WHITEPAPER

Employing multi-analyte profiling to deepen our understanding of cancer biology

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Introduction

With the dawn of novel immune-targeted therapeutics layered on top of complex combinations of chemo and targeted therapies for cancer, there is an increased requirement for sophisticated analytical approaches to understand mechanism of action, drug interactions, clinical response, and the array of potential side effects. While genome sequencing has transformed our understanding of cancer biology and revealed novel therapeutic targets, it alone is not the panacea. The case studies presented in this White Paper highlight the importance of utilizing multiple strategies to characterize how the regulation of critical cellular pathways impacts many aspects of therapeutic efficacy. They also highlight the challenges in deploying multiple analytical methodologies given the complexity of integrating analysis, sample input requirements, and technical limitations. For NanoString, the challenges highlighted in these case studies inspired the development of novel methodologies that both expand and simplify the multi-analyte analysis of tumors and their microenvironment.

Determining a direct relationship between protein and mRNA expression levels can often be problematic. Many studies have revealed that the correlation between gene expression and reciprocal protein abundances in the cell are poor. In particular, this can be due to differences in the level of regulation between the transcript and the protein product, including protein stability and half-life (Maier et al., 2009; Payne, 2015). Despite the low correlation demonstrated in many studies, the assumption is often that differences in gene expression in each biological study directly reflect differences in protein expression. While we know that biological regulation results in discordant RNA and protein expression, it is often difficult to design experiments to test these discordant results and determine where the regulation occurs and how. Schwanhausser et al. (2011) performed a global analysis of protein levels versus mRNA using Next Generation Sequencing (NGS), NanoString's digital barcode technology, and Mass Spectrometry in NIH3T3 mouse fibroblasts. Using metabolic pulse labeling (SILAC) and 4-thiouridine incorporation, both protein and mRNA half-lives were calculated, respectively. Proteins were, on average, 5 times more stable (median $t_{1/2}$ = 46 h) than mRNA ($t_{1/2}$ = 9 h). There was no correlation between mRNA and protein half-lives (R^2 = 0.02). Of the 5,028 genes that were identified at both the mRNA and protein levels, proteins were on average 900 times more abundant than the corresponding mRNA and protein concentrations spanned 5 orders of magnitude. Despite this spread, a log-log plot of mRNA versus protein showed a R^2 value of 0.41. This study is one of many that demonstrates the critical importance of profiling both RNA and protein for a complete understanding of the biology of your sample.

Correlation of NanoString to Protein Methodologies: Why analyze both?

Most published multi-analyte work tends to investigate just one analyte at a time employing complex data analysis techniques to merge divergent data sets *in silico*. Focusing on 1,500+ publications utilizing NanoString technology, roughly a third of these measure RNA on the nCounter® Analysis System and protein expression using other platforms. In this White Paper we will review the current literature investigating the utility of NanoString gene expression assays and how they correlate to reciprocal protein results using either flow cytometry, western blotting, or immunohistochemistry (IHC)-based approaches. This collection of publications and case studies seeks to better understand how drugs and novel modulators affect the immune system and other pathways with the ultimate goal of potentially identifying ways to better treat disease and minimize unwanted drug side effects.

