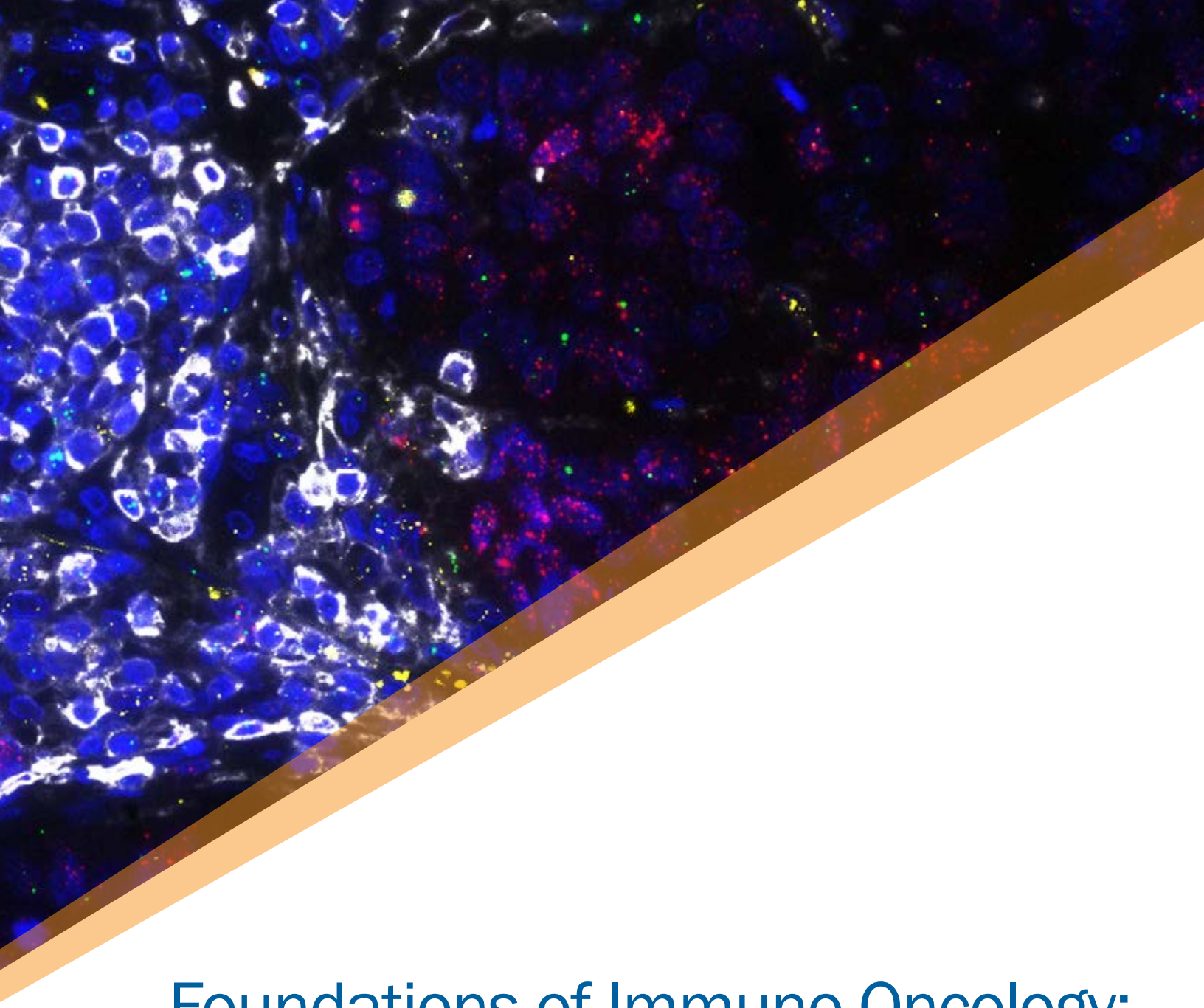


Foundations of Immuno-Oncology: Advancing Basic Science & Translational Research

bio-techne®

Introduction

Immuno-oncology (I-O) represents an especially dynamic area of cancer research. Aimed at developing novel strategies for therapeutic intervention, cancer immunologists systematically investigate the complex interactions taking place between the tumor and host immune system. The field has progressed tremendously since the pioneering work of Coley[1] and Ehrlich,[2] and today immunotherapy is hailed as one of the most promising approaches in the fight against various cancer types.

In the more than 20 years since the launch of the field's first commercial success, rituximab, great progress has been made with several immunotherapies developed and clinically tested. Monoclonal antibodies, checkpoint inhibitors, small molecules, cytokines, adoptive cell transfers, and other cell-based therapies have all made their way into the clinic. Many additional agents are under pre-clinical and early clinical investigation, including oncolytic viruses, antibody-drug conjugates, therapeutic and preventative vaccines, various small molecules, adjuvants, and bispecific antibodies.

As the pace of discovery and development from bench to bedside continues to amplify, the need for sensitive tools, accurate methods, and efficient technologies will continue to materialize. Nonetheless, success in this research area can be impeded by deep-rooted and intertwined cellular interactions, signaling pathways, mechanisms of tumor immune evasion, and the potentially grave adverse responses observed with immunotherapy. Moreover, these factors are further compounded by often sparingly available, time-sensitive, and highly heterogeneous samples from which meaningful conclusions are demanded. Such challenges are inherent to the biology of the field, but they should not be further complicated by the technical approach you choose.

Bio-Techne unites the world-class brands of R&D Systems, Novus Biologicals, Tocris Bioscience, ProteinSimple, and Advanced Cell Diagnostics, Inc., to uncomplicate your workflow and better support the I-O research community. In this eBook, we investigate immunotherapeutic approaches that are at the forefront of the field for targeting solid tumor types and their associated challenges. Within each interactive chapter, you'll also learn more about our diverse portfolio of solutions and capabilities that range from trusted gold standard reagents and kits to innovative technologies that are helping researchers advance their discoveries to the clinic.

Contents

References

1. Coley, WB, The treatment of malignant tumors by repeated inoculations of erysipelas: with a report of ten original cases, 1893; *Clinical Orthopaedics and Related Research*, 262: 3–11.

2. Ehrlich, P, Über Den Jetzigen Stand Der Karzinomforschung, 1909; *Ned Tijdschr Geneeskd*, 5: 273–90.

Our Story

Bio-Techne's roots are in the midwestern state of Minnesota. Our legacy brand, R&D Systems, was born here in 1976 when we churned the previous Land O' Lakes factory space into a biological products company that has grown up to be a world-leading supplier of hematology controls, cytokines, recombinant proteins, antibodies, and immunoassays. Yearning to produce far-reaching and complete solutions for scientists across the board, today, that same site is also a corporate home to a family of life science research and clinical diagnostic brands. We're a blended bunch. But by converging on our strengths, the Bio-Techne bloodline now extends across market lines and across the globe with unique product and instrument portfolios, EPIC employees, and diverse expertise.

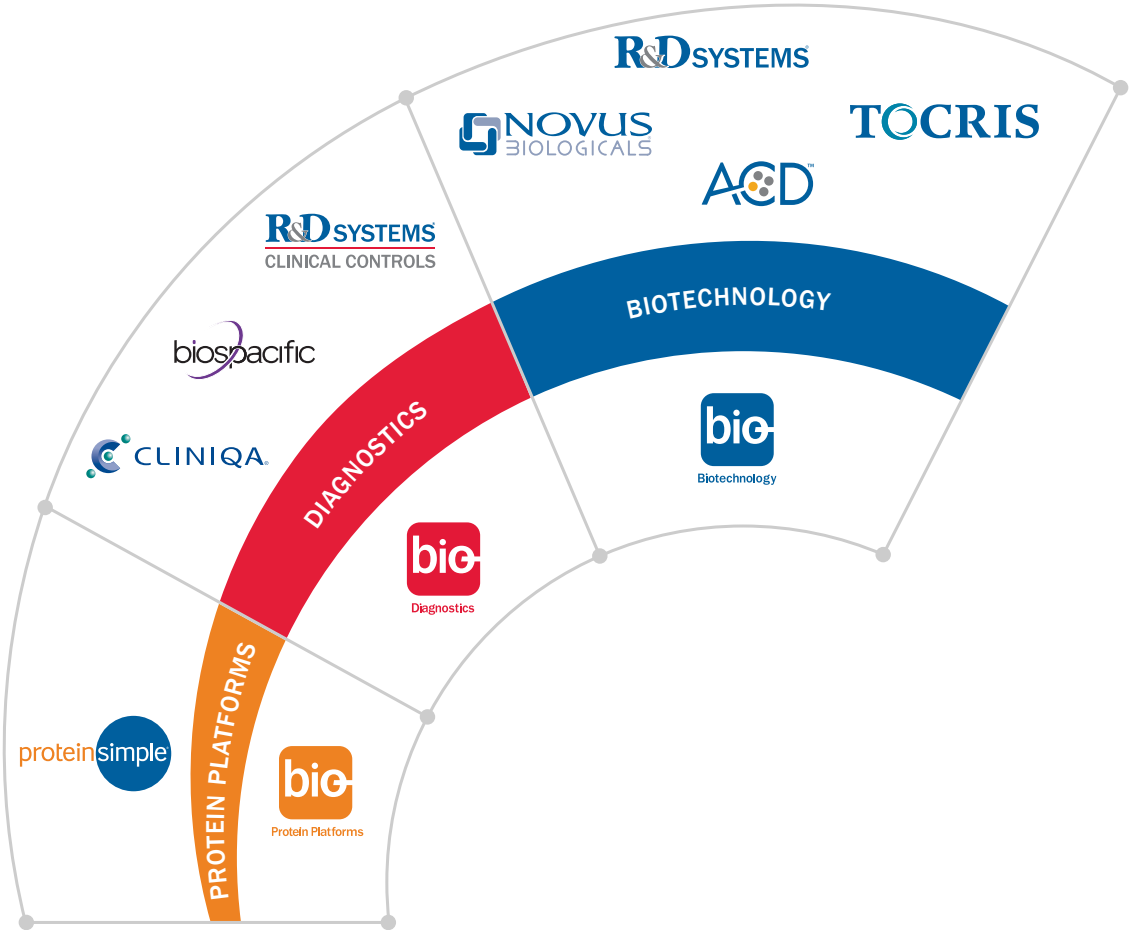
Click here to watch
this video to learn more about our story.

bio-techne brands

Click here to learn
more about the Bio-Techne brands.

Our Mission

We are committed to advancing immuno-oncology research and unlocking its full clinical potential by providing solutions to workflow challenges that exceed the expectations for product quality, instrument performance, and comprehensive support. Through these efforts, we aim to ultimately deliver renewed hope for patients with advanced cancer.



Chapter 1:
Exploring the Tumor Microenvironment

As an immunosuppressive or tolerant zone, the tumor microenvironment is an integrated network of various cell types including cancer, stromal, vascular, and immune cells (**Figure 1**). Tumor cells display aberrant cell growth and signaling patterns to create an environment that favors evasion of the immune system. Such permissive conditions are required for tumor growth, angiogenesis, progression, and metastasis, and immense efforts are underway to counteract these adaptations. However, the complex interplay between cells makes the tumor microenvironment a particularly difficult area of investigation that requires advanced tools for deeper analysis.

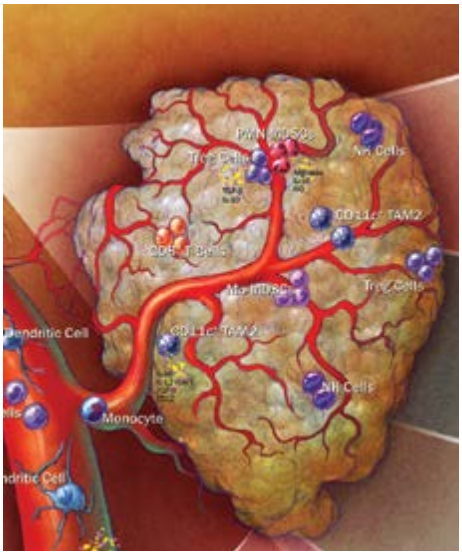


Figure 1. Diverse Cell Populations in the Tumor Microenvironment. The tumor microenvironment is an integrated network of various cell types including cancer, stromal, vascular, and immune cells.

Cancer Cells and Non-Cancer Cells Contribute to the Tumor Microenvironment

Many cell types are thought to contribute to the generation of an immunosuppressive tumor microenvironment including cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), mesenchymal stem cells, vascular endothelial cells, and lymphatic endothelial cells[1].

Cancer-Associated Fibroblasts

CAFs are thought to have several pro-tumor actions in the tumor microenvironment which have been reviewed by Tao et al[2]. These occur via a reciprocal relationship between CAFs and tumor cells. Tumor cells stimulate fibroblast activation via the secretion of growth factor and adhesion molecules including TGF-β, EGF, PDGF, FGF2, CXCL12, ICAM 1, and VCAM1. In return, CAFs promote tumor growth by the secretion of FGF, HGF, secreted frizzled-related proteins, IGF1, and other molecules such as integrin α11 and syndecan-1. CAFs also stimulate angiogenesis via a PDGF-stimulated increase in VEGF secretion. Finally, there is also evidence that suggests CAFs may play a role in metastasis and resistance to cancer therapy.[2]

Tumor-Associated Macrophages

TAMs are involved in tumor-promoting angiogenesis, fibrous stroma deposition, metastasis formation, and inhibition of T cell responses. Both M1- and M2-polarized macrophages have been identified in the tumor microenvironment. Several molecules are thought to participate in TAM-induced T cell immune suppression, including VEGF and TGF-β[3].

Myeloid-derived Suppressor Cells

MDSCs are a heterogeneous population of immature myeloid progenitor cells that fail to differentiate into granulocytes, macrophages, and dendritic cells (DCs). As with other cells in the tumor microenvironment, cross-talk between tumor cells and MDSCs is important for the inhibition of anti-tumor immunity. The anti-tumor immunity mediated by MDSCs occurs in response to intercellular cytokines and regulatory proteins released by the tumor and other recruited cells[4]. Looking for information on MDSCs? Read our MDSC Guide.

Understanding Immunosuppressive Myeloid Cells and Immunotherapy Using Proteome Profiler™ Antibody Arrays

Watch this on-demand webinar to hear Xin Lu, Ph.D., discuss his research aimed at understanding the anti-tumor efficacy of the dual immune checkpoint blockade and tyrosine kinase inhibition.

Regulatory T Cells

Tregs are a heterogeneous subset of $CD4^+$ T cells with suppressive properties that play a central role in maintaining immune homeostasis and self-tolerance, dampening inflammation, and preventing autoimmunity. Tregs also represent an important suppressive cell population within tumors, where their function is to inhibit the activities of $CD4^+$ and $CD8^+$ effector T cells, Natural Killer (NK) cells, Natural Killer T (NKT) cells, and antigen-presenting cells. Looking for more information on Tregs? Read our Regulatory T Cells Guide.

Click here to access our MDSC Guide.

B Cells

Although B cell association with the tumor microenvironment is correlated with a good prognosis, there are instances where B cells inhibit tumor-specific cytotoxic T cell responses[1]. Looking for more information on B cells? Download our B Cell Guide.

Click here to access our Regulatory T Cell Guide.

Dendritic Cells

DCs are key mediators of the innate and adaptive immune responses due to their wide-spanning antigen-presenting effector functions. DCs display major histocompatibility complex molecules and costimulatory receptors upon pathogen recognition to capture, process, and present antigens to naïve T cells. This action results in the production of polarizing cytokines that then promote pathogen-specific effector T cell differentiation and activation. In addition, DCs can promote self-tolerance by secreting tolerogenic cytokines that induce the differentiation of regulatory T cells. Because of their capacity to regulate T cell responses, there is considerable interest in DCs as potential therapeutic targets. Interested in learning more about DCs? Download our DC Guide.

Click here to download our B Cell Guide.

Click here to download our DC Guide.

Tumor-Associated Neutrophils

Although tumor-associated neutrophils (TANs) have been shown to have both pro- and anti-tumor effects as reviewed by Balkwill et al.[1], the pro-tumor phenotype predominates. In fact, anti-tumor TANs (N1) are thought to be converted to pro-tumor TANs (N2) during tumor progression.

Examining Complex Cellular Interactions in the Tumor Microenvironment

There are many challenges to monitoring immune responses in the context of the tumor microenvironment, according to Wargo et al[5]. They include limited clinical samples, heterogeneity in cellular behavior, complex collection paradigms, and the personalization of treatment strategies. Analytic techniques need to consider the pros and cons of using archival versus fresh tissue, the use of core biopsies versus surgical dissection/resection tissue, and finally, single versus longitudinal sampling of cancer tissue.

Toolbox Tips

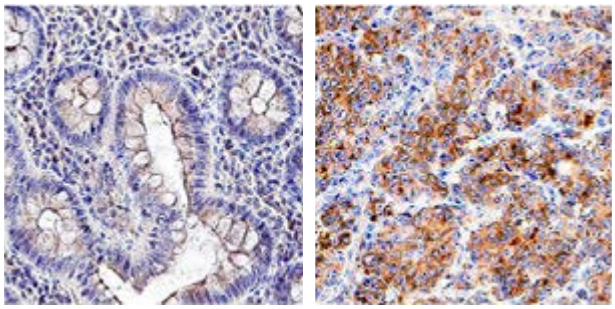
Click here to access our helpful protocol for immunohistochemistry using paraffin embedded tissue sections.

Characterization of Cell Type and Morphology of the Tumor Microenvironment

Sometimes the best place to start is to merely look at the tumor. What are the histological features? Are immune cells infiltrating the area? Histological or pathological analyses using antibodies against predictive biomarkers or checkpoint targets can be used to assess the likelihood of a successful therapeutic intervention (**Table 1**). **Figure 2** illustrates the detection of PD-L1/B7-H1 in immersion-fixed, paraffin-embedded sections of a normal human colon (left panel) and human colon cancer tissue (right panel).

BTN/BTNL	LAG-3*	PD-1*	TIM-3*
CTLA-4*	LILRA	PD-L1*	TIGIT
IDO	LILRB	PDL-2*	

Table 1. Example Antibodies for Tumor Microenvironment Targets. Antibodies against immune checkpoint targets, checkpoint target candidates or biomarkers for immuno-oncology studies. *CyTOF® ready antibodies available.



