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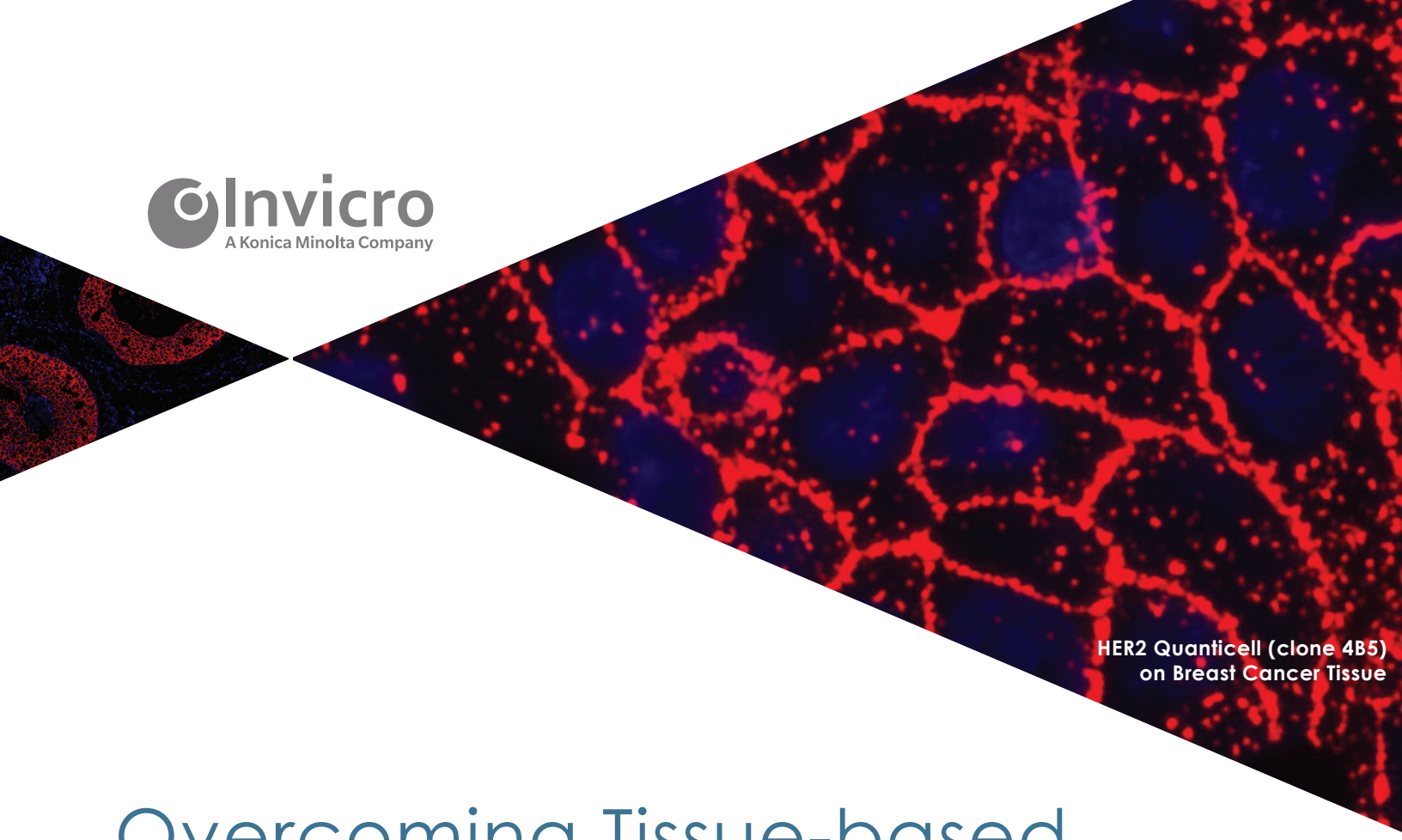
HER2 Quanticell (clone 4B5)
on Breast Cancer Tissue

Overcoming Tissue-based Biomarker Detection Challenges with Quanticell™

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Introduction

Immunohistochemistry (IHC) is a widely used technique in surgical and diagnostic pathology to identify the presence, location and semi-quantitatively assess specific markers at the tissue level. In our current era of precision medicine, tissue-based biomarker profiling by IHC is frequently used to select patients likely to benefit from targeted therapies. For example, in breast cancer, it is routinely used to evaluate the presence and relative amount of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) to guide clinical treatments decisions. Unlike blood-based and genomic biomarkers, IHC provides spatial information of target expression in specific cell types as well as subcellular locations.