Case Study #1

To date, treatment of BRAF-mutant metastatic melanoma with mitogen-activated protein kinase (MAPK) pathway targeted therapies (BRAF/MEK inhibitors) and immune checkpoint inhibitors has revolutionized disease management and improved outcomes for patients with advanced stage disease. Acquired resistance to MAPK inhibitor therapy, however, typically develops in most patients within a year and multiple mechanisms have been identified that lead to resistance. Understanding these mechanisms of resistance is critical for the development of more effective therapeutic strategies in BRAF-mutant melanoma (Welsh et al., 2016). Other preclinical and limited clinical findings have further suggested that immunomodulatory effects in the tumor microenvironment and on circulating immune cells by a BRAF inhibitor alone and the combination of a BRAF inhibitor with a MEK inhibitor is context-dependent. To investigate this further, Liu et al. (2015), addressed the immunological effects of both the BRAF and MEK inhibitors dabrafenib and trametinib, respectively. The study focused on whether these drugs potentiate or antagonize the activity of immunomodulatory antibodies such as PD-1, PD-L1, and CTLA-4 and if these effects were context dependent. They assessed the immunological effects of dabrafenib and trametinib at clinically relevant exposure concentrations on both immune and tumor cells *in vitro* and *in vivo* and tested their anti-tumor efficacy in combination with immunomodulatory antibodies in immune-competent syngeneic mouse models. Gene expression profiling using the comprehensive NanoString nCounter GX Human Immunology v2 panel showed that when human CD4+/CD8+ T cells were activated, only trametinib and not debrafenib could partially decrease the expression levels of a subset of cytokines and chemokines (e.g. IL-1, IL-2, IL-8, IL10, TNFα, CCL2) and activation/regulation markers (e.g. CD69, CD25, PD-1, CTLA-4) when added prior to or simultaneously with T cell activators (Figure 1a). However, these data did not translate *in vivo* using an immunocompetent mouse tumor model. Instead, combinations of trametinib with immunomodulators targeting PD-1, PD-L1, or CTLA-4 in a CT26 model were more efficacious than any single agent. The combination of trametinib with anti-PD-1 increased tumor-infiltrating CD8+ T cells in CT26 tumors. Concurrent or phased sequential treatment, defined as trametinib lead-in followed by trametinib plus anti-PD-1 antibody, demonstrated superior efficacy compared with anti-PD-1 antibody followed by anti-PD-1 plus trametinib (Figure 1b).

Could some of these differences be explained by merely addressing gene expression changes? The determination of the surface and cytoplasmic expression of characteristic

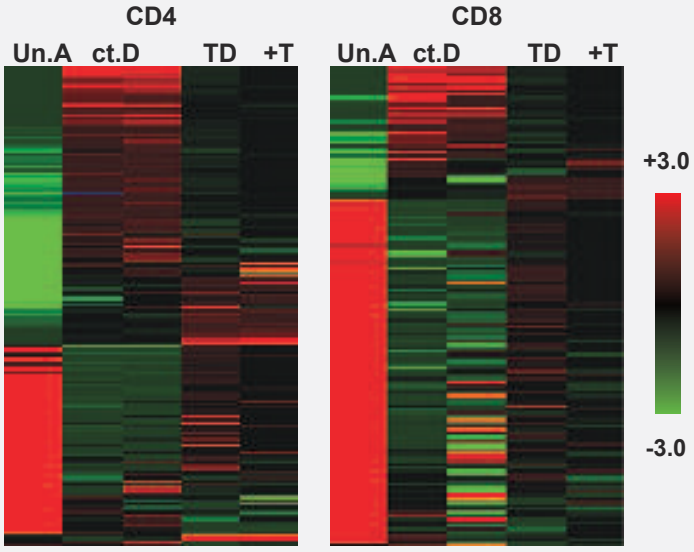

FIGURE 1a: Heatmap from representative genes. NanoString nCounter GX Human Immunology v2 panel was used. Dabrafenib (D) (300 nM), trametinib (T) (10 nM), or D+T (300 nM/10 nM) were added concurrently with CD3/CD28 activation beads to CD4+ and CD8+ T cells for 24 hours.

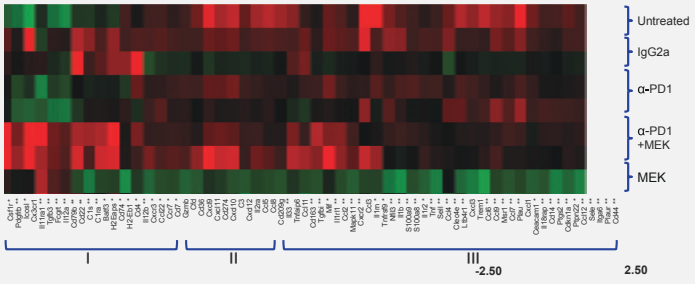

FIGURE 1b: Heatmap generated by clustering of 77 genes with ≥1.5-fold of tumor gene expression changes by any treatment group: Untreated; IgG2a, nonspecific isotype control for anti-PD-1; α-PD-1, anti-PD-1 antibody treatment; MEK, T treatment.