Normal Tissue Cancer

Figure 2. PD-L1/B7-H1 Expression in Human Colon and Colon Cancer Tissue. PD-L1/B7-H1 was detected in immersion-fixed, paraffin-embedded sections of a normal human colon (left panel) and human colon cancer tissue (right panel) using Goat Anti-Human PD-L1/B7-H1 Antigen Affinity-purified Polyclonal Antibody (R&D Systems, Catalog # AF156) at 5 µg/mL overnight at 4 °C. Tissue was stained using the Anti-Goat HRP-DAB Cell & Tissue Staining Kit (brown; R&D Systems, Catalog # CTS008) and counterstained with hematoxylin (blue). Specific staining was localized to cell membranes and cytoplasm.

Investigators are increasingly focused on combination therapies as the boundaries of single-target immune checkpoint blockade therapy narrow. How might we predict the efficacy of simultaneously targeting multiple signaling and/or immune checkpoint targets in the tumor microenvironment? Mass cytometry (CyTOF) is becoming an important tool in the armament against many cancer types. CyTOF uses antibodies conjugated with heavy metal ion tags instead of fluorochromes as a variation of flow cytometry.

Examination of Complex Spatial Relationships Among Different Cell Types Within the Tumor Microenvironment

Depending on your experimental setup, you may want to visualize cell types and states in a highly specific and sensitive manner, at the single-cell level and in an intact environment in order to provide morphological context. The RNAscope® assay is a valuable approach to visualize and evaluate various cell types and states present in the local tumor environment by detecting key functional molecules such as cytokines and chemokines in addition to cell lineage markers.

To characterize lymphoid and myeloid immune cell types and states in the tumor microenvironment of ovarian cancer, serial formalin-fixed, paraffin-embedded (FFPE) tissue samples were examined using the RNAscope 2.5 HD Duplex assay (Advanced Cell Diagnostics, Inc., Catalog # 322500) (**Figure 3**). Gene expression patterns for key functional markers *FOXP3*, *IFNγ*, *CXCL10*, and *CCL22* were combined with cell lineage markers *CD4*, *CD8*, *CD68*, and *CD163*. Double positives for *IFNγ* and *CD8*, *FOXP3* and *CD4*, *CXCL10* and *CD68*, and *CCL22* and *CD163* are indicative of cytotoxic T lymphocytes (CTL), Tregs, M1-like macrophages, and M2-like macrophages, respectively. Also, the recruitment of *CXCL10*-expressing immune cells can be observed in the context of *IFNγ* expression, and the presence and abundance of Tregs ($FOXP3^+CD4^+$) can be evaluated in relation to the expression of chemokine *CCL22* (**Figure 3**).

This technique can be combined with immunohistochemistry for an enhanced understanding of the regulation of expression. In **Figure 4**, lymphoid immune cell types are visualized in the tumor microenvironment of FFPE tissue biopsies of non-small cell lung cancer, combining RNAscope LS Multiplex Fluorescent ISH Assay (Advanced Cell Diagnostics, Inc., Catalog # 32800) for *IFNγ*, *FOXP3*, and *CD4* with immunohistochemistry (IHC) staining for CD8 (GeneTex, Inc., GTX75393). In this experiment, co-expression of *CD4* and *FOXP3* mRNA within the same cell is indicative of Treg cells (green arrows) and $IFN\gamma^+CD8^+$ CTLs are highlighted by red arrows (**Figure 4**).

Considering the highly plastic immune conditions of the tumor microenvironment, together with a spectrum of maturation and polarization, the RNAscope ISH immune-profiling approach may bring both novel insights for key contributing immunosuppressive factors and ways to facilitate intervention through cancer immunotherapy.

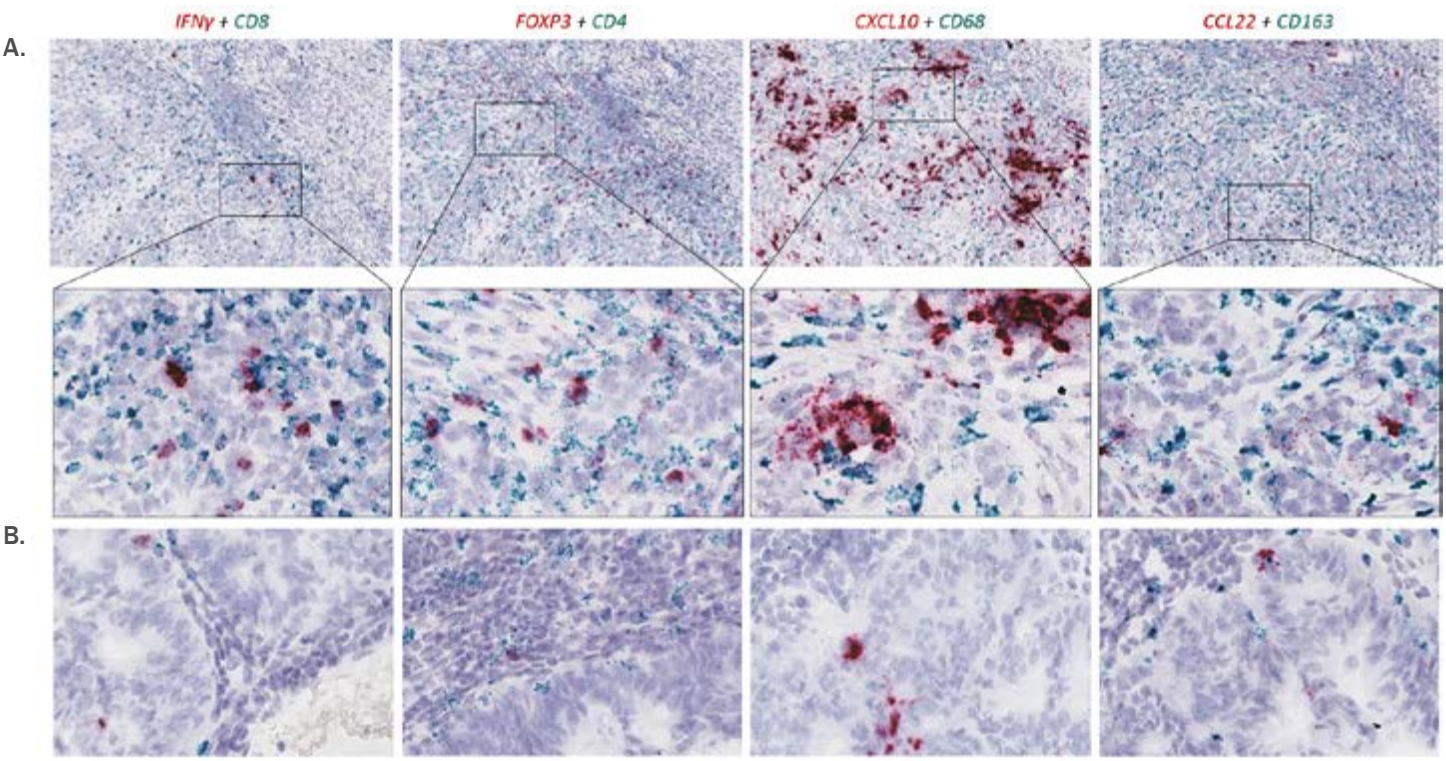


Figure 3. Characterization of Lymphoid and Myeloid Immune Cell Types and States in the Tumor Microenvironment Ovarian Cancer using the RNAscope 2.5 HD Duplex Assay on Formalin-Fixed, Paraffin-Embedded Tissue Sections. Representative images for immune cell infiltration in a highly inflamed ovarian tissue sample with abundant infiltration are shown of both lymphoid and myeloid immune cell types (**A**), and a tissue sample characterized by a limited number of infiltrating immune cells (**B**).

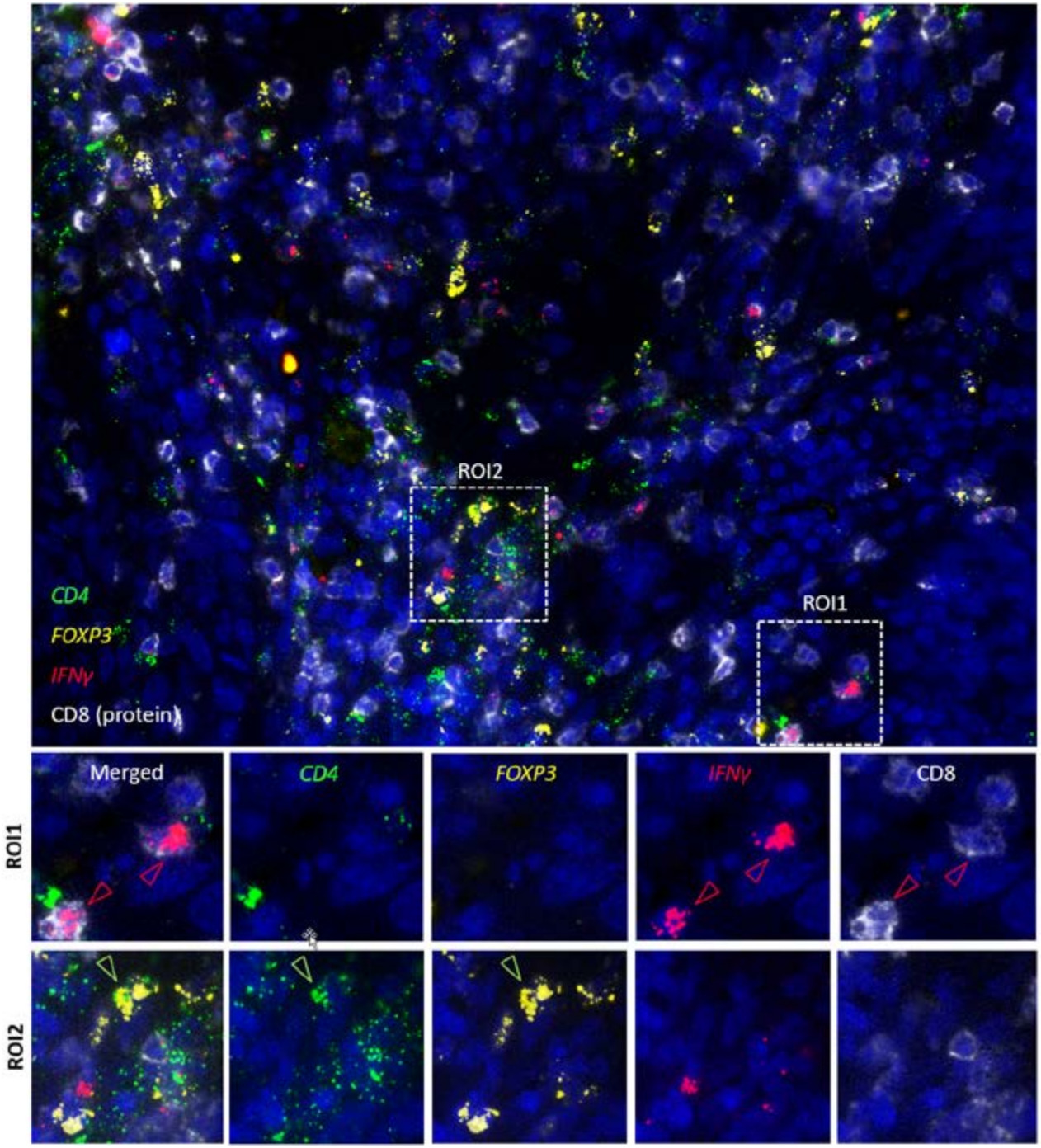


Figure 4. Visualization of Lymphoid Immune Cell Types in the Tumor Microenvironment of Non-Small Cell Lung Cancer in Formalin-Fixed, Paraffin-Embedded Tissue Samples Using the RNAscope LS Multiplex Fluorescent ISH Assay in Combination with Immunohistochemistry. Co-expression patterns indicative of immune cell types are visualized by ISH for three RNA targets followed by IHC for one protein. Here, probes for *CD4* mRNA (green), *FOXP3* mRNA (yellow), and *IFNγ* mRNA (red) were combined with CD8 antibody. Green arrows point out individual cells with co-localization of *CD4* and *FOXP3* mRNA, indicative of Treg cells. *IFNγ*$^+$CD8$^+$ CTLs are highlighted by red arrows.

Analysis of Subpopulations of Cells in the Tumor Microenvironment: Working with Limited Samples

The complexity of the tumor microenvironment presents unique challenges to immuno-oncology researchers. To analyze subpopulations of cells within this microenvironment, you may need to first isolate specific cell types to create samples that are highly enriched for your cells of interest. Isolation of specific cell subpopulations can be done through flow sorting or laser capture microdissection (LCM), both of which result in samples that may be too limited to analyze with traditional proteomic analysis tools.

Simple Western™ assays only need 3–10 µL of sample to measure protein abundance and/or post-translational modifications, and they do it all in a capillary. Dr. David Rosenberg at the Center for Molecular Medicine at the University of Connecticut Health Center used LCM to isolate aberrant crypt foci (ACF) with *KRAS* and *BRAF* mutations associated with colorectal cancer[6]. Before implementing Simple Western assays in their research, their proteomic analysis was limited to routine histological and immunohistological analysis due to small sample sizes. ACF biopsies were subjected to LCM, separated using the Simple Western Charge Assay and probed for total ERK1/2, pERK1, ppERK1, pERK2, and ppERK2. ERK1 and ERK2 phosphorylation of normal ACF samples were compared to *WT BRAF/KRAS* ACF, *KRAS*, and *BRAF* tissue. Increased levels of pERK1 and ppERK1 were observed in *WT BRAF/KRAS* and *KRAS* tissue only, whereas pERK1, ppERK1, pERK2 and ppERK2 levels were increased in all tissue samples (**Figure 5**). Simple Western assays gave Dr. Rosenberg a method that increased the informative potential of a single biopsy specimen which may ultimately enable his team to finally understand disease progression in their ACF samples at a molecular level.

Highly enriched or sparingly available samples can be challenging to measure with flow cytometry because of low epitope availability, poor flow antibodies or challenges during assay design and implementation. The Milo™ Single-Cell Western platform enables the analysis of protein expression heterogeneity in your enriched flow-sorted or sparingly available samples. As few as 10,000 cells can be loaded onto Milo's scWest chips to capture single cells for further protein expression analysis. Single-Cell Westerns on Milo were used by scientists at the University of North Carolina, Chapel Hill to analyze dissociated tissue biopsied from mouse small intestinal epithelium and flow-sorted to enrich for *SOX9-EGFP*-expressing cells. Cells were loaded onto scWest chips, and Milo was used to measure both *SOX9* and *GFP* expression along with a β-tubulin control in this sparingly available sample (**Figure 6A**). This analysis revealed that *SOX9* expression varied more than 30-fold across the cells analyzed (**Figure 6B**).

Learn More

Application Note: Breaking Laser Capture Microdissection Sample Size Road Blocks with Simple Western

Click here to read the full Application Note.

From Your Peers

"We collect samples from laser microdissection and were using our entire sample on just one regular Western blot. With Wes, we can do multiple assays with one sample collection."

- Mary Howell, Laboratory Coordinator, Department of Internal Medicine, East Tennessee State University

Click here to read the full story.

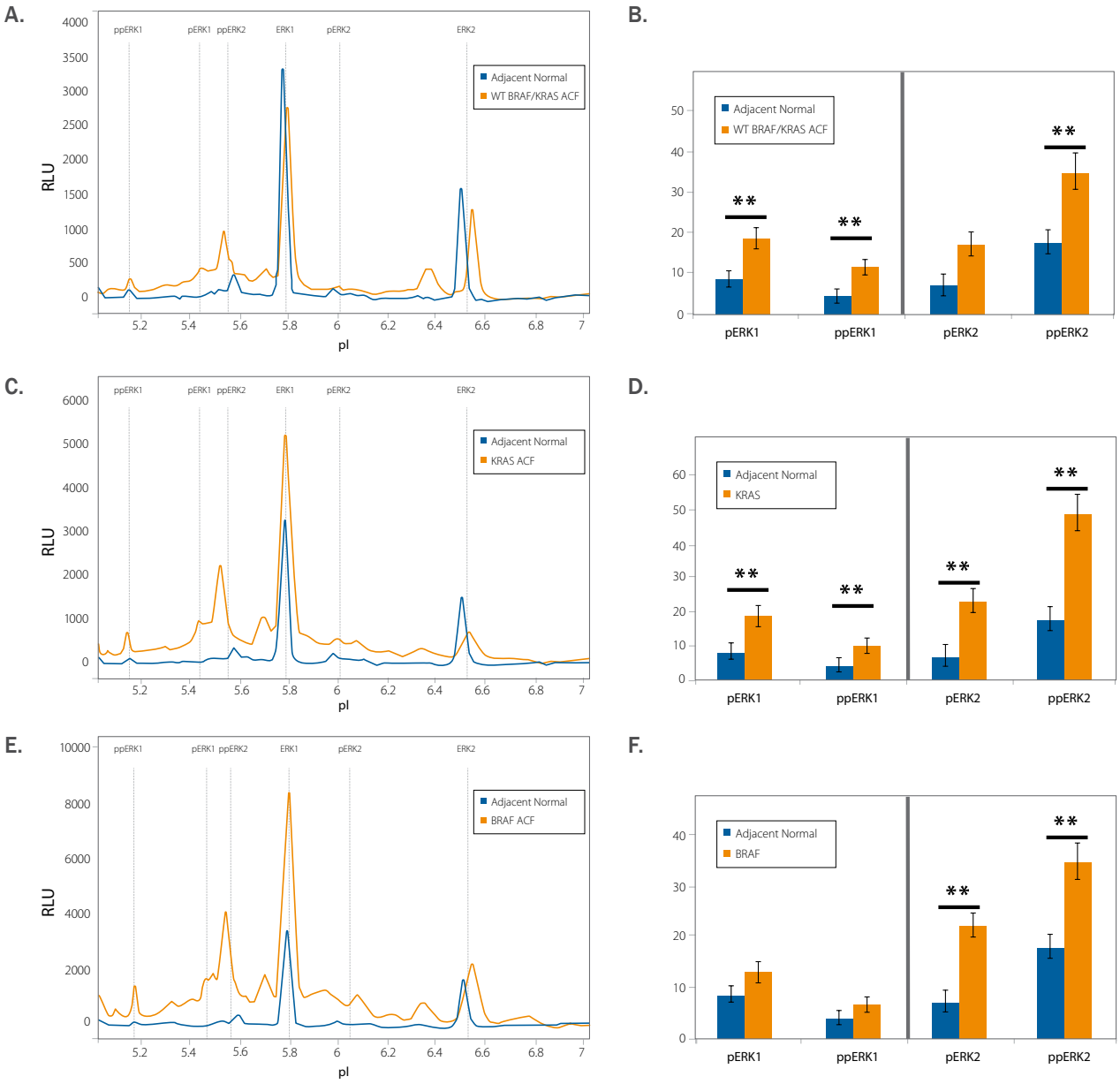


Figure 5. ERK1/2 Detected in Aberrant Crypt Foci. *WT KRAS/BRAS* (**A** and **B**), *KRAS* mutant (**C** and **D**) and *BRAF* (**E** and **F**) LCM samples (orange) were compared to adjacent normal tissue (blue) using Simple Western Charge. Phospho-ERK1 levels were only elevated in *WT BRAF/KRAS* and *KRAS* mutants, while pERK2 levels were increased in all ACF samples.