IHC can also identify protein expression within the context of the cellular microenvironment, such as localization within the tumor margins or association with specific cell types. Furthermore, it can be multiplexed, allowing interrogation of several markers on the same tissue section. With the emergence of targeted immunotherapies, multiplex IHC can provide valuable information about the tumor microenvironment and may offer insight on how to significantly improve outcomes of cancer patients through combination therapies.

Immunotherapies, including activating and inhibitory T cell receptors, adoptive cell transfer methods, chimeric antigen receptor T cells (CAR-Ts), T cell receptor (TCR)-modified T cells, antibody-drug conjugates (ADCs) and bispecific antibodies are actively being explored in the research and clinical setting. To harness the full therapeutic potential of these agents, it is essential to understand the relationship between tumor cells and their microenvironment, including the immune repertoire, cancer-associated fibroblasts and vasculature. In addition, certain limitations of traditional IHC need to be addressed, such as lack of precise quantification, limited sensitivity to detect low levels of proteins, pre-analytical influences and inter-laboratory variability. The Quanticell technology employs a novel fluorescent nanoparticle detection system referred to as phosphor-integrated dots (PIDs). This detection system is quantitative and greatly enhances signal detection without the noise introduced by traditional IHC amplification techniques. The Quanticell technique, therefore, allows for significant gains in detection sensitivity with a much wider dynamic range. In this technical white paper, we provide an overview of this novel technology along with case studies to demonstrate how Quanticell can be applied across a broad range of translational research applications.

Overview of Quanticell Technology

Quanticell is a nanoparticle-based detection technology developed by Konica Minolta Precision Medicine that represents a major paradigm shift in quantitative IHC approaches.[1] Yet, the assay is straightforward to implement and follows the classic IHC workflow to enable rapid assay development and optimization (Figure 1A). Target analytes, including antigens or therapeutic agents, such as peptides and therapeutic antibodies are detected using unconjugated primary antibodies. The primary antibody can be an *in vitro* diagnostic, research use only (RUO) or custom-designed antibody. After incubation with the primary antibody, a biotin-conjugated secondary antibody is introduced and visualized with the streptavidin-coated PID particles. The lower limit of detection (LLOD) is significantly improved, and the dynamic range is much greater in comparison to traditional IHC. Importantly, this assay does not require a signal amplification step, thus maximizing signal while minimizing background noise. PID visualization and quantitation is done using a standard optical system and dedicated image-processing software. The number of PID particles is calculated based on the observed combined fluorescent intensity of the PID particles. Previous studies have established the relationship between the number of PID particles as detected by scanning electron microscopy and the fluorescent intensity of bright spots derived using high-pass-filtered images. The expression levels of target proteins in tissues is reported as the average PID number/cell or the average PID number/unit area (PID score/100 μm^2).

Technical Advantages of Quanticell

PIDs are advanced, custom-synthesized nanoparticles (Figure 1B) exhibiting unique optical properties that produce fluorescent emissions 30,000× brighter than conventional molecular probes. For example, the red PIDs, with a controlled diameter of ~130 nm, contain approximately 100,000 perylene diimide dye molecules, thereby provide highly stable fluorescence with minimal photobleaching. The surface of PID particles have a dense streptavidin calyx (~2,500 streptavidin molecules/PID) linked via polyethylene glycol chains to significantly increase the detection accuracy, sensitivity and dynamic range (10^{-6} to 10^{-1} mM).