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proteins by flow cytometry is a common method for the diagnosis of various diseases and immunophenotyping represents an important tool in the diagnosis and staging of patients. The authors utilized multi-color flow cytometry in their studies and confirmed cell surface changes in the expression of CD69, CD25, PD-1, OX40, and CTLA-4 with trametinib, however, the expression levels of CD69 and OX40 were above non-activated T cells. On tumor cells, dabrafenib and trametinib up-regulated HLA molecules and melanoma antigen MART1 expression, and down regulated immune-suppressive factors such as PD-L1 (Figure 2), VEGF, and IL-8 etc in BRAF V600E melanoma cells. Further protein exploration using immunohistochemistry techniques revealed that treatment with trametinib alone and in combination with anti-PD-1 antibody led to 70–75% inhibition of phospho-ERK in the tumor, thus demonstrating effective MAPK signaling inhibition by trametinib in the CT26 tumor model in immunocompetent mice. This study effectively demonstrates the utility of a multi-analyte approach to support clinical exploration of dabrafenib and/or trametinib in combination with specific immunomodulatory antibodies.

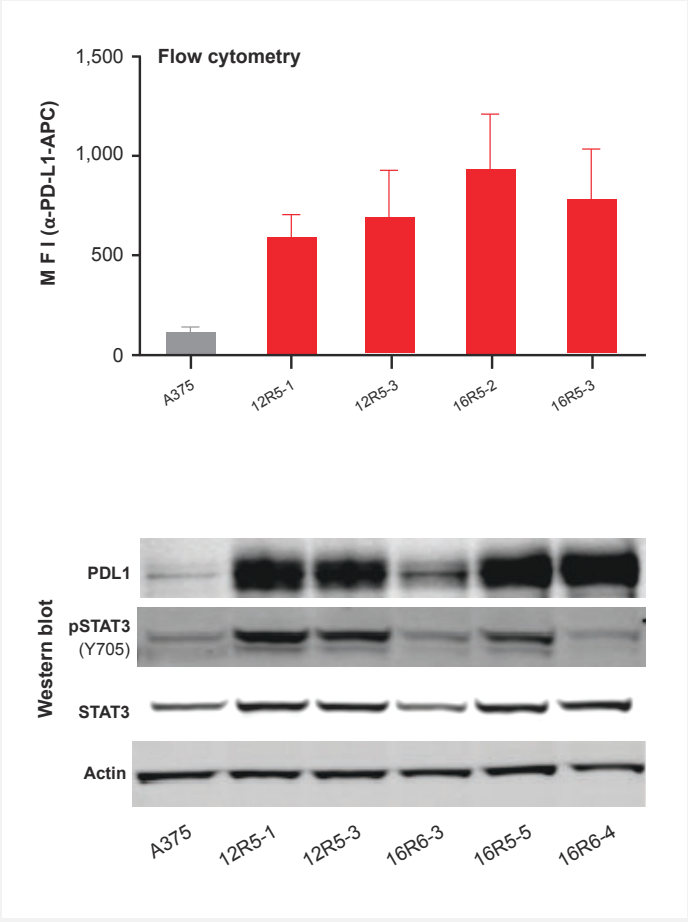

FIGURE 2: Immunomodulation by D and T in A375 BRAF mutant melanoma cells. PD-L1 protein expression from flow cytometry and Western Blot analyses in A375 parental and D acquired resistant cell lines.

Case Study #2

Chimeric antigen receptor T cell (CART)-based therapies have been recently adopted for the treatment of B cell related cancers, among others. The premise of CART therapy is to extract a patient's T cells from blood and engineer the cells to recognize and kill cancer before reinfusing the cells back into the patient. This occurs by modifying the cells with a viral vector to induce expression of an artificial, or chimeric, receptor specific for a particular cancer-associated antigen, which in most cases is CD19, an antigen expressed in B cell-related cancers. Lawrence Cooper's lab in Houston adopted a similar strategy but instead used a non-viral system called "Sleeping Beauty," which relies on a transposon derived from fish to integrate any desired gene into the genome. The system employs electroporation to introduce elements of the Sleeping Beauty system into T cells. The Cooper lab demonstrated a need for their pre-clinical experimental approach to investigate immune cell function to support their current "first-in-human" Phase I clinical trial of CAR+ T cells. The trial is for an investigational therapy that utilizes Receptor tyrosine kinase-like orphan receptor-1 (ROR1), which is expressed on sub-populations of B cell malignancies and solid tumors but not by healthy B cells or normal post-partum tissues. A major advantage of targeting ROR1 over the current T cell therapies targeting CD19 is that recipients would not deplete B cells and develop hypogammaglobulinemia, thereby mitigating the risk for impaired humoral immunity. The group selected for T cells expressing CAR through co-culture with γ-irradiated activating and propagating cells (AaPC) that co-expressed ROR1 and co-stimulatory molecules by using a NanoString custom CodeSet, "Lymphocyte codeset array," and multi-panel flow cytometry. To assess