Individual cells can also be placed into the microwells on an scWest chip using a micromanipulator to do Single-Cell Western analysis on exceedingly rare cells like circulating tumor cells (CTCs). Elly Sinkala and colleagues from the University of California, Berkeley, used this method to detect eight protein targets in CTCs, enabling a detailed analysis of cellular profiles and the characterization of distinct biophysical phenotypes[7]. The precision and detection sensitivity of the assay eliminated the need for post-isolation cell culture and offered a "new approach to examining CTCs, with relevance spanning from understanding CTC biology to monitoring an individual's response to therapy," according to the paper authors.

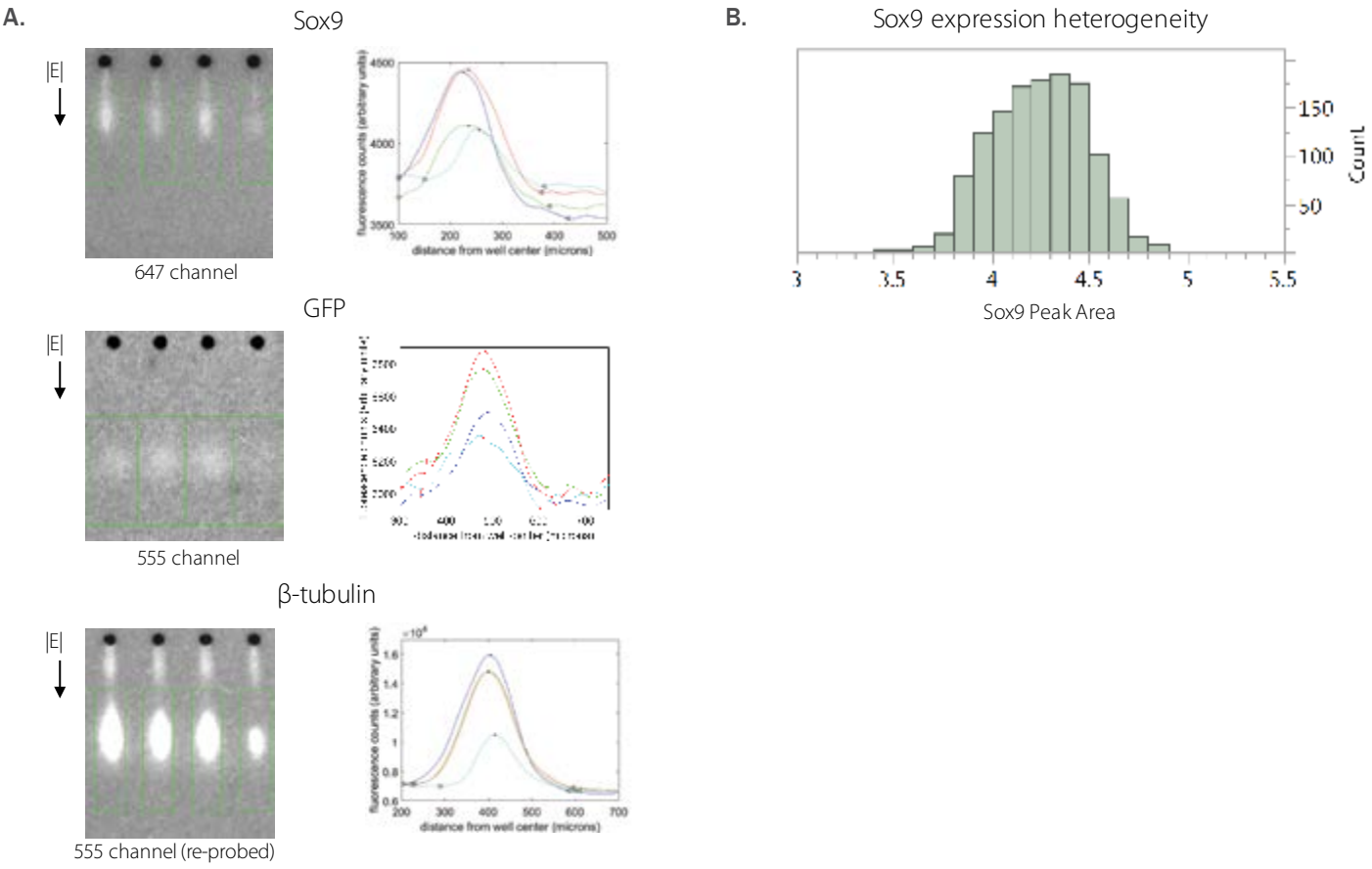


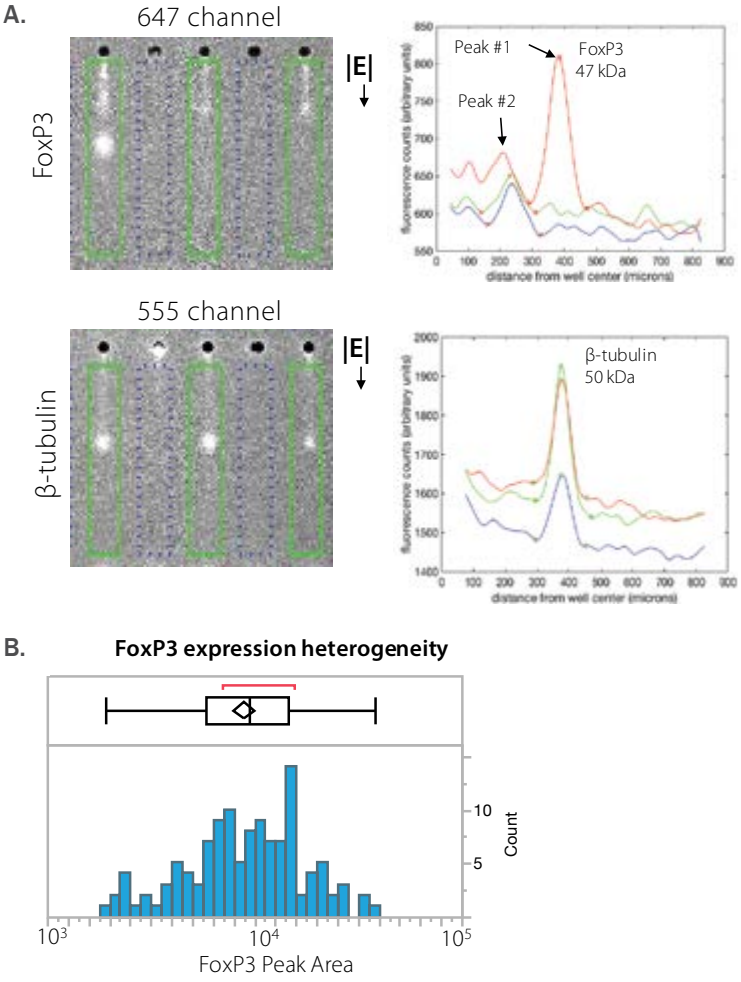
Figure 6. Single-Cell Westerns on Milo using *SOX9-EGFP*-Sorted Cells from Mouse Small Intestinal Epithelium. Analysis of *SOX9*, *GFP*, and β-tubulin expression using Milo (**A**). *SOX9* expression varied more than 30-fold across the cells analyzed (**B**).

Detection of Protein Targets Not Easily Measured with Flow Cytometry

Tregs express the transcription factor FOXP3, which is commonly used to identify this cell type. However, it can be challenging to measure FOXP3 using flow cytometry because there are limited high-quality flow-validated FOXP3 antibodies, and the nuclear location of FOXP3 can present challenges to assay implementation. Because Milo lyses the cells and uses one streamlined workflow to measure single-cell protein expression, the standard workflow enables detection of FOXP3 in peripheral blood mononuclear cells (PBMCs). **Figure 7A** shows FOXP3 and β-tubulin expression in three single cells. Fluorescence intensity plots for all three single-cell separations highlighted in green show clear peaks are detected for both FOXP3 and β-tubulin. FOXP3 expression varied by almost 10-fold across the individual PBMCs analyzed (**Figure 7B**). Single-Cell Westerns on Milo also enable precise enumeration of the number of cells that express the target-of-interest. In this case, 8% of the PBMCs express FOXP3, suggesting that 8% of the mixed sample of PMBCs are Tregs (**Figure 7C**).

This FOXP3 analysis also revealed the existence of a second peak running at a higher molecular weight (**Figure 7A**). This signal may be indicative of biologically relevant

information; for example, the peak could be caused by FOXP3 dimerization, interaction with other regulatory proteins or it could also reflect subcellular localization of FOXP3, as FOXP3 can be found in both the cytoplasm and the nucleus. Interestingly, it appears that these two FOXP3 peaks are orthogonally expressed—20% of cells stain positive for either Peak 1 or Peak 2, whereas only 1% stain positive for both peaks (**Figure 7D**). This novel observation relies on Milo's ability to resolve these two protein peaks in the molecular weight sizing separation, which could not be made with flow cytometry because it relies on immune binding alone for target detection. Although Tregs and other immune cell subtypes have traditionally been assayed via flow cytometry, the molecular weight sizing information provided by Single-Cell Westerns provides extra validation that the signals measured can be attributed to the target-of-interest. The ability to measure FOXP3 and identify Tregs within clinically relevant samples such as PBMCs could be key to the development of the next generation of immunotherapies with unprecedented efficacies.



C.

FoxP3+

β-tubulin+ Count Total %	False	True	Total
False	60 4.86	24 1.94	84 6.80
True	1054 85.34	97 7.85	1151 93.20
Total	1114 90.20	121 9.80	1235

D.

FoxP3 Peak #2+

FoxP3 Peak #1+ Count Total %	False	True	Total
False	984 79.68	130 10.53	1114 90.20
True	106 8.58	15 1.21	121 9.80
Total	1090 88.26	145 11.74	1235

Figure 7. Representative *FOXP3* and β-tubulin Expression from a Peripheral Blood Mononuclear Cell Sample. A second peak running at a higher molecular weight was observed in cells expressing *FOXP3* (**A**). *FOXP3* expression varied by almost 10-fold across the individual PBMCs analyzed (**B**). Analysis of cell heterogeneity for FOXP3 and β-tubulin shows the number and percent of positive and negative cells in the sample (**C**). Analysis of cell heterogeneity for *FOXP3* peak 1 and peak 2 shows the number and percent of positive and negative cells in the sample expressing one or the other peak (**D**).

References

1. Balkwill, RF, et al., The tumor microenvironment at a glance, 2012; *Journal of Cell Science*, 125: 5591–6.

2. Tao, L, et al., Cancer associated fibroblasts: An essential role in the tumor microenvironment, 2017; *Oncology Letters*, 14: 2611–20.

3. Frankel, T, et al., The role of tumor microenvironment in cancer immunotherapy. In: Kalinski P. (eds) Tumor Immune Microenvironment in Cancer Progression and Cancer Therapy. *Advances in Experimental Medicine and Biology*, 2017; 1036. Springer, Cham.

4. Ostrand-Rosenberg, S, and Fenselau, C, Myeloid derived suppressor cells: immune suppressive cells that impair antitumor immunity and are sculpted by their environment, 2018; *The Journal of Immunology*, 200: 422–31.

5. Wargo, JA, et al., Monitoring immune responses in the tumor microenvironment, 2016; *Current Opinion in Immunology*, 41: 23–31.

6. Drew, DA, et al., Nanoproteomic analysis of extracellular receptor kinase-1/2 post-translational activation in microdissected human hyperplastic colon lesions, 2013; *Proteomics*, 13: 1428–36.

7. Sinkala, E, et al., Profiling protein expression in circulating tumour cells using microfluidic western blotting, 2017; *Nature Communications*, 8: 14622.

Chapter 2:
Immune Checkpoints: Identification and Next Generation Targets

Immune checkpoints consist of a range of immunoregulatory pathways that enhance or moderate immune responses. Crucial in the checkpoint process are co-stimulatory and co-inhibitory molecules or proteins that show remarkable promise as therapeutic targets for cancer. Some examples include members of the B7, CD28, TIM, CD226 families, and others such as Galectin-9, LAG3, and CD155/PVR.

Immune checkpoint therapy targeting the PD-1/PD-L1 pathway is a new paradigm in cancer treatment with long-lasting clinical benefits across multiple types of cancer. However, most patients are resistant or will relapse after their initial response. Therefore, understanding the mechanisms behind both types of resistance is critical to overcoming current immuno-therapy challenges. Characterizing the tumor microenvironment for immune checkpoint inhibitor or biomarker expression with single-cell and spatial resolution can provide critical insight into new immunotherapeutic strategies and identify new predictive biomarkers for stratifying patients most likely to benefit from immunotherapies.

A great deal of immune checkpoint research has focused on T cell co-signaling because this mechanism is crucial for the T cell response to cancer. Want to learn more? Check out R&D System's interactive guide to T cell co-signaling.

The detection and accurate analysis of biomarkers are critical to immunotherapy research and treatment paradigms. The checkpoint protein PD-1 can be analyzed in a variety of tissue types using traditional techniques such as Western blotting, flow cytometry and immunohistochemistry (IHC) using human PD-1 antibody (R&D Systems, Catalog # AF1086) (**Figure 1**).

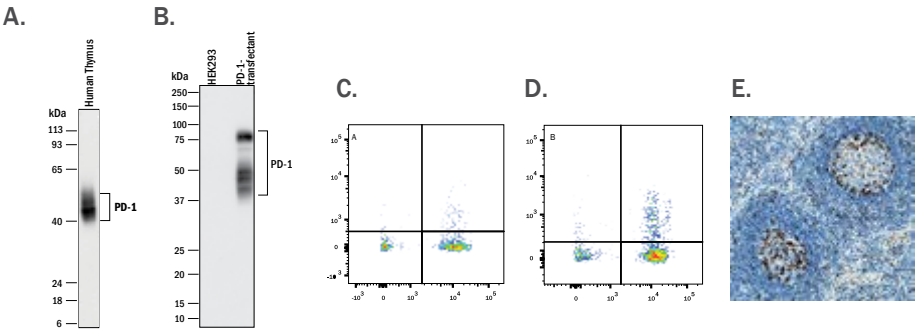
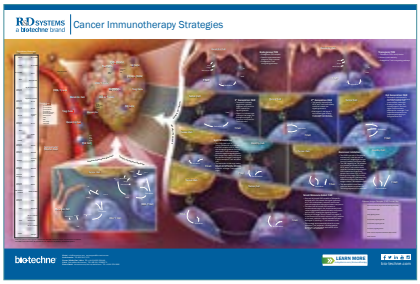


Figure 1. Detection of PD-1. Detection of human PD-1 by Western blot (**A, B**) in human thymus tissue lysate (**A**) and HEK293 human embryonic kidney cell line either mock transfected or transfected with human PD-1 (**B**). Detection of PD-1 by flow cytometry in untreated (**C**) or treated (**D**) human PMBCs. PD-1 expression in immersion-fixed, paraffin-embedded sections of human lymph tissue (**E**).

Cancer Immunotherapy Strategies



Click here to download or request your copy of R&D Systems Cancer Immunotherapy Poster.

Quantitative Assays for Measuring Checkpoint Expression

Accuracy and consistency are important prerequisites for assessing checkpoint targets or putative biomarkers. R&D Systems Quantikine® ELISAs are the gold standard for ELISAs. They are complete, fully validated, ready-to-run sandwich ELISAs that are designed to provide the highest levels of specificity, accuracy, precision, and sensitivity in analyte quantification. Kit performance relies heavily on the selection of quality antibody pairs and rigorous in-house testing throughout the development process including component and kit stability, sensitivity, linearity, recovery, intra- and inter-assay precision, and cross-reactivity and interference testing with related analytes to confirm assay specificity. This stringent validation testing is used to optimize assay performance and verify that each kit will provide reproducible results both well-to-well and lot-to-lot. By carefully considering each of these variables before the product is released, scientists at R&D Systems take every step possible to ensure that Quantikine ELISA Kits will provide customers with reliable, consistent results without the need for further assay optimization. Click here to learn more!

For example, the Quantikine Human/Cynomolgus Monkey PD-L1/B7-H1 Immunoassay is a 4.5-hour, solid-phase ELISA designed to measure human/cynomolgus monkey B7-H1 in cell culture supernates, cell lysates, serum, plasma, and urine (**Figure 2**). It contains HEK293-expressed recombinant human B7-H1 and accurately quantitates the recombinant factor. Results obtained using natural human/cynomolgus monkey B7-H1 showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural human/cynomolgus monkey B7-H1.

