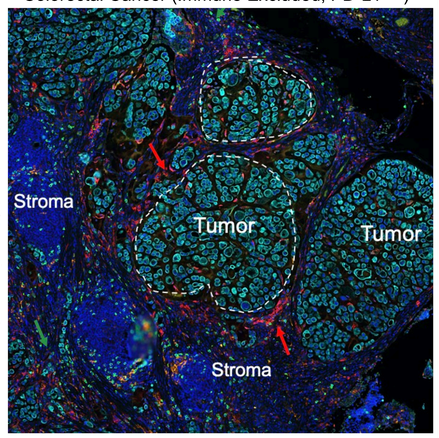
| Antibody | Expressed on | Fluoro-phore | Position in IHC panel |
|---|---|---|---|
| CD8 | Cytotoxic T cells | 520 | 1 |
| PD-L1 | Various cell types including tumor cells & immune cells | 570 | 2 |
| Pan-Cytokeratin | Tumor cells | 690 | 3 |
| TREM2 | TAMs | 620 | 4 |

Lung Cancer (Immune Excluded; PD-L1+)




CD8+ (green; 6%) – PD-L1+ (yellow; 37%) – TREM2+ (red; 10%)

Colorectal Cancer (Immune Excluded; PD-L1^Low)




CD8+ (green; 3%) – PD-L1+ (yellow; 4%) –TREM2+ (red; 25%)

PanCK (cyan) - DAPI (blue)

## Using Image Analysis to Understand Spatial Localization of TREM2 in Relation to Other Markers in the Tumor Microenvironment

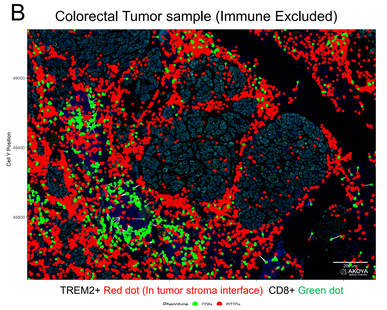
A

| Tissue Category | Percentage of Total Cells | | | | |
|---|---|---|---|---|---|
| | CD8+ | TREM2+ | PD-L1+ | PANCK+ | TREM2+/PD-L1+ |
| **Tumor** | 2% | 2% | 0% | 81% | 0% |
| **Stroma** | 5% | 44% | 7% | 3% | 4% |
| **Total** | 3% | 25% | 4% | 37% | 2% |

Using our multiplex IHC assay we can study the composition of different cell phenotypes, including TREM2, in the tumor and stroma compartments. This is exemplified in table **(A)** above for 1 colorectal tumor sample. The same colorectal tumor sample was also used to perform a "nearest neighbor" analysis as shown in the image and table to the right **(B)**. In this example of an immune excluded tumor we see that TREM2 is highly expressed in the stroma lining the tumor nests and CD8 also is mainly present in the stroma. The median distance is thus much larger between a TREM2+ cell and CD8+ cell in the tumor compartment as compared to the stroma compartment.

B

Colorectal Tumor sample (Immune Excluded)




TREM2+ Red dot (In tumor stroma interface) CD8+ Green dot

| Nearest Neighbor Distances from TREM2+ to CD8+ (microns) | | | |
|---|---|---|---|
| Tissue Category | Min | **Median** | Max |
| Tumor | 8.51 | **333.46** | 1657.03 |
| Stroma | 3.00 | **31.02** | 762.00 |

## Summary & Acknowledgements

- TREM2 expression using the TREM2 IHC assay demonstrated that TREM2⁺ TAMs were highly enriched in the TME of the prioritized solid tumor indications currently being pursued in the PY314 phase 1a clinical trial.
- The monoplex TREM2 IHC assay is successfully being used on FFPE archival tumor tissues from enrolled patients to determine TREM2 expression.
- The multiplex IF assay is offering insights into the localization of TREM2⁺ TAMs and their spatial relationship with other cells types present in the TME to determine what immune composition will be more favorable for patient response to PY314 therapy.
- **We want to thank the PY314 R&D and clinical teams at Pionyr Immunotherapeutics and our collaborators at Mosaic CAP-CLIA IHC Laboratories (Forest Lake, CA) for their work on the optimization and validation of the anti-TREM2 IHC assay and for screening FFPE tumor tissues of patients enrolled in the PY314 Phase 1 clinical trial.**