the transcriptional profile of CAR+ T cells following numeric expansions with AaPC and cytokines, RNA lysates were profiled (Figure 3a) for expression of a selected group of lymphocyte genes. Genes were segmented by transcription factors (Figure 3a, Left), genes associated with survival, co-stimulation, and trafficking (Figure 3a, Center), and genes associated with effector function (Figure 3a, Right). Similarly, a multiplexed flow cytometry methodology was used to validate the NanoString assay by examining the surface expression of canonical T cell markers to determine the frequencies of memory populations (Figure 3b). This approach highlights the utility of both RNA and protein experimental approaches to demonstrate that, in this case, the surface phenotype of ROR1-specific CAR+ T cells was corroborated and indicated that these cells have desirable characteristics for fighting ROR1+ malignancies. One aspect to consider is that as part of the proteomic analysis by flow cytometry, the study was limited to looking at several co-stimulatory molecules as well as priming and activation associated proteins. Casting a wider net by investigating other cell surface or intracellular protein expression levels to determine the function of these novel modified T cells may prove beneficial in limiting potentially harmful side effects.

Case Study #3

Case Study #2 focused on novel immunotherapy targeting ROR1, which is one of a number of Receptor Tyrosine Kinases (RTK) that when aberrantly activated can be a driving force of human carcinogenesis. As a result, many small-molecule inhibitors targeting RTK have been developed for cancer therapy. RTK are transmembrane-type receptors with cytoplasmic tyrosine kinase domains, which transduce extracellular signals to a variety of intracellular signaling cascades, such as RAS-ERK, PI3K-AKT, IP3-Ca2+, and DAG-PKC. Importantly, they represent a large superfamily that are classified into the EGFR, FGFR, INSR ROR, and EPH groups (Katoh, 2016). The fibroblast growth factor receptors (FGFR) are involved in the regulation of cell survival, proliferation, differentiation, and motility during embryogenesis, adult-tissue homeostasis, and carcinogenesis. They have, therefore, become an attractive target for novel treatment options in tumors where they are aberrantly expressed. Several small-molecule FGFR kinase inhibitors are currently in clinical development. One such inhibitor is AZD4547, which is a novel and selective inhibitor of the FGFR1, 2, and 3 tyrosine kinases that has been shown in many studies to inhibit proliferation in a dose-dependent manner, specifically in cancer cell-lines