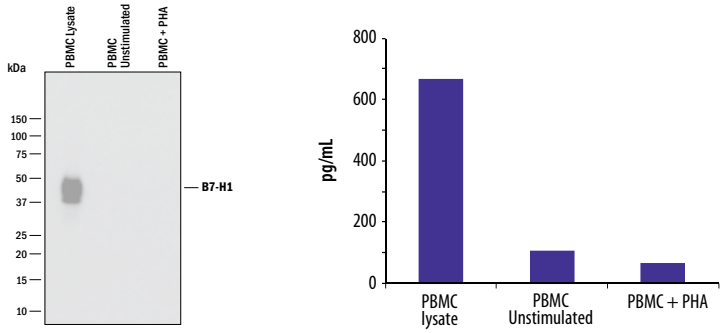


Figure 2: The Quantikine ELISA Detects Endogenous B7-H1/PD-L1 in Human Peripheral Blood Mononuclear Cells. Lysates from human peripheral blood mononuclear cells (PBMCs) cultured overnight in RPMI-10% FBS, and conditioned media from human PBMCs unstimulated or stimulated with Phytohemagglutinin (PHA) for six days were analyzed by Western Blot and Quantikine® ELISA. Samples were resolved under reducing SDS-PAGE conditions, transferred to a PVDF membrane, and immunoblotted with the detection antibody used in this kit. The Western Blot band intensity shows a direct correlation with ELISA sample values. The conditioned media samples are below the level of detection in the Western Blot.

R&D Systems Quantikine® ELISAs are a powerful tool for reliably and quantitatively assessing soluble B7-H1/PD-L1 levels. Read the R&D Systems white paper, Soluble B7-H1/PD-L1 Levels in Multiple Cancer Subtypes: High Sensitivity Measurement by Immunoassay, **Click here to learn more.**

Traditional ELISAs are not always sensitive enough to detect endogenous levels of PD-L1 (**Figure 3**). Ella's Simple Plex PD-L1 assay overcomes this challenge, allowing for the detection of single-digit picogram levels of endogenous PD-L1 with single-digit coefficient of variations.

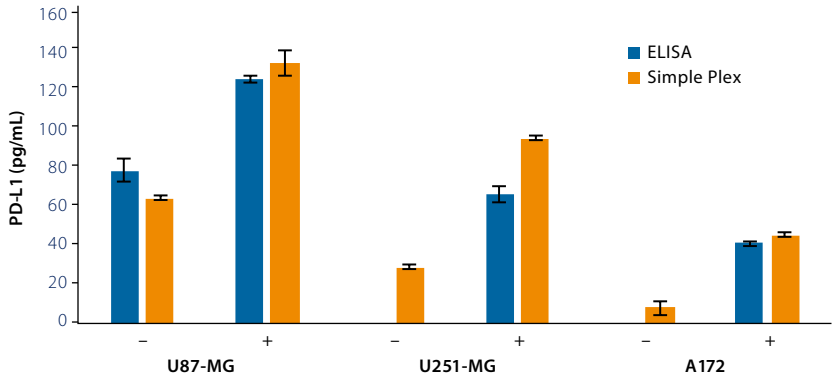


Figure 3. Traditional Sandwich ELISA and Simple Plex Assays Both Detect Similar Fold Increases in PD-L1 Expression After Phorbol 12-Myristate 13-Acetate Treatment. The Simple Plex assay detected PD-L1 in all samples, but the traditional ELISA did not detect PD-L1 expression in control samples below its lower limit of quantitation.

Learn More

Application Note: High Fidelity Detection of Endogenous PD-L1 at Low Picogram Levels with Simple Plex Assays.
Click here to read the Application Note.

To better understand immune checkpoint targets, you may need to move beyond traditional techniques like IHC to tools that can characterize single-cell expression profiles of immune checkpoint targets such as the RNAscope technology. The RNAscope technology enables rapid and efficient detection of co-expression profiles of any target mRNAs including checkpoint and cell-specific markers, with single-molecule sensitivity and high specificity in formalin-fixed, paraffin-embedded (FFPE), fresh frozen, and fixed frozen tissue samples.

Non-small cell lung cancer FFPE samples were examined using the RNAscope Duplex 2.5 HD assay (Advanced Cell Diagnostics, Inc., Catalog # 322500) to characterize the single-cell co-expression profiles of multiple therapeutic checkpoint targets. **Figure 4** illustrates the assay and data analysis workflow for a non-small cell lung cancer (NSCLC) checkpoint study.

In this NSCLC study, the expression of *PD-L1* mRNA was visualized in combination with other checkpoint markers (*PD-L2*, *TIM3*, *LAG3*, and *CTLA-4*) in individual cells within the morphological tissue context (**Figure 5**). Arrows point out single cells co-expressing two checkpoint markers offering possible insights into resistance mechanisms and combinatorial checkpoint therapies. Also, different expression levels of *PD-L1* and *PD-L2* mRNA were detected across tumor cells pointing to a heterogeneous expression pattern for these two checkpoint markers in tumor cells (**Figure 6**).

On the Trail of Immuno-Oncology Monitoring with Luminex and Simple Plex

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to learn how Luminex and Simple Plex can screen and validate multiple targets accurately for immuno-oncology.

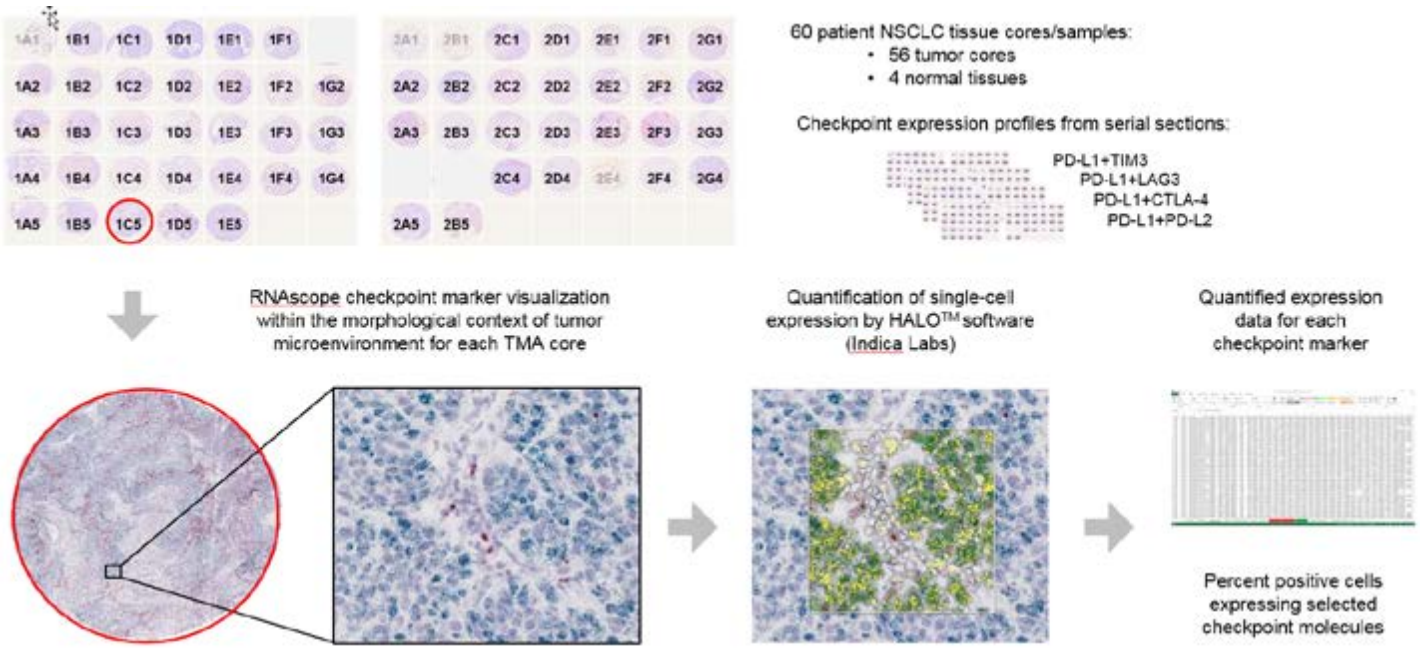

Figure 4. Schematic of the RNAscope Assay Workflow and Possible Data Analysis for the Non-Small Cell Lung Cancer Checkpoint Study.

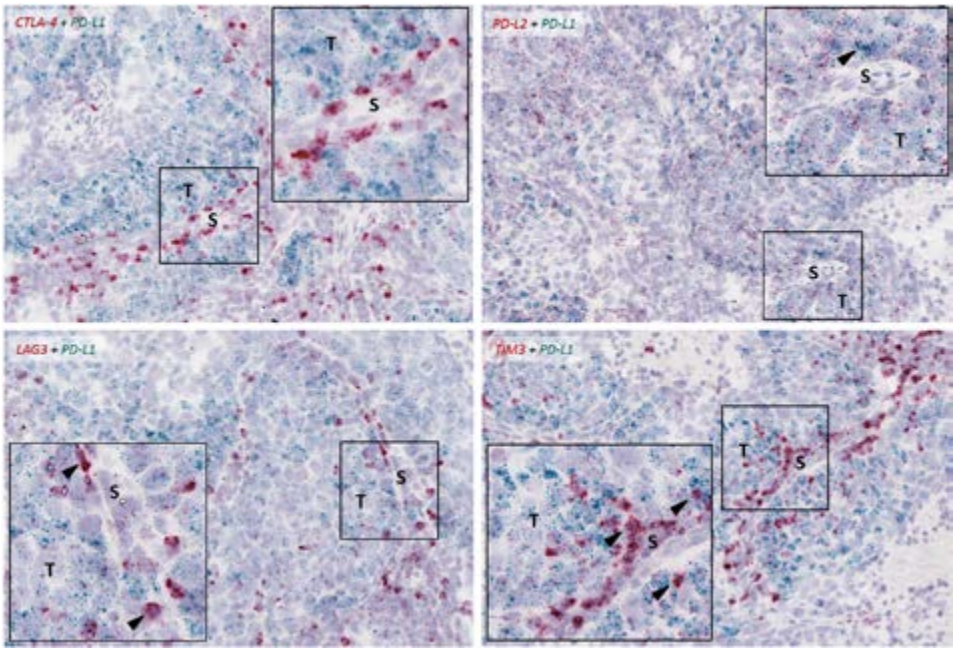

Figure 5. Expression and Single-Cell Co-Expression Profiles of Therapeutic Checkpoint Targets in Non-Small Cell Lung Cancer Formalin-Fixed, Paraffin-Embedded Tissue Samples Using the RNAscope 2.5 HD Duplex Assay. The expression of *PD-L1* was visualized in combination with other checkpoint markers (*PD-L2*, *TIM3*, *LAG3*, and *CTLA-4*) in individual cells within the morphological tissue context. Arrows indicate cells with co-expression of checkpoint targets.

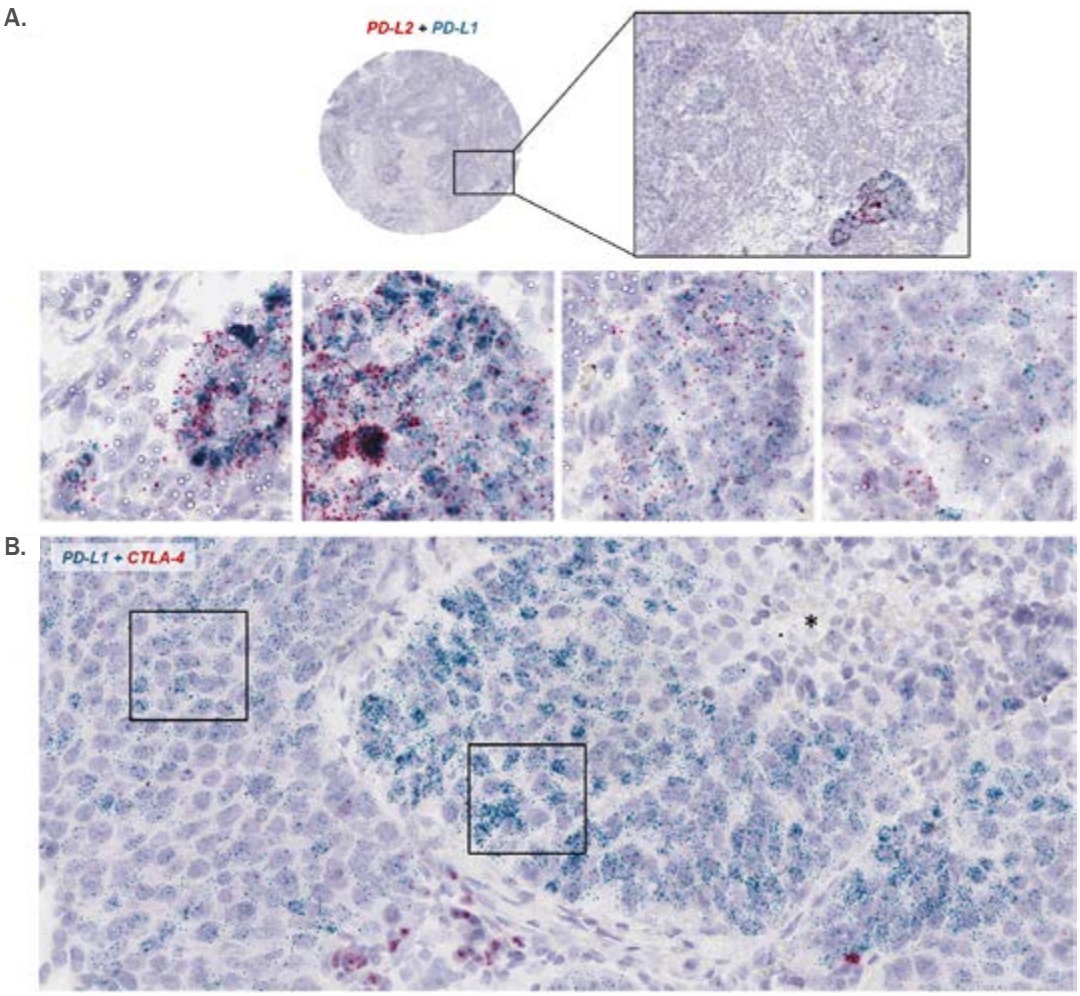

Figure 6. Visualization of Heterogeneous Expression of Immune Checkpoint Markers in the Tumor Microenvironment of Non-Small Cell Lung Cancer Formalin-Fixed, Paraffin-Embedded Tissue Samples Using the RNAscope 2.5 HD Duplex Assay. Heterogeneous expression for both *PD-L1* (*green*) and *PD-L2* (*red*) in tumor cells is shown in four higher magnification insets selected from the area of interest in the FFPE tissue core (**A**). Different levels of *PD-L1* mRNA expression (*green*) are detected across tumor cells pointing to a heterogeneous expression pattern for *PD-L1* (**B**). Tumor areas with high and low *PD-L1* expression are indicated by the rectangles. Note, no *PD-L1* expression is detected in the necrotic area (*).

This technique can also be combined with IHC as demonstrated by the simultaneous RNAscope ISH detection of three checkpoint targets *PD1*, *TIM3*, and *PD-L1* followed by IHC for CD45 (Agilent Technologies, Inc., M070129-2) as an immune cell marker in human FFPE lung cancer tissue (**Figure 7**).

These applications demonstrate the utility of the highly specific and sensitive RNAscope ISH platform in helping to understand the mechanisms adopted by cancer cells to evade the host immune surveillance and ultimately developing resistance against checkpoint blockades.

In addition, multiple publications have used the RNAscope assay for the mRNA detection of checkpoint marker *PD-L1* across a variety of applications in human cancers. Schalper et al. performed a retrospective analysis on two sets of tissue microarrays with 636 stage I–III breast carcinoma patient samples using the

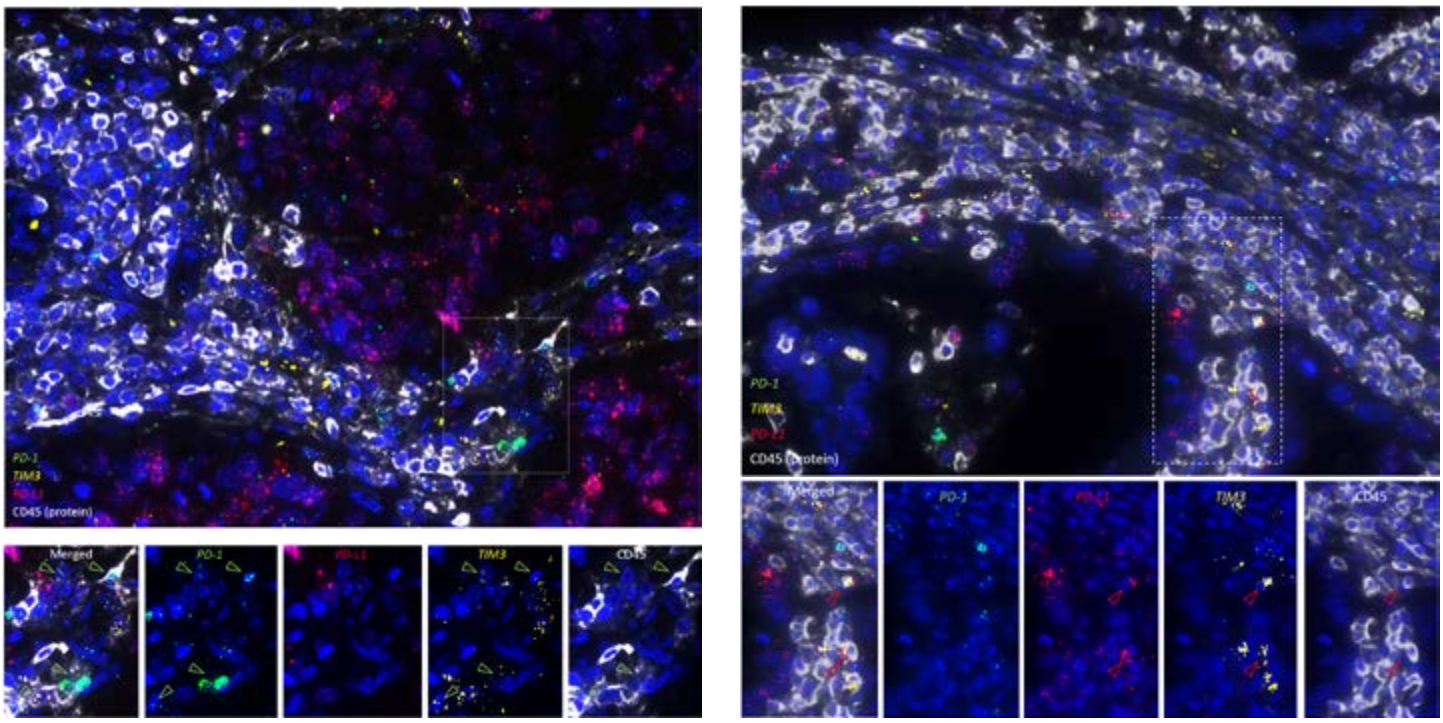

Figure 7. Immune Checkpoint Co-Expression Analysis at the Single Cell Level in Formalin-Fixed, Paraffin-Embedded Human Lung Cancer Using the RNAscope LS Multiplex Fluorescent *In Situ* Hybridization Assay Combined with Immunohistochemistry. The RNAscope Multiplex Fluorescent assay was used to simultaneously detect three checkpoint targets *PD1* (green), *TIM3* (yellow), and *PD-L1* (red) followed by IHC for CD45 as an immune cell marker. Co-expression is shown in single cells of *PD-1* and *TIM3* (green arrows) and *PD-L1* and *TIM3* (red arrows).

fluorescent RNAscope assay and showed that *PD-L1* mRNA expression was identified within the tumor (cytokeratin-positive) compartment of nearly 60% of breast tumors[1]. More specifically, *PD-L1* mRNA expression levels were correlated with increased tumor-infiltrating lymphocytes and better clinical outcome (recurrence-free survival), illustrating the possible value of *PD-L1* mRNA expression as a prognostic biomarker in breast carcinomas and the use of the RNAscope assay as a reproducible, antibody-independent, and compartment-specific method of mRNA detection. Research by Lee et al. has shed light onto the mechanism adopted by long-lived CD44+ tumor-initiating cells to selectively evade host immune responses by preferential expression of *PD-L1*, and therefore, provides a rationale for adjuvant PD-1/PD-L1 axis blockade in head and neck squamous cell carcinoma[2]. The RNAscope 2.5 HD Duplex assay was used to demonstrate the co-localization of *PD-L1* and *CD44* mRNA, and these ISH results confirmed flow cytometry and RT-PCR findings showing higher expression of PD-L1 in CD44+ cells. Dolled-Filhart et al. used the RNAscope assay to support the development of a prototype IHC assay for the anti–PD-L1 antibody clone 22C3. A comparison of the distribution pattern for PD-L1 protein expression (IHC) and *PD-L1* mRNA (ISH) confirmed the specificity of clone 22C3 within NSCLC cell pellets and intact human tonsil tissue samples[3].