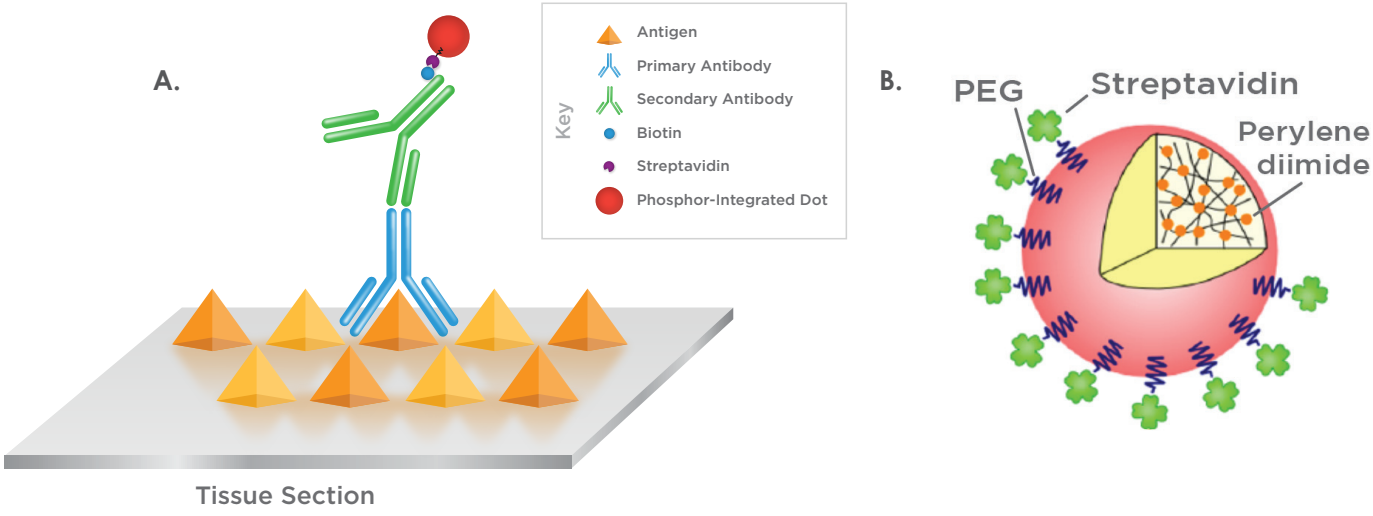

Figure 1. Overview of Quanticell technology. (A) The standard antibody-based workflow relies on an indirect detection method, whereby a primary antibody is added followed by a biotinylated secondary antibody to detect target antigens in tissue. The signal is detected using streptavidin-coated fluorescent PID nanoparticles. **(B)** PIDs are stable and highly fluorescent particles that have approximately 2,400 streptavidin molecules linked via polyethylene glycol (PEG) chains on its surface to provide significantly higher reactivity with secondary antibodies.

The Quanticell assay is able to detect analytes that are not visualized by conventional IHC methods with a 300-fold greater linear dynamic range, thus offering a potentially quantitative assay that extends well beyond the detectable range observed with conventional IHC methods. To benchmark a new Quanticell assay, orthogonal techniques, such as flow cytometry, ELISA and/or liquid chromatography mass spectroscopy (LC-MS) have been used to assess the sensitivity, specificity and accuracy (assay concordance). The Quanticell assay is performed using conventional automated histology equipment, thus reducing assay variance and enabling a high throughput workflow. The sample types already validated with the Quanticell assay include formalin-fixed paraffin-embedded tissue and fresh frozen tissue. The Quanticell assay is amenable to multiplexing with chromogenic substrates, or other fluorescent probes to provide comprehensive biomarker profiling solutions that may be able to improve patient stratification and response monitoring.