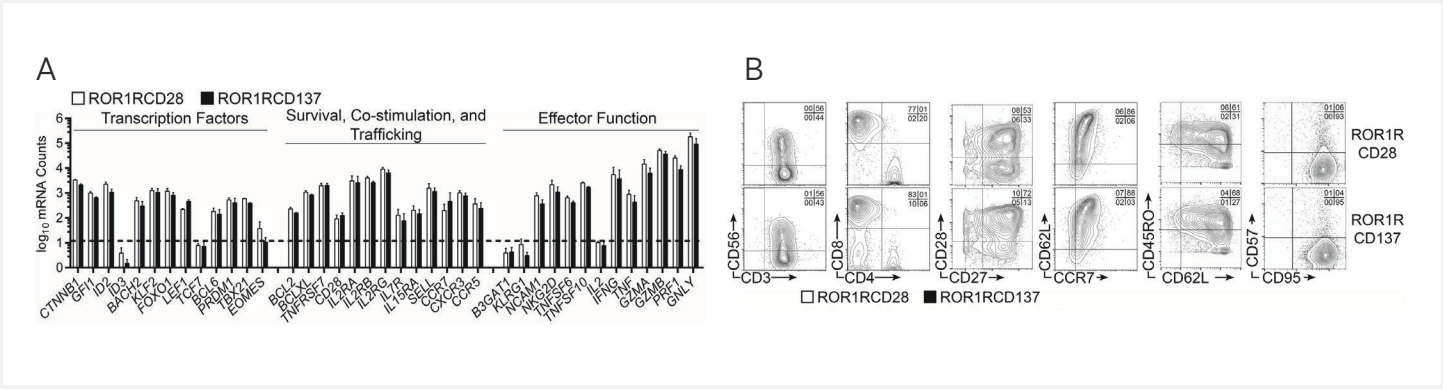

FIGURE 3: Lymphocyte transcriptional profile and memory markers expressed on CAR+ T cell surface. After 29 days of expansion on clone#1 AaPC/IL-2/IL-21, ROR1RCD28 and ROR1RCD137 cells were lysed for mRNA expression analysis using NanoString or phenotyped for T cell surface markers by flow cytometry. (a) RNA lysates were profiled for expression of a selected group of lymphocyte genes with non-enzymatic digital multiplex array of mRNA transcripts (NanoString) where transcription factors are shown on the left, genes associated with survival, co-stimulation, and trafficking are shown in the center, and genes associated with effector function are shown on the right. The dashed line represents the limit-of-detection calculated by mean +2xSD of negative controls. (b) Flow cytometry of ROR1RCD28 and ROR1RCD137 T cells showing co-staining for CD3 and CD56, CD4 and CD8, CD28 and CD27, CD62L and CCR7, CD45RO and CD62L, or CD95 and CD57 in cells gated for CAR expression based staining with Fc-specific antibody. ROR1RCD28+ T cells are shown in the top panels and ROR1RCD137 T cells are displayed in the bottom panels. One of 3 representative donors is displayed and quadrant frequencies are shown in the upper right corners.

known to overexpress FGFR. A study by Delpeuch et al. (2016) comprehensively investigated the effects of AZD4547 on the FGFR pathway. One of the current limitations are the number of available assays that detect direct and specific inhibition of FGFR signaling, e.g., phosphorylation of FGFR or phosphorylation of Fibroblast growth factor receptor substrate 2 (FRS2). These assays are also limited by antibody quality and compatibility with assay platforms with potential clinically applicability. Additionally, clinical tissue is often only available as formalin-fixed paraffin-embedded (FFPE) material and limited in quantity, restricting the number of potential protein biomarkers that can be investigated by IHC analysis. As a result, only limited protein analysis could be achieved using cells lines and xenograft material in this study. Analysis by western blotting of the phosphorylation of two known key downstream mediators of FGFR signaling, FRS2 and ERK, demonstrated target engagement, and further consideration of ERK signaling using IHC approaches was limited by the availability and quality of the antibodies (Figure 4a,b). Transcript biomarker analysis using qPCR approaches was refined and triaged for NanoString analysis, which allowed a broader pathway output overview due to the multiplex capacity and high dynamic range of the assay. To confirm consistency in transcript biomarker modulation between the two platforms, RNA expression from xenograft models was analyzed using both qPCR and NanoString. Dynamic changes of key transcript biomarkers showed a high level of correlation and consistency across both platforms, demonstrating these transcript biomarkers can be transferred reliably to NanoString's nCounter platform to better enable clinically-relevant research (Figure 5a). However, to investigate if some of the key transcripts could serve as pharmacodynamic (PD) biomarkers, and be detected at adequate levels, their baseline level expression was