Characterization of immune checkpoint expression and infiltrating immune cells in the tumor microenvironment by RNAscope ISH

In this on-demand webinar

Annelies Laeremans, Ph.D., discusses *in situ* visualization of immune checkpoint targets at the single-cell level in the tumor microenvironment of NSCLC and ovarian cancer archived FFPE tissue samples using the highly specific and sensitive *in situ* hybridization assay RNAscope.

Explore Next Generation Immune Checkpoint Targets

The blockade of immune checkpoint molecules such as CTLA-4 and PD-1/PD-L1 is a promising therapeutic approach for cancer treatment. Unfortunately, blocking CTLA-4 or PD-1/PD-L1 is not universally successful across different cancer types and patients. Co-targeting multiple checkpoint molecules simultaneously could improve the effectiveness of this strategy. For this reason and others, investigators are also examining next-generation immune checkpoint targets.

Butryophilins

Butryophilins are a novel class of B7-related type 1 transmembrane proteins. These proteins have co-stimulatory or co-inhibitory effects on T function, raising the possibility that they are potential immunotherapy drug targets. R&D Systems is the exclusive source of bioactive Butyrophilin proteins and antibodies. Learn More!

VISTA/VSIG

B7 family members and their receptors play a central role in the regulation of T cell responses through T cell co-stimulatory and co-inhibitory pathways that constitute very attractive targets for the development of immunotherapeutic drugs. Recent work from R&D Systems scientists has shown that V-Set and Immunoglobulin domain-containing 3 (VSIG-3) is a novel ligand of the V domain-containing Ig suppressor of T cell activation (VISTA). In this study, we report that VSIG-3/IGSF11 is a ligand of the B7 family member VISTA/PD-1H that inhibits human T cell functions through a novel VSIG-3/VISTA pathway. An extensive functional ELISA binding screening assay reveals that VSIG-3 binds the new B7 family member VISTA but does not interact with other known members of the B7 family. Furthermore, VSIG-3 inhibits human T cell proliferation in the presence of T cell receptor signaling. VSIG-3 significantly reduces cytokine and chemokine production by human T cells including IFN-γ, IL-2, IL-17, CCL5/RANTES, CCL3/MIP-1α, and CXCL11/I-TAC. Anti-VISTA neutralization antibodies attenuate the binding of VSIG-3 to VISTA, as well as VSIG-3-induced T cell inhibition. Thus, we have identified a novel B7 pathway that can inhibit human T cell proliferation and cytokine production. This unique VSIG-3/VISTA co-inhibitory pathway may provide new strategies for the treatment of human cancers, autoimmune disorders, infection, and transplant rejection, and may help in the design of improved vaccines.

B7-related Butryophilins as Immune Modulators

In this on-demand webinar
Christian Erickson, B.S., discusses novel immunomodulatory proteins with structure and functions similar to the B7 family.

Immune Modulation by Butyrophilin 1A1 (BTN1A1)

Download this poster
to learn how BTN1A1 acts as a co-inhibitory molecule to modulate T cells through an unknown receptor on the surface of T cells. BTN1A1 may be a potential target of immune checkpoint molecules for therapeutic purposes.

VSIG-3/IGSF11 is a Ligand of VISTA/PD-1H and Inhibits Human T Cell Function

Download this poster
to learn how VSIG-3 was identified as a novel ligand for VISTA and how the engagement of VSIG-3 with VISTA on activated T cells inhibits T cell proliferation as well as cytokine and chemokine production.

A Role for VSIG3 in Acquired Resistance to Anti-PD-1 Therapy in Metastatic Melanoma?
Read our blog post!

LILRA/B

Leukocyte immunoglobulin-like receptors A/B (LILRA/B) are expressed on several cell types including B cells, natural killer (NK) cells, and T cell subsets among others. LILRAs are stimulatory while LILRBs are inhibitory[4]. The LILRB subset contains immunoreceptor tyrosine-based inhibitory motifs also found on other promising immune checkpoint targets such as PD-1, BTLA, and TIGIT. Given their structure and role in immune cell function, it is not surprising that these proteins represent potential checkpoint targets. Learn More!

Metabolic Checkpoint Inhibitors

Finally, elements of the kynurenine pathway are potential immune checkpoint targets[5]. Indoleamine 2,3-dioxygenase 1 (IDO1) and Tryptophan 2,3-dioxygenase 2 (TDO2) catalyze the first step of the kynurenine pathway responsible for the conversion of tryptophan to N-formyl-kynurenine then kynurenine. High levels of IDO/TDO activity in tumor cells are thought to contribute to lowered extracellular tryptophan levels and an associated immunosuppressive environment. Additional downstream enzymes in the pathway include Kynurenine 3-Monooxygenase, and Kynurenine Aminotransferases (KAT), and Kynureninase. KAT, specifically, converts kynurenine to kynurenic acid. Both kynurenine and kynurenic acid are agonists for aryl hydrocarbon receptor, a transcription factor that either directly or indirectly leads to the activation of immunosuppressive regulatory T cells and dendritic cells. Indeed, both IDO and TDO-2 are recognized for their potential as immune checkpoint targets and are currently being evaluated for clinical applications. R&D Systems has a wide variety of high-value enzymes from the Kynurenine Pathway including TDO2 (**Figure 8**). Learn More!

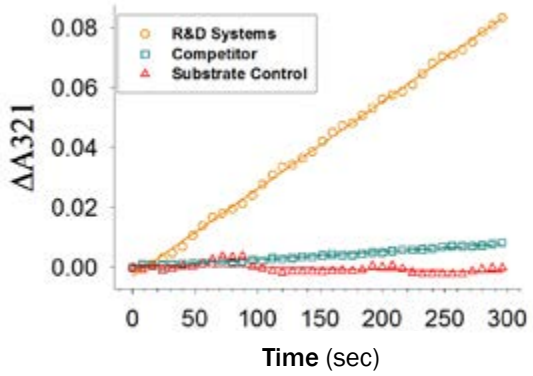

Figure 8. Recombinant Human TDO2 is Measured by its Ability to Oxidize L-tryptophan to N-formyl-kynurenine. The activity (orange) is approximately 10-fold greater than the competitor's TDO2 (green).

References

1. Schalper, KA, et al., *In situ* tumor PD-L1 mRNA expression is associated with increased TILs and better outcome in breast carcinomas, 2014; *Clinical Cancer Research*, 20: 2773–82.

2. Lee, Y, et al., CD44+ cells in head and neck squamous cell carcinoma suppress T-cell–mediated immunity by selective constitutive and inducible expression of PD-L1, 2016; *Clinical Cancer Research*, 22: 3571–81.

3. Dolled-Filhart, M, et al., Development of a prototype immunohistochemistry assay to measure programmed death ligand-1 expression in tumor tissue, 2016; *Archives of Pathology & Laboratory Medicine*, 140: 1259–66.

4. Van der Touw, W, et al., LILRB receptor-mediated regulation of myeloid cell maturation and function, 2017; *Cancer immunology, immunotherapy*: CII, 66.8: 1079–87.

5. Weng, T, et al., Recent discovery of indoleamine-2,3-dioxygenase 1 inhibitors targeting cancer immunotherapy, 2018; *European Journal of Medicinal Chemistry,* 143: 656–69.

Chapter 3:
Therapeutic Monoclonal Antibodies: Analysis & Characterization

Monoclonal antibodies (mAbs) have emerged as first-line therapeutic agents for various cancer types. In addition to their large mass and complex structure, mAbs are varied in their origin, makeup, effector function, and delivery. Therefore, mAbs require thorough formulation development, including characterization, quantitation, and preservation. Quality control is critical to maintaining not only the product's quality, but also safety—mAbs are sensitive to the manufacturing process, and precision during manufacturing is essential to avoid variations in the product protein.

Regulatory agencies require that all biopharmaceutical companies monitor critical quality attributes for the commercialization of any mAb therapeutic product. Accurately doing so is dependent on the sensitivity and resolution of your analytical system. During mAb development, the analysis should afford high sensitivity to post-translational modifications such as glycosylation and phosphorylation and should adequately resolve the size/fragment heterogeneity often inherent in complex protein therapeutics.

Charge Variant Analysis of Monoclonal Antibodies

Imaged capillary isoelectric focusing (icIEF) technology is widely regarded as the standard methodology for separating proteins or peptides based on their isoelectric points (pIs). During icIEF, a capillary is filled with the study sample and an ampholyte mixture. Upon the application of an electric field, the ampholytes form a pH gradient in the capillary. The charged protein migrates through the pH gradient in response to the applied voltage until it reaches the pH value that is equivalent to the protein's pI, at which point the migration stops due to a net neutral charge. In this way, each protein in a sample becomes "focused" according to its specific pI. The entire capillary is then imaged to generate a charge heterogeneity profile of the sample under study. pI values and relative peak areas are examined to create sample profiles. icIEF profiling can be applied throughout the manufacturing process of mAbs from scale-up to formulation development and stability testing to quality assurance of lot-to-lot consistency, ensuring the necessary quality attributes of the mAb.

The value of icIEF technology was validated by an interlaboratory study with ten participating biopharmaceutical companies, where the use of icIEF for mAb charge heterogeneity was evaluated[1]. The study determined the technique showed good precision with relative standard deviation values of <0.8%, and the authors reported: "Reproducible quantitative values of the apparent pI and the percent peak area of the charged variants were obtained using different analysts, different instruments of the same equipment model, and different ampholytes lots." The authors concluded, "These results validate the appropriate use of imaged capillary

isoelectric focusing in the biopharmaceutical industry in support of process development and regulatory submissions of therapeutic antibodies."

The Maurice™ system brings icIEF technology to the next level by vastly simplifying the charge heterogeneity analysis workflow. There's no transfer line, capillary and switch valve maintenance, or tedious cartridge installation procedures at the beginning of the run. The cartridge, samples, and reagents are simply loaded into Maurice, batch parameters are then set up, and the preparation is finished by hitting Start.

In addition to UV detection applied in earlier icIEF systems, Maurice's native fluorescence detection for cIEF measures the fluorescence emission of tryptophan's aromatic group. It's label-free, so time is not spent optimizing protein labeling or dealing with the background noise that results when a label unconjugates from the protein. Baselines are significantly cleaner and less sensitive to ampholyte interference, allowing for more options when optimizing the pH gradient. To determine the linear dynamic range of Maurice's cIEF fluorescence mode, an mAb was serially diluted two-fold from 125 µg/mL down to 0.5 µg/mL in 1X Sample Buffer (**Figure 1**). The total peak area was extremely linear across 2-logs with an R^2 of 0.999.

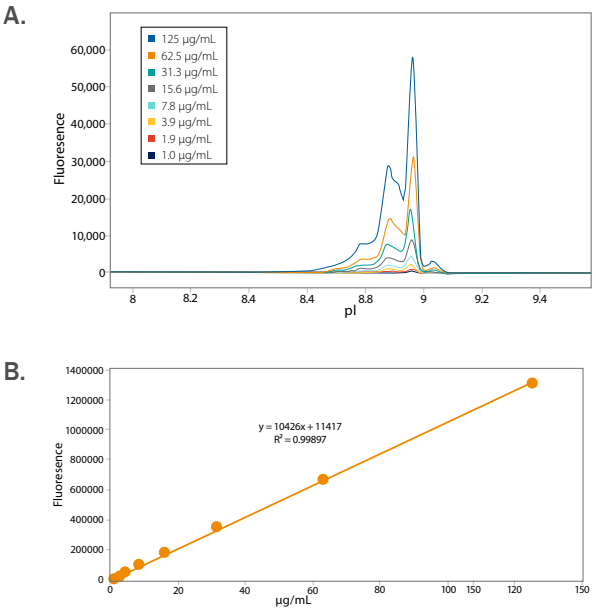

Figure 1. Monoclonal Antibody Analysis with Maurice. A monoclonal antibody was serially diluted from 125 µg/mL down to 0.5 µg/mL to demonstrate the linearity of fluorescence on Maurice (top). Linear regression of the total peak area demonstrates 2-log linearity with an R^2 of 0.999 (bottom).

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Application Note: Improving Charge Variant Analysis with Maurice Native Fluorescence
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Figure 2 illustrates data equivalency for cIEF analysis of mAb11 on Maurice, iCE3, and iCE280 systems. Six peaks were detected with a main peak at 7.2 around 0.15 absorption units (Figure 2). The pI values were extremely consistent, with no variation regardless of the instrument used for analysis (Table 1). The coefficient of variations (CVs) for all systems were ≤0.2%. Peak composition percentages were all within 2%, even for the minor peaks, demonstrating data equivalency across systems (Table 2). The iCE280 and iCE3 systems and Maurice, using the cIEF absorbance mode, were precise with CVs for peaks >10% peak composition, all under 11.5%. Data generated on Maurice were particularly precise with CVs ≤2.4% for peaks greater than 10% composition.

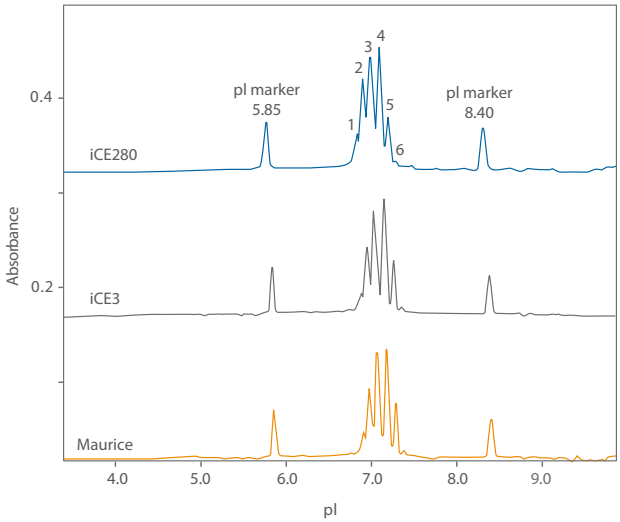

Figure 2. Maurice, iCE3, and iCE280 Data Equivalency for cIEF Charge Heterogeneity Absorbance Assays

Learn More

Application Note: Maurice, iCE3, and iCE280 Data Equivalency for cIEF Charge Heterogeneity Absorbance Assays

Click here to read the Application Note.

AVERAGE pI, (n=36)			
PEAK	iCE280 (% CV)	iCE3 (% CV)	MAURICE (% CV)
1	6.9 (0.1%)	6.9 (0.1%)	6.9 (0.0%)
2	7.0 (0.1%)	7.0 (0.1%)	7.0 (0.0%)
3	7.1 (0.2%)	7.1 (0.1%)	7.1 (0.1%)
4	7.2 (0.1%)	7.2 (0.1%)	7.2 (0.0%)
5	7.3 (0.1%)	7.3 (0.0%)	7.3 (0.0%)
6	7.4 (0.2%)	7.4 (0.1%)	7.4 (0.0%)

Table 1. mAb11 pI Values, with % Coefficient of Variations in Parenthesis, Across All Three iCE Systems.

AVERAGE % PEAK COMPOSITION, (n=36)			
PEAK	iCE280 (% CV)	iCE3 (% CV)	MAURICE (% CV)
1	7.8% (14.5%)	6.2% (11.8%)	7.1% (9.0%)
2	20.5% (11.5%)	18.5% (4.0%)	18.4% (2.4%)
3	32.1% (6.0%)	32.7% (2.2%)	32.5% (1.4%)
4	28.2% (3.4%)	30.0% (1.9%)	29.5% (1.0%)
5	10.4% (7.1%)	11.5% (3.2%)	11.2% (1.3%)
6	1.0% (18.5%)	1.1% (10.0%)	1.3% (7.5%)

Table 2. mAb11 Average % Peak Composition, with % Coefficient of Variations in Parenthesis, Across All Three iCE Systems.

Characterization of Monoclonal Antibody Size Variants

mAb fragmentation and size migration assessments have evolved from gel (SDS-PAGE) to capillary electrophoresis (CE-SDS). CE-SDS is a high-throughput analytical technology equipped with quantitative data integration that has effectively reduced turnaround time during screening and development. CE-SDS separation and analysis with Maurice can assess the purity and identity of therapeutic monoclonal antibodies using a much simpler workflow that results in highly robust, high-quality data.