Selected Application Examples

1. Improved Stratification of HER2-low Patients

The are several FDA-approved companion diagnostic IHC assays available to identify invasive breast cancer patients eligible for HER2 targeted therapy. Based on current clinical ASCO/CAP guidelines and package inserts, an IHC score of 3+ with uniform intense membrane staining in >10% of the invasive tumor cells is considered a HER2 positive case. However, such HER2 positive population only represents approximately 15% of invasive breast cancer patients and up to 25% of patients with metastatic disease. There have been several next-generation anti-HER2 therapies developed since the introduction of trastuzumab. The most recent advances have included the development of HER2 ADCs. The clinical trials have focused on response rate and improved progression free survival in patients who developed resistance to anti-HER2 therapy. Trastuzumab deruxtecan (T-DXd) has shown an impressive

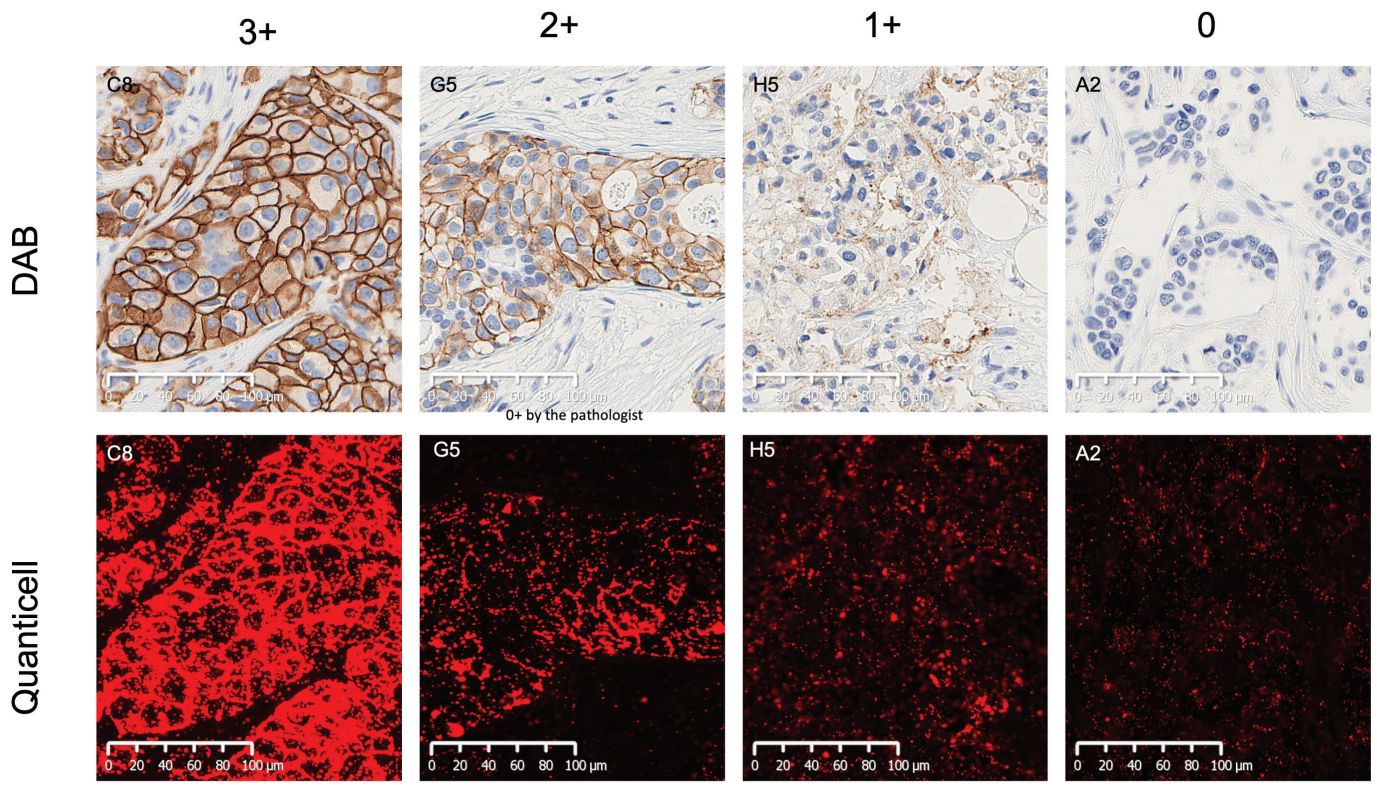

Figure 2. Comparison of HER2 DAB-IHC versus Quanticell assay. Four selected cores from the human breast tumor tissue array are shown. Core A2, with IHC score of 0 showed a positive PID score (above baseline) and identified as "false negative" by DAB-IHC and "true positive" by Quanticell, thus allowing a more objective and quantitative scoring of HER2 expression.