The study sample is loaded into a capillary and proteins are separated by size using an entangled polymer separation matrix. Separation is monitored in real-time as peaks pass a single-point detector. The peak relative migration time is compared with an internal standard or in relation to any designated peak. The reduced IgG method provides fast, consistent baseline resolution of the non-glycosylated heavy chain and heavy chain in only 25 minutes (**Figure 3A**). The non-reduced IgG method is optimized for IgG fragment detection and only requires a 35-minute separation time to resolve all IgG fragments and glycosylated/non-glycosylated intact IgG (**Figure 3B**).

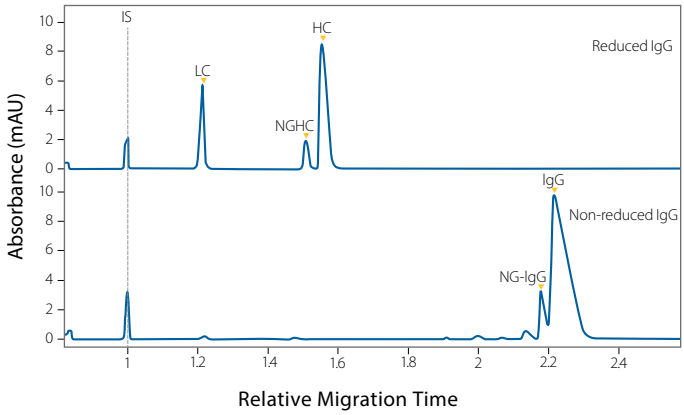

Figure 3. Separation of the Reduced (top) and Non-Reduced (bottom) Maurice IgG Standard with the 10 kDa Internal Standard. The IgG standard was reconstituted to 1 µg/mL with 1X Sample Buffer containing SDS. Samples were reduced with β-ME and non-reduced samples alkylated with IAM before heat denaturing at 70 °C for 10 minutes. Reduced and non-reduced samples were separated for 25 and 35 minutes, respectively.

References

1. Salas-Solano, O, et al., Robustness of iCIEF methodology for the analysis of monoclonal antibodies: an interlaboratory study, 2012; *Journal of Separation Science*, 35: 3124–9.

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Application Note: Sizing-up IgG with Maurice's CE-SDS Application

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Let Maurice help you:

- Resolve molecules with complex charge profiles
- Develop reproducible platform methods in a day
- Analyze samples at low concentrations to prevent aggregation
- Meet all your 21 CFR compliance needs

Watch Webinar

In this webinar

our panelists discuss the application of Maurice for biopharmaceutical analysis including an evaluation of Maurice for biopharmaceutical charge and size profiling; a comparability study of Maurice, iCE3, and a conventional CE system; applications to fluorescence detection and non-glycosylated mAb quantitation; and the characterization of lipid nanoparticles

Chapter 4:
Small Molecules & Immuno-Oncology

Recent clinical successes in targeting the immune system for the treatment of cancer have focused on the use of biologicals which include antibodies, proteins, engineered cells, and oncolytic viruses. However, small molecules directed at the immune system offer several advantages over biologicals; they can target intracellular targets that protein-based therapeutic agents cannot access, they have high oral bioavailability, and they reach high levels of distribution in the local tumor microenvironment. Additionally, small molecules can regulate immunosuppressive cell types such as TAMs and DCs, which are not directly regulated by checkpoint inhibitors. **Table 1** presents a selection of cancer immunology products available from Tocris Bioscience.

Cat No.	Product Name	Description	
3700	Imiquimod	TLR7 agonist	TRL7 activation induces the release of proinflammatory cytokines and activation of NF-κB signaling. Imiquimod activates antigen presenting cells and antitumor cellular immune response.
5698	1-Methyl-D-tryptophan	IDO inhibitor	IDO activity in tumors inhibits proliferation and induces apoptosis of T cells. Inhibition with 1-Methyl-D-tryptophan enhances the antitumor immune response of T cells *in vitro*.
5794	LM 10	TDO inhibitor	TDO is expressed in tumors and prevents immune rejection of tumor cells. Inhibition with LM 10 restores the ability of mice to reject TDO-expressing tumors.
5872	AZ 10397767	CXCR2 antagonist	CXCR2 ligands recruit neutrophils to tumors. Antagonism with AZ 10397767 reduces recruitment both *in vitro* and *in vivo* and is associated with slower growing tumors.
5945	2′,3′-cGAMP sodium salt	STING agonist	2′,3′-cGAMP binds to a stimulator of interferon genes (STING) to activate the innate immune response. Causes repolarization of TAMs to antitumor phenotype.
6007	INCB 024360-analog	IDO inhibitor	Inhibition of IDO with INCB 024360-analog enhances antitumor immune response and inhibits tumor growth *in vivo*.
6083	PSB 12379	Ecto-5′-nucleotidase (CD73) inhibitor	CD73 catalyzes the hydrolysis of extracellular AMP to adenosine, which causes local immunosuppression via adenosine receptors. Inhibition with PSB 12379 causes an immune response in the tumor microenvironment.

Table 1. A Selection of Cancer Immunology Products Available from Tocris Bioscience.

Immune cell signaling is a mechanism that can be easily modulated with small molecules and some different pathways show promise as potential targets. Toll-like receptors are expressed on antigen presenting cells and trigger a pro-inflammatory response upon ligand binding. Agonists at TLR7 and TLR8 have shown promise in preclinical results[1], and subsequently, the TLR7 agonist Imiquimod (Tocris, Catalog # 3700) has been approved as a topical monotherapy for basal cell carcinoma[2]. These small molecules have antitumor effects mediated by the activation of dendritic cells and natural killer cells to kill tumor cells, and their antitumor effects are also mediated by the suppression of T cells[3].

Chemokines and their receptors play a critical role in the immune response in cancer, and as G-protein-coupled receptors, chemokine CXC receptors are a druggable target for oncotherapy. AMD 3100 (Tocris, Catalog # 3299) is a CXCR4 antagonist, and in preclinical investigations, it switches the inflammatory response from a Th2 to a Th1 type response, thus promoting a pro-inflammatory environment[4]. Also (±)-AMG 487 (Tocris, Catalog # 4487) has been shown to inhibit lung metastasis in a mouse model of metastatic breast cancer[5].

Amino acid metabolism is a conserved pathway that is involved in the regulation of the immune response. The IDO family of dioxygenases (IDO1, IDO2, and TDO), responsible for the conversion of tryptophan to kynurenine and additional metabolites have shown promise as therapeutic targets in cancer. Multiple immunosuppressive roles have been shown for IDO, which ultimately impair immune recognition and promote tumor growth. Inhibition of IDO/TDO was one of the first small molecule-based strategies proposed for the induction of the immune response in cancer[6]. In preclinical investigations 1-Methyl-D-tryptophan (Tocris, Catalog # 5698), an IDO inhibitor, enhanced the antitumor and antiviral immunoresponse of CD8 positive T cells *in vitro*[7] and reduced tumor volume in mice with xenografts overexpressing IDO[8]. Additionally, LM 10 (Tocris, Catalog # 5794) reduces the growth of TDO expressing mastocytoma tumors in mice[9].

In their comprehensive review Adams et al. further discuss the targeting of immune response pathways with small molecules, outlining the rationale for their use and currently available small molecule treatments[1].

References

1. Adams, JL, et al., Big opportunities for small molecules in immuno-oncology, 2015; *Nature Reviews Drug Discovery*, 14: 603–22

2. Geisse, J, et al., Imiquimod 5% cream for the treatment of superficial basal cell carcinoma: results from two phase III, randomized, vehicle-controlled studies, 2004; *Journal of American Academic Dermatology*, 50: 722–33.

3. Pradere, JP, et al., The yin and yang of Toll-like receptors in cancer, 2014; *Oncogene*. 33: 3485–95.

4. Hogaboam, CM, et al., The therapeutic potential in targeting CCR5 and CXCR4 receptors in infectious and allergic pulmonary disease, 2005; *Pharmacology & Therapeutics*, 107: 314–28.

5. Walser, YC, et al., Antagonism of CXCR3 inhibits lung metastasis in a murine model of metastatic breast cancer. 2006; *Cancer Research*, 66: 7701–7.

6. Mellor, AL, Dunn, DH, IDO expression by dendritic cells: tolerance and tryptophan catabolism, 2004; *Nature Reviews Immunology*, 4: 762–74.

7. Rytelewski, M et al., Supression of immunodominant antitumor and antiviral CD8+ T cell responses by indoleamine 2,3-dioxygenase, 2014; *PLoS One*, 9: e90439.

8. Nakamura, N, et al., Effects of indoleamine 2,3-dioxygenase inhibitor in non-Hodgkin lymphoma model mice, 2015; *International Journal of Hematology*, 102:327–34.

9. Pilotte, L, et al., Reversal of tumoral immune resistance by inhibition of tryptophan 2,3-dioxygenase, 2012; *Proceedings of the National Academy of Sciences of the United States of America*, 109: 2497–502.

Chapter 5:
Cell-based Therapeutics: Research and Translational Approaches

Immune cell therapies and stem cell therapies are receiving increasing amounts of attention for their use as treatments for cancer. Perhaps the most attention has focused on cancer T cell therapies, where the patient's cells are used in the treatment. A common workflow includes harvesting cells from the patient, enriching the cells of interest followed by genetically modifying and expanding the cells *ex vivo*. The cells, now reprogrammed for enhanced anti-tumor activity, are then reintroduced into the patient. Some cell types used for immune cell therapy include tumor-infiltrating lymphocytes, transgenic T cell receptor cells, and chimeric antigen receptor (CAR) T cells.

Chimeric Antigen Receptor T Cells in Cancer Immunotherapy

CAR T cells refer to T cells which are genetically engineered to express CARs. CARs consist of four main components—a single-chain variable fragment (an extracellular target binding domain), a spacer domain, a transmembrane domain, and an activation or intracellular signaling domain. CAR T cells can recognize cell surface antigens in a human leukocyte antigen-independent manner. This characteristic enables CAR T cells to execute antigen-specific T cell activation, proliferation, and cytokine production, which drives the killing of the tumor cells (**Figure 1**). CAR T cells' ability to recognize a range of antigens in a non-major histocompatibility complex-restricted manner widens their range of clinical applications beyond cancer immunotherapy.

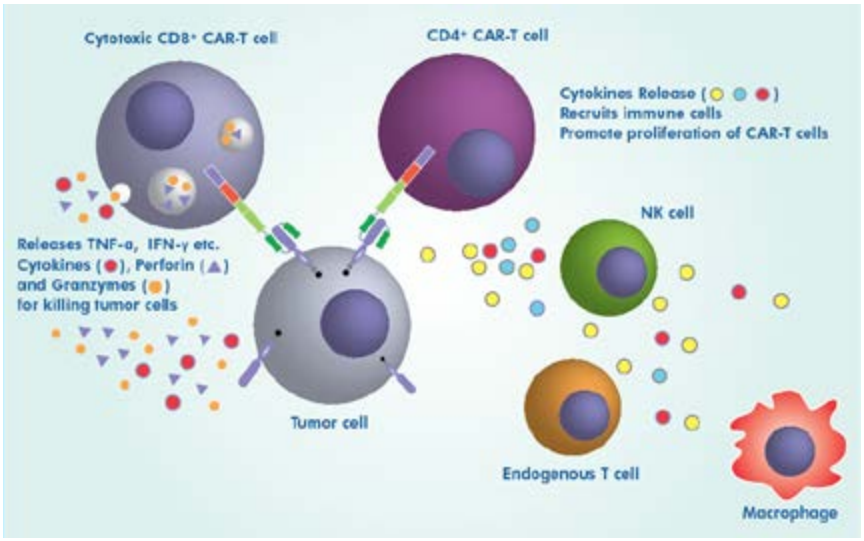
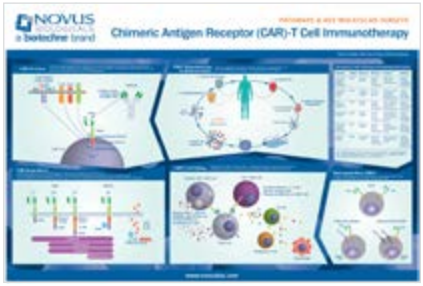


Figure 1. Mechanism of CAR T Killing Tumor Cells. For more details, review Novus Biologicals' poster, *Chimeric Antigen Receptor T Cells in Immunotherapy*, contributed by Marco Ruella, M.D.

CAR-T Cell Immunotherapy



Download Novus Biologicals' poster, **contributed by Marco Ruella, M.D., June Lab, University of Pennsylvania, PA,** for an overview of CAR structure, CAR T tumor cell killing, manufacturing of CAR T cells, generation of CAR constructs, next-generation CAR T cells, and more!

CAR T cell-based immunotherapy has shown impressive efficacy in hematological malignancies[1]. However, more research developments are warranted to tackle the challenges of applying CAR T cell immunotherapy to treat epithelial or solid malignancies. The latter represents a large group of cancers which often show resistance to immunotherapy by evading the immunologic attack. Major challenges in the application of CAR T cell treatment to solid tumors include insufficient CAR T cell activation, CAR T cell cytotoxicity, and physical/chemical barriers for CAR T cells in the tumor microenvironment. Genetically engineered CAR T cells and humanized tumor models hold great promise for the treatment of cancers through immunotherapy.

Tools for Cell Isolation

Following cell harvesting, the cells of interest need to be isolated and enriched before any experimental manipulation. In addition to cell viability concerns during the process, the purity of the population needs to be carefully managed to avoid contaminants, as viability and purity can both impact the efficacy and safety of the treatment. R&D Systems MagCellect™ technology is based on the use of ferrofluids or magnetic nanoparticles that have no magnetic memory (superparamagnetic). This feature protects purified cell populations against magnetic forces which may affect cell viability and interfere with downstream applications. By virtue of their small size (~150 nm diameter), MagCellect Ferrofluids behave like colloidal particles, easily remaining in solution and allowing for efficient diffusion kinetics in the binding reaction. **Figure 2** illustrates the enrichment of mouse splenocytes before (**A**) and after (**B**) separation of CD8$^+$ T cells using the MagCellect Mouse CD8$^+$ T Cell Isolation Kit. The purity of all isolated cells in this experiment was 92.39%.

Learn more about the principle of the MagCellect Assay here.

Looking for a MagCellect Kit for your research?
Click here to see our complete listing of kits.

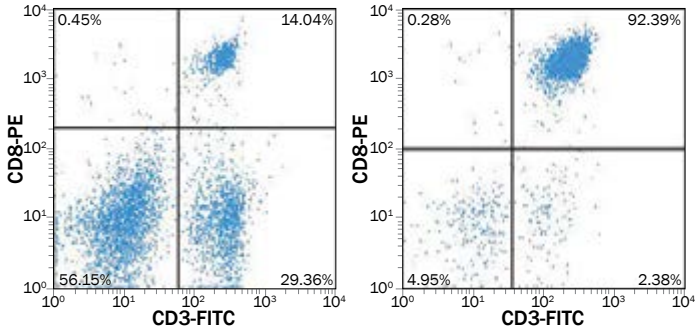


Figure 2. Enrichment of Mouse Splenocytes using the MagCellect Mouse CD8$^+$ T Cell Isolation Kit. Mouse splenocytes are shown before (**A**) and after (**B**) isolation of CD8$^+$ T cells using the Magcellect™ Mouse CD8$^+$ T Cell Isolation Kit (R&D Systems, Catalog # MAGM203). Dot plots reflect double staining of all viable cells using a FITC-conjugated Rat Anti-Mouse CD3 Monoclonal Antibody (R&D Systems, Catalog # FAB4841F) and a PE-conjugated Rat Anti-Mouse CD8 alpha Monoclonal Antibody (R&D Systems, Catalog # FAB116P).

Tools for Cell Expansion and Differentiation

Many cell therapy applications require the expansion and differentiation of a population to generate large numbers of cells for the treatment. Optimized kits can provide consistent and reliable results, ensuring efficient immune cell differentiation. CellXVivo™ Immune Cell Differentiation and Expansion Kits include optimized cytokine combinations and protocols for the differentiation and expansion of dendritic cells, T helper cell subtypes, Tregs, and B cells.

FOXP3⁺ Treg cells are a suppressive subset of CD4⁺ T cells that function to antagonize immune responses. *In vitro* differentiation of Treg cells from the larger naïve CD4⁺ T cell population provides increased numbers of Treg cells to facilitate downstream research. **Figure 3** illustrates the differentiation of human CD4⁺ T cells into Treg cells. Human peripheral blood naïve CD4⁺ T cells were incubated with reagents included in the Human Treg Cell Differentiation Kit (R&D Systems, Catalog # CDK006) for five days. Cells were fixed, permeabilized, and stained using the FlowX™ Human Regulatory T Cell Kit (R&D Systems, Catalog # FMC021), followed by flow cytometry analysis of FOXP3 and CD25 expression.

Learn more about the CellXVivo Immune Cell Differentiation and Expansion Kits

Click here to see our complete listing of kits.

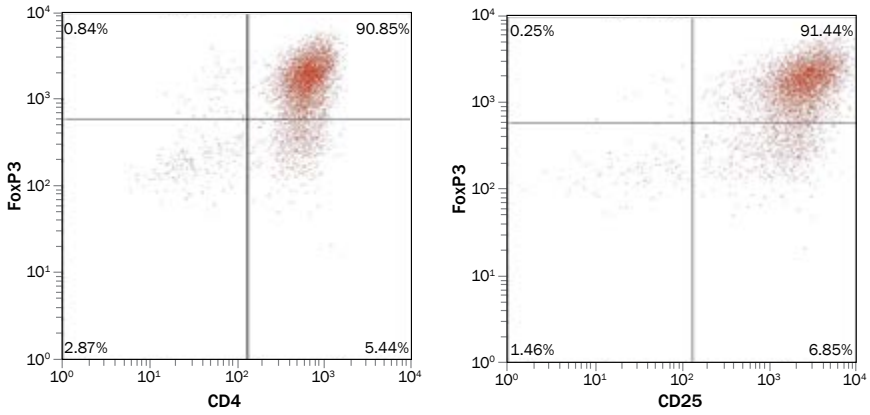


Figure 3. Differentiation of Human CD4+ T Cells into Treg Cells Confirmed by FOXP3 and CD25 Expression. Human peripheral blood naïve CD4+ T cells were incubated with reagents included in the Human Treg Cell Differentiation Kit for five days. Cells were fixed, permeabilized, stained, and analyzed by flow cytometry.

Tools for Cell Type Verification

Figure 4 illustrates the verification of Th1 cell identified using flow cytometry. Human peripheral blood naïve CD4⁺ T cells were differentiated using the reagents included in the Human Th1 Cell Differentiation Kit. The cells were stained with an APC-conjugated Mouse Anti-Human IFN-γ Monoclonal Antibody (R&D Systems, Catalog # IC285A) and a PE-conjugated Mouse Anti-Human IL-4 Monoclonal Antibody (R&D Systems, Catalog # IC204P) (A, B). The cells were stained with an APC-conjugated Mouse Anti-Human IFN-gamma Monoclonal Antibody (R&D Systems, Catalog # IC285A) and a PerCP-conjugated Mouse Anti-Human IL-17 Monoclonal Antibody (R&D Systems, Catalog # IC3171C) (C, D). Control cultures were used to place the quadrants.

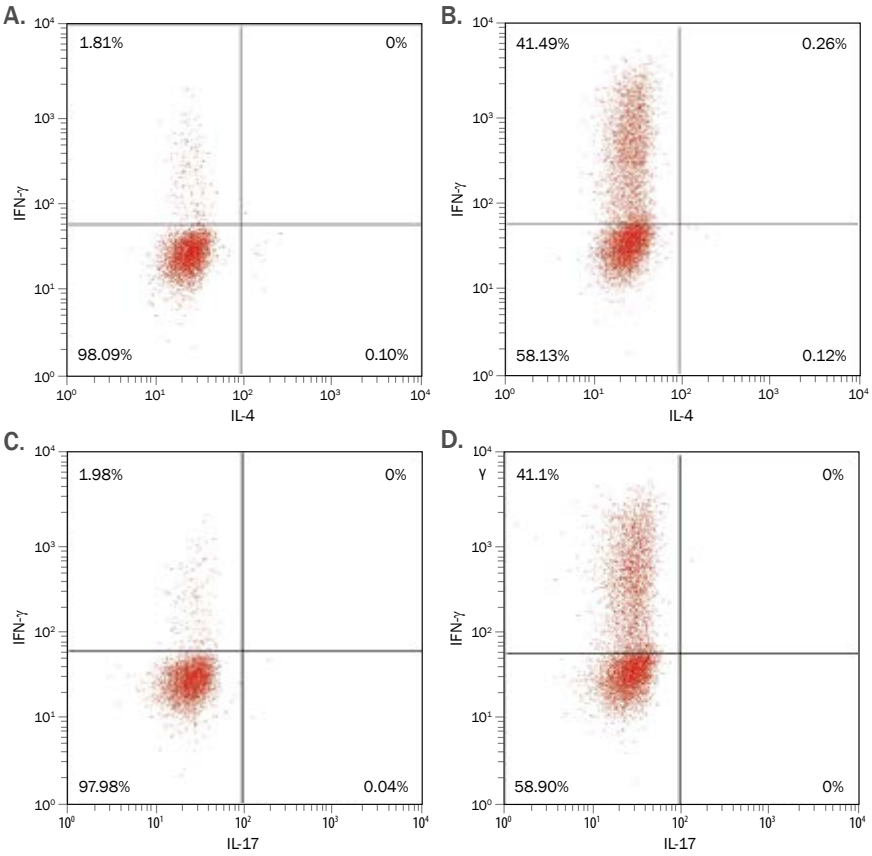


Figure 4. Verification of Th1 Cell Identity using Flow Cytometry. Human peripheral blood naïve CD4⁺ T cells without (**A**, **C**) and with (**B**, **D**) a five-day differentiation using the reagents included in the Human Th1 Cell Differentiation Kit.

Assessing the Efficacy of Cell Therapy in Experimental Model Systems

A variant of epidermal growth factor receptor, EGFRvIII is the most frequent mutation found in glioblastoma (GBM) patients[2]. Given its prominent role in GBM disease progression, numerous therapies are being developed to target EGFRvIII. Researchers at the University of Pennsylvania, Massachusetts General Hospital, and Novartis recently published their work using the RNAscope assay for the *in situ* characterization of CART-EGFRvIII in GBMs[3]. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediated antigen loss and induced adaptive resistance in patients with recurrent glioblastoma.

The authors used an RNAscope probe designed against the 3′ untranslated region (UTR) of their CAR design to examine the cellular localization of the CAR before and after infusion of CART-EGFRvIII cells in GBM patients. These CAR-directed probes specifically detected CAR⁺ cells *in situ* after CART-EGFRvIII infusion, but not pre-infusion. As such, RNAscope visually confirmed that CART-EGFRvIII cells travel to the tumor site of glioblastoma patients and resulted in on-target activity. RNAscope *in situ* analysis of the tumor microenvironment also revealed a higher post-infusion

infiltrate of IFNγ-positive activated T cells in GBM tissues. One of the key benefits of using the RNAscope technology for CAR detection is that unlike the CAR protein which in humans is indistinguishable from endogenous, the viral UTRs, and any other portion of the CAR design, provide unique tags that the RNAscope assay can detect to specifically visualize where the CAR is trafficked to in the tissue context.

Good Manufacturing Process-Grade Proteins for Cell Therapy Manufacturing

Immune cell therapies, stem cell therapies, and regenerative medicine, often grouped into the category of advanced therapy medicinal products, offer some of the more revolutionary and exciting new approaches for treating human disease. Because they utilize living cells or tissues as the therapeutic, the manufacturing process is vastly more complex than those of more traditional treatment methods. The use of high-quality media supplements such as growth factors and cytokines is paramount to ensuring safety, efficacy, and minimizing batch-to-batch variability. R&D Systems GMP-grade proteins are of the highest consistency and quality and help avoid technical issues during the manufacturing process (**Figure 5**). Click here to see a full list of GMP-Grade Proteins.

Quality Control of Formulations

An accurate determination of subvisible particles and protein aggregates is important to ensure the safety and efficacy of biopharmaceutical formulations due to immunogenicity concerns. Biopharmaceutical manufacturers are expected to characterize, monitor, and control subvisible protein particles and non-protein particles in their products. Accurately and precisely characterizing particle populations for a product is increasingly important as regulatory agencies require this as proof of a robust and reproducible manufacturing capability. This information helps establish comparability between lots of drugs as well as biosimilars and their originator molecules.

As traditional techniques such as light obscuration lack the sensitivity to distinguish translucent and potentially harmful protein aggregates, other options are needed. Micro-Flow Imaging® (MFI) offers several advantages over traditional techniques in the analysis of subvisible and visible particles in protein formulations as its image-based approach offers direct particle detection. The particle count, size, and morphological information provided enables novel and unique insights into particle characterization and quantification with just a single test. As a result, MFI systems can discriminate protein aggregates from the silicone micro-drops, air bubbles, and other contaminants commonly found in complex biopharmaceutical samples.

CAR-T Cell Target Selection Using Innovative *in situ* Hybridization Technology

Watch this webinar,
moderated by Courtney Anderson, Ph.D.,
featuring James B. Rottman, D.V.M., Ph.D., D.A.C.V.P.,
Senior Director of Translational Development
Bluebird Bio.

Engineering iPSC-derived Natural Killer Cells for Enhanced Efficacy of Cancer Therapies

In this on-demand webinar,
Bruce Walchek, Ph.D., discusses approaches to augment antibody-dependent cell mediated cytotoxicity by NK cells as a therapeutic approach to treat cancer.

Confirming Accurate Particle Counting and Sizing on MFI Systems

Click here to read the application note.

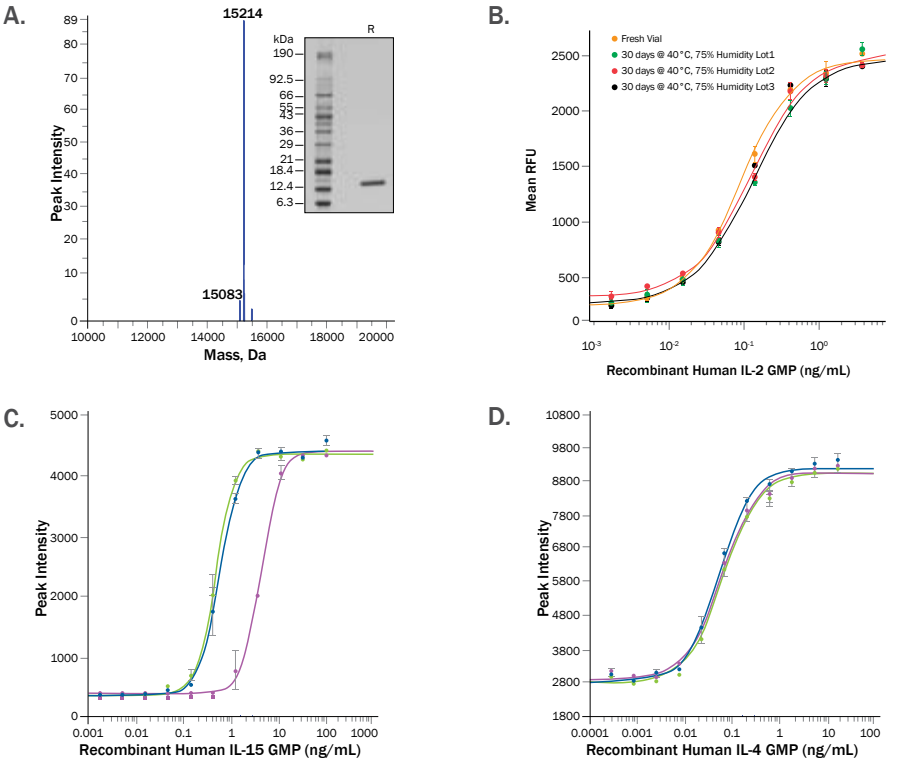


Figure 5. Analysis of R&D Systems Good Manufacturing-grade Cytokines. Our GMP cytokines have high purity standards (**A**). Electrospray Ionization mass spectrometry analysis of GMP Recombinant Human IL-3 (Catalog # 203-GMP). The labeled peaks at 15214 Da and 15083 Da correspond to the calculated molecular mass with the N-terminal Met, 15213 Da, and without the N-terminal Met, 15081 Da, respectively. Greater than 97% purity is also seen using SDS-PAGE under reducing (R) conditions and visualized by silver staining (inset). **Our GMP cytokines Undergo stressed and real-time stability testing** (**B**). The bioactivity of three different lots of GMP Recombinant Human IL-2 (Catalog # 202-GMP) was stressed for 30 days at 40 °C. The ability of these samples to induce the proliferation of CTLL-2 cytotoxic T cells was compared to a sample stored as indicated on the Certificate of Analysis. Overlapping bioactivity curves highlight reproducible stability over three different lots. **Our GMP cytokines are potent** (**C**). Two lots of GMP Recombinant Human IL-15 (Catalog # 247-GMP; green and blue) exhibit 10-fold greater activity in a proliferation assay using MO7e human megakaryocytic leukemic cells than the equivalent cytokine from another supplier (magenta). **Our GMP cytokines show strong lot-to-lot consistency** (**D**). GMP Recombinant Human IL-4 (Catalog# 204-GMP) stimulates the proliferation of TF-1 human erythroleukemic cells. Overlapping curves highlight reproducible bioactivity over three different lots.

References

1. Park, JH, et al., CD19-targeted CAR T-cell therapeutics for hematologic malignancies: interpreting clinical outcomes to date, 2016; *Blood*, 127:3312–20.

2. Gan, HK, et al., The EGFRvIII variant in glioblastoma multiforme, 2009; *Journal of Clinical Neuroscience*, 16:748–54.

3. O'Rourke, DM, et al., A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma, 2017; *Science Translational Medicine*, 9: eaaa0984.

Chapter 6:
Cytokines and Immunotherapy: Tools for Measuring Cytokine Release Syndrome

Cytokine Release Syndrome (CRS; also known as Cytokine Storm) is a potentially life-threatening adverse response to immunotherapy and involves the release of supraphysiological levels of inflammatory cytokines from activated lymphocytes or myeloid cells. CRS has been observed in patients who have received monoclonal or bispecific antibody infusions as well as those who received adoptive T cell therapy[1]. As investigators race to develop novel immunotherapy or combination therapies, it is critical that they anticipate adverse responses like CRS. Bio-Techne's brands provide many tools for measuring cytokine secretion.

Quantitative Assays for Measuring Cytokine Expression

Cytokines in supernatants or lysates can be measured simultaneously using the Proteome Profiler™ Antibody Array. This is a membrane-based antibody array for the parallel determination of the relative levels of selected human cytokines and chemokines. It requires no specialized equipment and eliminates the need for multiple Western blot experiments. Antibody array kits contain buffers, detection antibodies, and membranes spotted in duplicate with high-quality capture antibodies. The arrays utilize chemiluminescence or infrared antibody conjugates for detection. Thus, membranes can be assessed for changes in Cytokine levels in the same manner as traditional Western blots.

Proteome Profiler™ Antibody Arrays

Watch this video, to learn more.

In this example, THP-1 human acute monocytic leukemia cells were untreated or treated with 1 µg/mL of recombinant human IFN-γ (R&D Systems, Catalog #285-IF) for 16 hours and 1 µg/mL of lipopolysaccharide (LPS) for eight hours followed by analysis with the Proteome Profiler Human XL Cytokine Array Kit (**Figure 1**). Treatment with INF-gamma and LPS resulted in increased expression of IFN-gamma, IL-6, IL-8, IP-10, I-TAC, MCP-1, MIG, and MIP-1-alpha/MIP-1-beta.

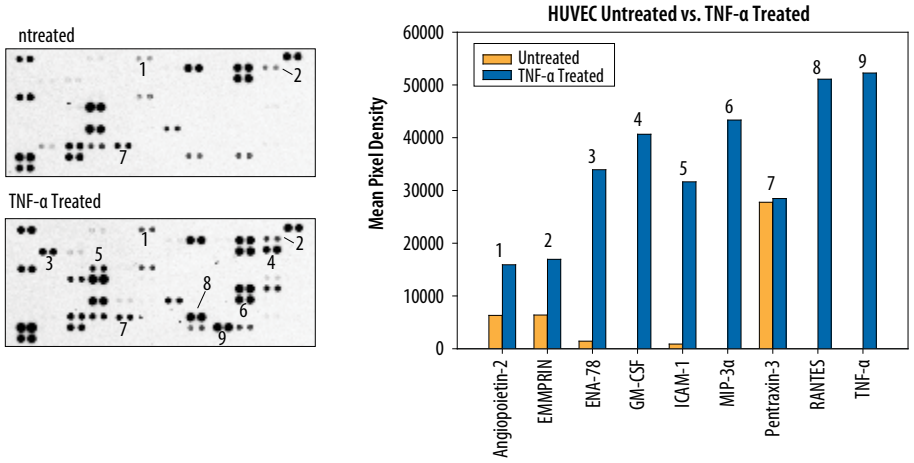


Figure 1. IFN-gamma and LPS Increase Cytokine Expression in THP-1 cells. THP-1 human acute monocytic leukemia cells untreated or treated with 1 µg/mL of recombinant human IFN-gamma for 16 hours and 1 µg/mL of LPS for eight hours.

Array	Number of Analytes
Human Cytokine Antibody Array	36
Mouse Cytokine Antibody Array	40
Rat Cytokine Antibody Array	29
Human XL Cytokine Antibody Array	105
Mouse XL Cytokine Antibody Array	111
Rat XL Cytokine Antibody Array	79

Table 1. Proteome Profiler Antibody Arrays for the Analysis of Cytokines

Bio-Techne offers Luminex® Assays and Luminex High-Performance Assays for simultaneously detecting and quantifying multiple target analytes in qualified complex sample types. These assays produce reproducible results for every target analyte. They require small sample volumes, are cost-effective, and allow researchers to collect more data in less time than other assays. Click here to learn more about Luminex.

Features of Luminex Assays:

- ✓ Broad selection
- ✓ Superior accuracy
- ✓ Flexible
- ✓ Easy to order

Why choose the R&D Systems Cytokine Discovery Luminex® High-Performance Assay over another Luminex vendor? Our assays are more accurate.

We assessed accuracy by comparing the linearity of dilution of our Luminex assay to that of several competitors (**Figure 2**, next page). Accurate assays yield a straight line on the graphs near 100% recovery because the analyte being measured is accurately detected as samples are diluted. If the line deviates far from 100% as samples are diluted, this indicates the assay is not accurately measuring the analyte of interest.

We currently offer more than 600 complete, ready-to-use Quantikine® ELISA Kits and 1,000 DuoSet® ELISA Development Systems for numerous different analytes and species including human, mouse, rat, canine, primate, and porcine. Choosing quality reagents that will lead to results you can trust is one of the more critical aspects of scientific research. R&D systems has several ELISA options for measuring cytokines depending on your needs (**Table 2**).

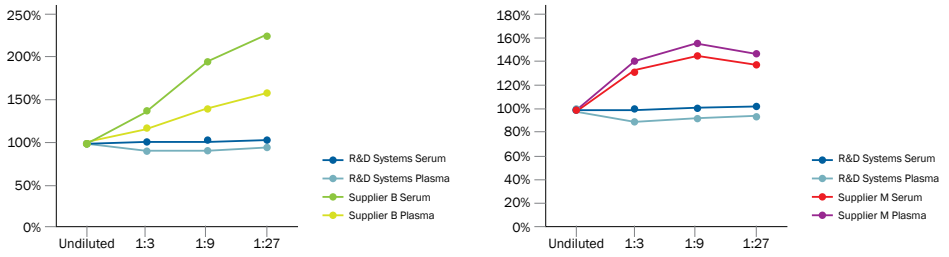


Figure 2. A Known Concentration of the IL-2 was Spiked into Serum or Plasma and Assayed by Three-Fold Serial Dilutions for Each XL Cytokine Discovery Luminex Performance Assay and for Each Supplier Luminex Assay. Sample values were back-calculated based on the standard curve, normalized to the initial sample dilution, and represented as % recovery vs. sample dilution.