60.9% response rate in heavily treated HER2 positive metastatic breast cancer patients.[2] Unlike trastuzumab emtansine (T-DM1), T-DXd cargo is a membrane-permeable topoisomerase I inhibitor facilitating bystander cytotoxicity that is independent of HER2 expression. Notably, T-DXd therapy recently provided a significant response rate of 37.1% in the therapy-resistant metastatic breast cancer population that displayed HER2 1+ and 2+ IHC score. Since 75% of metastatic breast cancer patients are HER2 negative, and at least 50% show 1+ and 2+ expression, it is critical to establish the LLOD for HER2 that still derives benefit from T-DXd therapy. The existing HER2 IHC assays lack sensitivity at low levels of expression and are specifically titrated to not show expression in normal breast ductal epithelial cells, which contain approximately 20,000 HER2 receptors. The Konica Minolta Precision Medicine team developed an early Quanticell assay that detected HER2 expression even in the luminal A/HER2 negative cell lines, MCF7 and T47D. Quanticell HER2 scores showed a strong linear correlation with FACS analysis in cell lines and demonstrated significant correlation with precision, sensitivity and reproducibility with conventional HER2 testing methods.[3] Moreover, we also developed a Quanticell assay that detects T-DXd and γH2AX to simultaneously score trastuzumab binding and tumor cytotoxicity.

In this case study (Figure 2), the performance of a HER2 Quanticell assay was compared to conventional IHC (Ventana, clone 4B5) on a 104 core breast tumor tissue array (BR20810, US Biomax). The results indicate that patients classified as 0+ by conventional IHC could be further stratified into "HER2 negative" and "HER2 low" phenotypes.[4] Quanticell has demonstrated the ability to stratify low levels of HER2 expression and may be a valuable tool for T-DXd therapy and other ADC drugs with similar mechanisms to move them into the clinic.

2. Assessing Tumor Drug Delivery for Optimizing Therapeutic Index of Trastuzumab

Understanding the heterogeneous distribution of systemically administered drugs, including ADCs can help improve therapeutic risk-benefit ratio of drugs that are in clinical trials.[5] Parameters, such as overexpression of surface antigen, diffusion kinetics and drug penetration into the tumor mass, presence of necrosis and the tumor microenvironment can affect drug pharmacology. IHC-based techniques that provide information on tumor drug pharmacokinetics (PK) can positively impact both drug development and "precision drug dosing" protocols.

This case study was designed to understand the distribution of trastuzumab in breast cancer xenografts.[6] HER2 positive (BT474) and HER2 negative (MDA-MB-231)

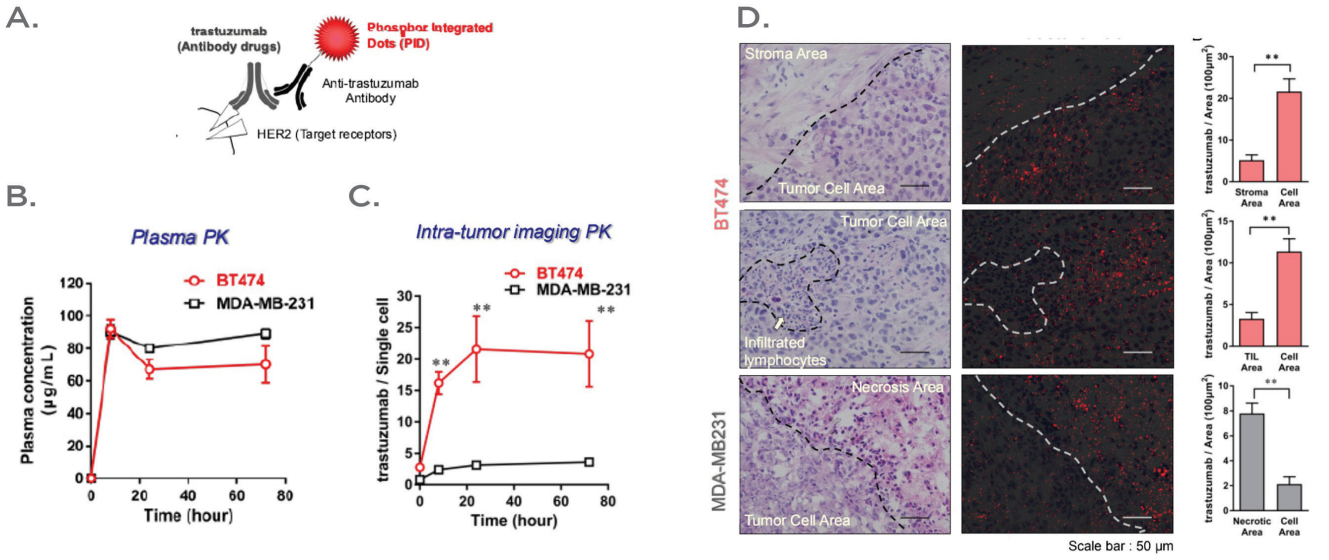

Figure 3. Detection of trastuzumab distribution by Quanticell assay. (A) Illustration of trastuzumab detection by Quanticell assay using a biotinylated anti-trastuzumab antibody. **(B)** Plasma and **(C)** Intra-tumoral PK analysis of trastuzumab in breast cancer (BT474, HER2+ or MDA-MB-231, HER2-) xenografts. **(D)** Trastuzumab distribution in stroma/tumor, immune-rich/tumor, and viable/necrotic areas with corresponding PID scores.

xenograft models were used and a trastuzumab Quanticell assay was evaluated at the whole tumor and cellular level (Figure 3A). PK analysis of trastuzumab in plasma showed similar distribution in both tumor models (Figure 3B), while intra-tumoral PK analysis revealed a more accurate and higher concentration of trastuzumab in BT474 tumors (Figure 3C). By performing region-specific analysis of the tumors, trastuzumab concentration was noted higher in the HER2 positive BT474 tumor cells and lower in the stroma and immune-rich regions. Also, a non-specific accumulation of trastuzumab within the necrotic region of the MDA-MB-231 xenografts was observed (Figure 3D). This study demonstrates how Quanticell can provide information on drug accessibility at the tumor tissue level, target binding, and downstream pharmacology.

3. Quantification of Extranuclear ERa in HER2 Negative Breast Cancer with Prognostic Application

ER is expressed in the majority of invasive breast carcinomas and is an important predictive and prognostic marker. IHC-based detection of ER protein is routinely used in the clinical setting and tumors are reported using a binary classification approach as either ER positive or ER negative. Assessment of ER expression by IHC is affected by both physiologic and laboratory processing methodologies.[7] Standard IHC analysis detects the presence of nuclear ER protein yet does not accurately reflect the functional activity of the ER pathway. As a result, Guo *et al* [8] developed an ERa Quanticell assay that measured total ERa, nuclear ERa and extranuclear ERa. They showed that high extranuclear-to-nuclear ERa (ENR) in breast cancer (hormone receptor positive and HER2 negative) correlates with a poor clinical outcome and served as an independent prognostic factor that can be used to predict response and resistance to endocrine therapy. This IHC-based pathway analysis can be extended to other signaling pathways to improve decision-making on designing personalized therapy protocols.[9]

Discussion

In the last decade, a rapid surge in the number of new drugs has revolutionized cancer therapy treatments in the clinic. To support new drug development programs, the Quanticell technology was developed to provide a highly sensitive, amplification-free detection technology that is more linear with a wider dynamic range. This technology has immediate potential as a translational research tool, particularly in the development of cancer immunotherapies and antibody-drug conjugates. We have demonstrated that Quanticell assays are uniquely suited for identifying low expressing biomarkers and can be multiplexed to understand useful readouts like bystander killing and immune contexture of the tumor microenvironment. Finally, the Quanticell technology can be deployed in clinical trials as a robust and reproducible CAP/CLIA validated exploratory endpoint.

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