ELISA	When to use it
Quantikine® Colorimetric Sandwich ELISAs	Gold Standard!
Quantikine® HS Colorimetric Sandwich ELISAs	Targets with low expression levels
Quantiglo® Chemiluminescent Sandwich ELISAs	Chemiluminescent Readout
Cell-Based ELISAs	Fluorescence measurement of (non) adherent cells
DuoSet® ELISA Development Systems	Do it yourself (DIY) ELISA, New 5-plate size

Table 2. R&D Systems ELISA Options for Measuring Cytokines.

Ella's Simple Plex assay is ideal for detecting cytokines from precious human samples and cancer cell lines. Simple Plex assays can measure targets at sub-picogram per mL levels in low volumes with a single-digit coefficient of variation. Scientists from Genentech examined the ability of Simple Plex assays on the Ella platform for detecting cytokines including IL-6, IL-8, and TNF-α and concluded that the platform was able to detect low endogenous level analytes with high sensitivity, and that Simple Plex assays are useful for assessing the mechanism of action of immuno-therapies and focused clinical validation of biomarker candidates[2].

Paulomi Aldo et al. examined the ability of Simple Plex assays to detect and quantify cytokines from human and mouse samples, comparing their results to conventional immunoassays such as ELISA and Luminex[3]. They concluded that "Simple Plex showed major advantages over these traditional plate-based immunoassay approaches for multiplexing in terms of required sample volumes, high sensitivity and dynamic range, coefficient of variation, and reproducibility." **Table 3** outlines available Simple Plex assays on the Ella immunoassay platform for CRS-markers.

CRP	IL-2Ra/CD25
GM-CSF	IL-6
Granzyme A	IL-6RA
Granzyme B	IL-10
INF-gamma	MCP-1/CCL2
IL-1 beta	TNF-alpha

Table 3. A Selection of Available Simple Plex assays for Cytokine Release Syndrome Markers.

The robust visualization of small secreted molecules like cytokines and chemokines at the RNA level offers the additional advantage of identifying the potential cellular sources for these small secreted signals. Coexpression patterns of cytokines/chemokines with key cell lineage markers could offer insights into the spatial relationships and interactions between various immune cell types together with information on their state (see Chapter 2: Tumor Microenvironment).

This is illustrated in glioblastoma formalin-fixed, paraffin-embedded tissue samples by the expression of *IFNγ* mRNA in individual $CD8^+$ (IHC) cells (**Figure 3**). Additional examples are shown in **Figure 4** for *IL10/CD163*, *IL12/CD68* (glioblastoma); and *CXCL10/CD68*, and *CCL22/CD163* (ovarian cancer). Also, *IL6* and *IL6ST* as a part of the cytokine receptor complex are detected on the same tissue section using the RNAscope® assay.

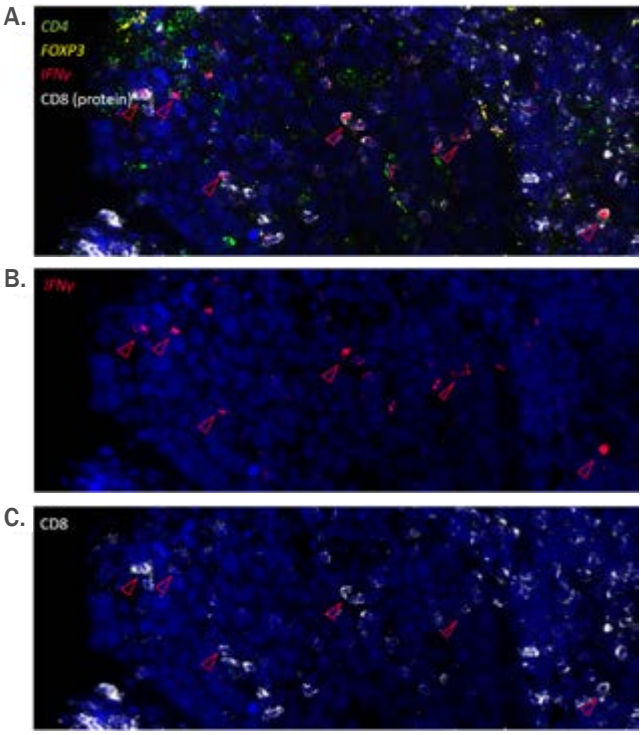


Figure 3. *In Situ* Detection of IFNγ Using the RNAscope LS Multiplex Fluorescent ISH assay in Combination with IHC for CD8 Cell Lineage Marker. Overlay for *IFNγ* mRNA (red), *CD4* mRNA (green), and *FOXP3* mRNA (yellow) with CD8 antibody (white) (**A**). Single channel images for *IFNγ* mRNA (**B**) and CD8 protein (**C**). Arrows indicate single cells with co-expression of *IFNγ* and CD8.

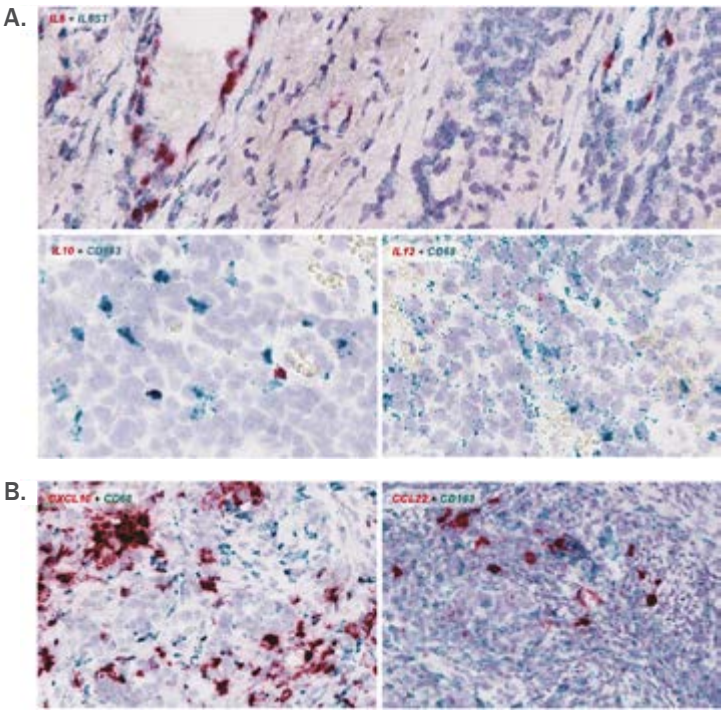


Figure 4. *In Situ* Detection of Cytokines in the Tumor Microenvironment of Glioblastoma and Ovarian Cancer FFPE Tissue Samples Using RNAscope 2.5 HD Duplex Assay. Interleukins *IL6*, *IL10*, and *IL12* (**A**) and chemokines *CXCL10* and *CCL22* (**B**) are detected (red) in combination with *CD163* and *CD68* cell lineage markers, and IL6ST (green) in glioblastoma (**A**) or ovarian cancer (**B**) tumors.

References:

1. Lee, DW, et al., Current concepts in the diagnosis and management of cytokine release syndrome, 2014; *Blood*, 124: 188–95.

2. Gupta, V, et al., Bioanalytical qualification of clinical biomarker assays in plasma using a novel multi-analyte Simple Plex™ platform, 2016; *Bioanalysis*, 8: 2415–28.

3. Aldo, P, et al., Simple Plex™: a novel multi-analyte, automated microfluidic immunoassay platform for the detection of human and mouse cytokines and chemokines, 2016; *American Journal of Reproductive Immunology*, 75: 678–93.

Chapter 7:
Tools to Advance Your Immuno-Oncology Research

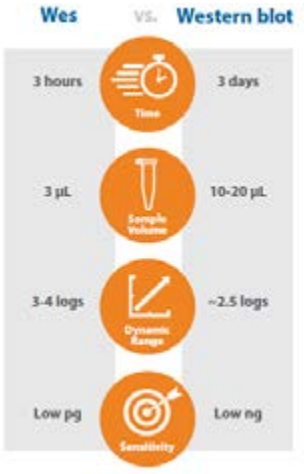
Bio-Techne unites the world-class brands of R&D Systems, Novus Biologicals, Tocris Bioscience, ProteinSimple, and Advanced Cell Diagnostics to better serve the scientific community. Bio-Techne supports the immuno-oncology workflow with trusted and innovative solutions.

Meet Wes

Simple Western™ systems (ProteinSimple) reinvent how Western blots are done and automate all assay steps from protein separation, immunoprobing, detection, and analysis of data. They also deliver quantitative, reproducible data in hours instead of days. There is no cutting of individual strips, repeated washing steps or incubations. With Simple Western assays, just pipette the sample and reagents into the wells of an assay plate, set up the run and press Start.

Simple Western assays are automated, capillary-based immunoassays that solve many of the challenges that come with traditional Westerns. There are four Simple Western systems to choose from; all produce very reproducible data with intra-assay coefficient of variations (CVs) <15%. Wes provides relative quantitation on up to 25 data points in just three hours with only 30 minutes of hands-on time for setup. If higher throughput is needed, Sally Sue™, Peggy Sue™, and NanoPro 1000 provide 96 data points overnight and only need about an hour of hands-on time.

How does Wes compare to traditional Westerns?



- ✓ He's quantitative
- ✓ He can handle your throughput
- ✓ He only needs 3 µL of sample
- ✓ And best of all, he's fast

Want to learn how Wes works?

Click here to watch a video to see how Wes can process up to 25 samples in three hours. The short video shows the step by step process, from sample loading to signal detection.

From Your Peers

"Some of the proteins we wanted to detect are low in abundance, and it's really hard to get a good amount of protein for traditional Western blot. The low protein concentrations required by Wes makes it easier to save precious samples ensuring protein detection."

–Debbie Hicks, Ph.D., Faculty Fellow, Wolfson Childhood Cancer Research Centre, Northern Institute for Cancer Research, Newcastle University

Click here to learn more.

Single-Cell Western
Meet Milo

Milo (ProteinSimple) is the world's first Single-Cell Western platform. He measures protein expression in thousands of single cells in a single run to allow sample heterogeneity profiling. With a wide range of applications from profiling target protein expression heterogeneity and the percent of cells that are target-positive, measuring protein isoform heterogeneity, to simplifying phospho-flow signaling studies, measuring gene editing efficiency and validating single-cell RNA-Seq data, Milo will provide answers on a single-cell level.

Want to learn how Milo works?

He measures protein expression in thousands of single cells in a single run. Load the cell suspension, and the scWest chip will capture approximately 1,000 single cells. Milo then does a fast, one-minute SDS-PAGE separation on each single-cell lysate on-chip. Next, probe using some favorite conventional Western antibodies to measure up to four proteins per cell simultaneously. Click here to watch a short video to see how Milo can automate 1,000 single cell separations in just four hours.

From Your Peers

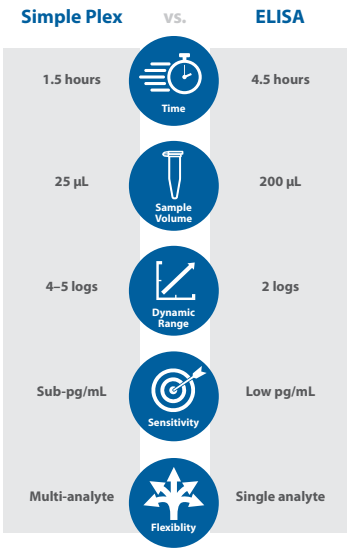
"I was looking at two different isoforms of a protein, and it was important to know if the cells were expressing just one versus the other or both in a given cell. That was tricky until Milo came. We're also getting more relevant information because Milo allows us to look at the expression of this protein in tissue biopsies."

–Prashant Vijay Thakkar, Ph.D., Postdoctoral Associate, Department of Medicine, Weill Cornell Medicine

Click here to read more.

Ella & Simple Plex Assays
Meet Ella

Simple Plex™ assays, automated on the Ella immunoassay platform (ProteinSimple), offer a microfluidic alternative to ELISA that lets you validate serum biomarkers faster, with inter-assay reproducibility in the single-digit CVs and sensitivity in the low pg/mL. Setup takes 10–15 minutes and results are ready in just one hour, allowing serum samples to be screened 3–6X faster than a traditional ELISA.



How do Simple Plex assays compare to traditional ELISAs? Traditional ELISAs can need up to 300 µL of the sample to generate triplicate data, but with Simple Plex assays on Ella, triplicate data results are received with just 25 µL of sample. Ella uses fluorescence detection, so she also stretches the potential applications of sample sets, providing less assay variability, better sensitivity, a 3–5 log dynamic range, and low single-digit CVs. Our 32×4 (32 samples/four analytes) and 16×4 (16 samples/four analytes) formats simultaneously run up to four analytes per run and get up to 128 triplicate data results in under 90 minutes. **Table 1** outlines a selection of available Simple Plex immuno-oncology biomarker assays.

Want to learn more about ELLA?

Click here to watch a video to see how Ella can process up to 72 samples in 75 minutes.

The short video shows the step by step process, from sample loading to analyzed data.

BAFF	PD-L1	HE4/ WFDC2	MDC/CCL22
BLC/BCA-1/CXCL13	PIGF	IFN-gamma	TARC
c-MET/HGF R	TGF-beta	IL-2	VEGF-C
CA 19-9	TIM-3	IL-4	Amphiregulin
CA125	TRAIL	IL-6	MIP-1 beta
ErbB2/HER-2	VEGF-A	IL-10	MIP-3 alpha/CCL20
GM-CSF	Angiopoietin 1	IL-17A	Thrombospondin-1
HVEM	Angiopoietin 2	IL-18	TNF-beta
LAG-3	BCMA/TNFRSF17	IP-10	

Table 1. A Selection of Available Simple Plex Immuno-Oncology Biomarker Assays.

RNAscope® ISH Technology

The RNAscope technology (Advanced Cell Diagnostics, Inc.; ACD) is an advanced *in situ* hybridization assay that allows for the visualization of single-cell gene expression, targeting RNA sequences directly in tissues. The proprietary double-Z probe design, in combination with ACD's patented signal amplification and background suppression technology, enables highly specific and sensitive detection of target RNAs in formalin-fixed, paraffin-embedded, fresh frozen, and fixed frozen cells and tissues, with each dot representing a single RNA transcript.

RNAscope signal amplification is achieved by a cascade of hybridization events (**Figure 1**):

- Step 1: Approximately 20 double-Z target probes hybridize to the RNA target (targeting approximately 1 kb region).
- Step 2: Pre-amplifiers hybridize to the 28-base binding site formed by each double-Z probe. Hybridization of only one Z probe will inhibit binding of the pre-amplifier, thereby providing high specificity.
- Step 3: Amplifiers recognize multiple binding sites on each preamplifier.
- Step 4: Labeled probes containing a fluorescent molecule or chromogenic enzyme bind to the numerous binding sites on each amplifier. This signal amplification system increases assay sensitivity.

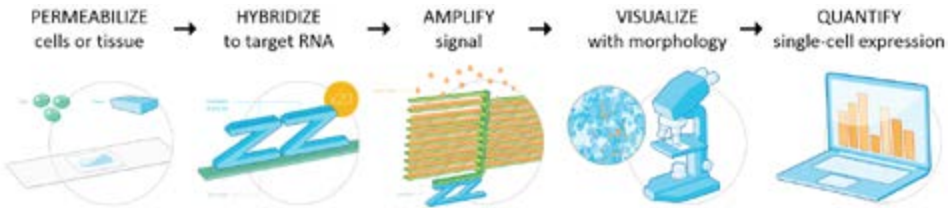


Figure 1. RNAscope *In Situ* Hybridization Assay Workflow.

From Your Peers

"Ella allows us to rapidly quantify biomarkers from hundreds of cryopreserved samples and correlate them to specific outcomes. Now we're ready to validate that what we identified in frozen plasma samples correlates to fresh blood samples taken at the point-of-care. Ella's simplicity and reproducibility will allow us to reliably test samples across different sites, enhancing triage especially in remote locations of developing countries where access to trained healthcare professionals is unlikely."

–Aleks Leligdowicz, M.D./Ph.D.,
Department of Medicine, University of Toronto

Click here to read more.

Click here to watch a short video on RNAscope *in situ* hybridization.
The multiplexing capabilities of the chromogenic (up to two targets) and fluorescent (up to four targets) RNAscope assays and the ability to combine the RNAscope ISH technology with immunohistochemistry provide pivotal information to gain deeper insights into tumor immunology and the complex and heterogeneous tumor microenvironment.

Custom Services

When your work demands unique reagents or scientific support, turn to the decades of product development legacy behind Bio-Techne's trusted brands. R&D Systems Custom Services delivers quality products and tools that enable scientific discovery and progress that leads to improved therapeutics and diagnostics. We offer a wide variety of custom solutions to help scientists attain their research goals, advance life sciences research, and support clinical breakthroughs. Together with a dedicated project manager, our expert scientists, quality assurance team, and world-class technical support we will deliver solutions exactly tailored to bring you success faster and more economically.

Click here to watch a short video about Bio-Techne custom services.

We will work with you to ensure success:

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- ✓ Consult with our expert scientists.
- ✓ Document milestones throughout the project.
- ✓ Initiate and communicate project progression.
- ✓ Deliver your world-class product or service.

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- Antibody Custom Services: Generate custom monoclonal or polyclonal antibodies from immunogen design to purified antibody specificity screening by partnering with our expert development team.
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- Luminex Custom Services: Leverage our extensive inventory of antibodies and proteins to add missing analytes to your multiplex panel quickly.
- Immunoassay Custom Services: Partner with the scientists that develop Quantikine ELISAs to optimize your assay or translate across platforms.
- Biomarker Testing Services: Allow our dedicated assay service group to analyze your precious samples using our gold-standard assays.
- Chemistry Services: Trust our world-class chemistry team to deliver unique compounds and screening panels.
- Custom Compound Libraries: Create a unique compound library, designed to meet your exact screening requirements.
- Monoclonal Antibody Panels: Take advantage of tens of thousands of monoclonal antibodies that are not listed in our catalog.
- Cell Culture Media Services: Cell culture media is important. Let our experts make it and test it for you.