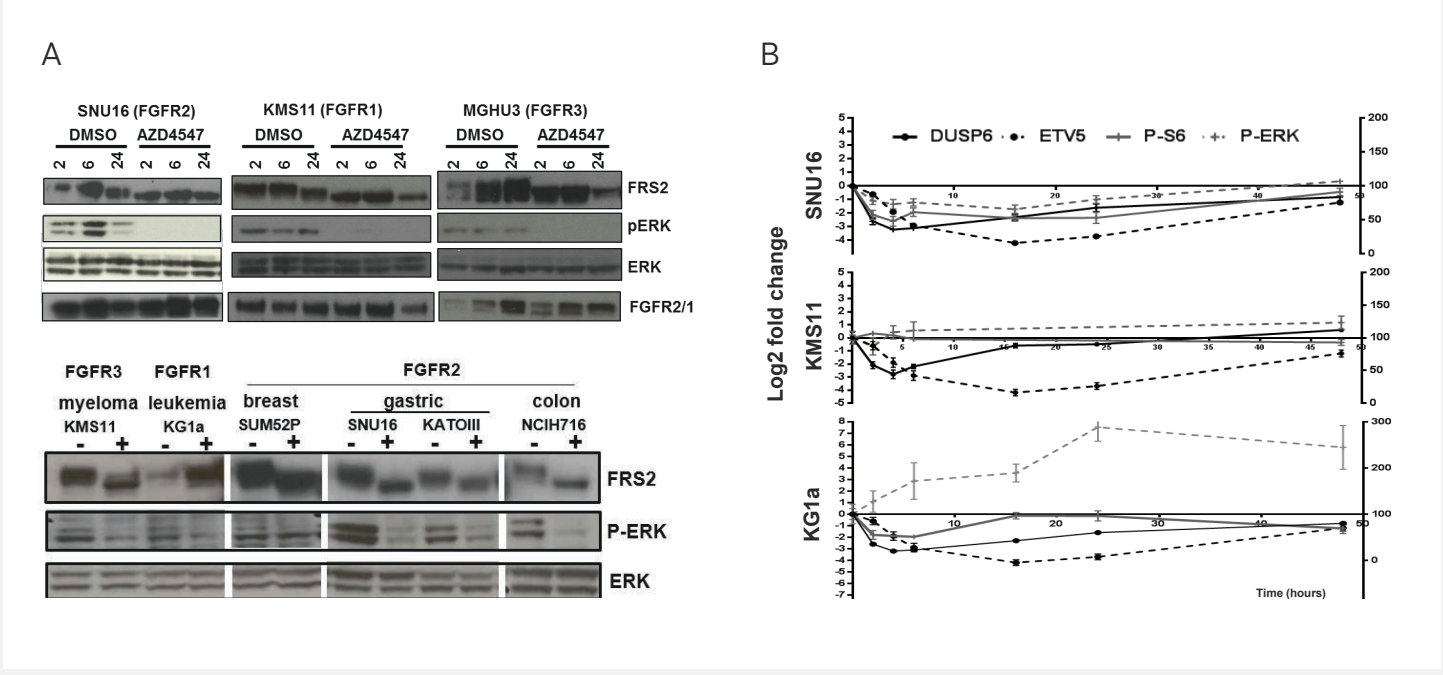

FIGURE 4: FGFR pathway modulation in FGFR1, 2, or 3 dysregulated and control cell lines treated with AZD4547. (a) Cell lysate were analyzed by Western Blot for phosphorylation of FRS2 and ERK, over time, on a selection of FGFR1, 2, and 3 cell lines represented. Similarly, cell lysate after 6 hours treatment of a larger cell line panel with FGFR1, 2, or 3 dysregulation or with similar tissue background but no FGFR deregulation is represented. (b) FFPE sections of all xenograft models (SNU16, KG1a, KMS11) were stained for IHC with phospho-ERK (grey plain line) and phospho-S6 (grey dotted line) antibodies and quantified (right y-axis) and compared to log2 fold change (left y-axis) of DUSP6 (black plain line) and ETV5 (black dotted line) over time (x-axis).

assessed in 195 gastric cancer tumors by NanoString analysis. The data as indicated in Figure 5b supports the potential of several key transcripts to be evaluated as PD biomarkers of FGFR inhibition. The challenge presented in this publication was the limit in addressing protein biomarkers that could be used for analysis of FGFR pathway modulation in clinical tumor tissue due to antibody specificity and quality issues for proximal markers. These PD biomarkers such as phospho-ERK and phospho-S6 could only be accommodated by semi-quantitative IHC assays on clinical tumor tissue.

Having the ability to perform a single integrated assay that analyzes mRNA, protein, protein post-translational modifications, and even SNV from limited amounts of clinical specimens, now made possible with the nCounter® Vantage 3D™ DNA:RNA:Protein Solid Tumor Assay, is the ideal PD analytical platform.

Conclusion

The research studies described in this White Paper highlight the importance of analyzing both RNA and protein to gain a deeper understanding of mechanism of action and response to a variety of cancer therapeutics. Each case study also highlights the challenges of such endeavors where it is necessary to integrate and extrapolate from disparate technologies and their associated data outputs. Studying RNA or protein alone can lead to incomplete or incorrect biological conclusions, a limitation that can be overcome by stringent study of both analytes at the same time from the same biological specimen. Analysis of multi-analyte data, especially in situations of discordance, will benefit from increasing the numbers of measured analytes, limiting variables in sample processing, and simplifying data outputs, thus increasing the pace of discovery.

To learn more about how 3D Biology™ Technology enables simultaneous analysis of DNA, RNA, and protein from a single sample on the nCounter platform, visit 3d.nanostring.com.

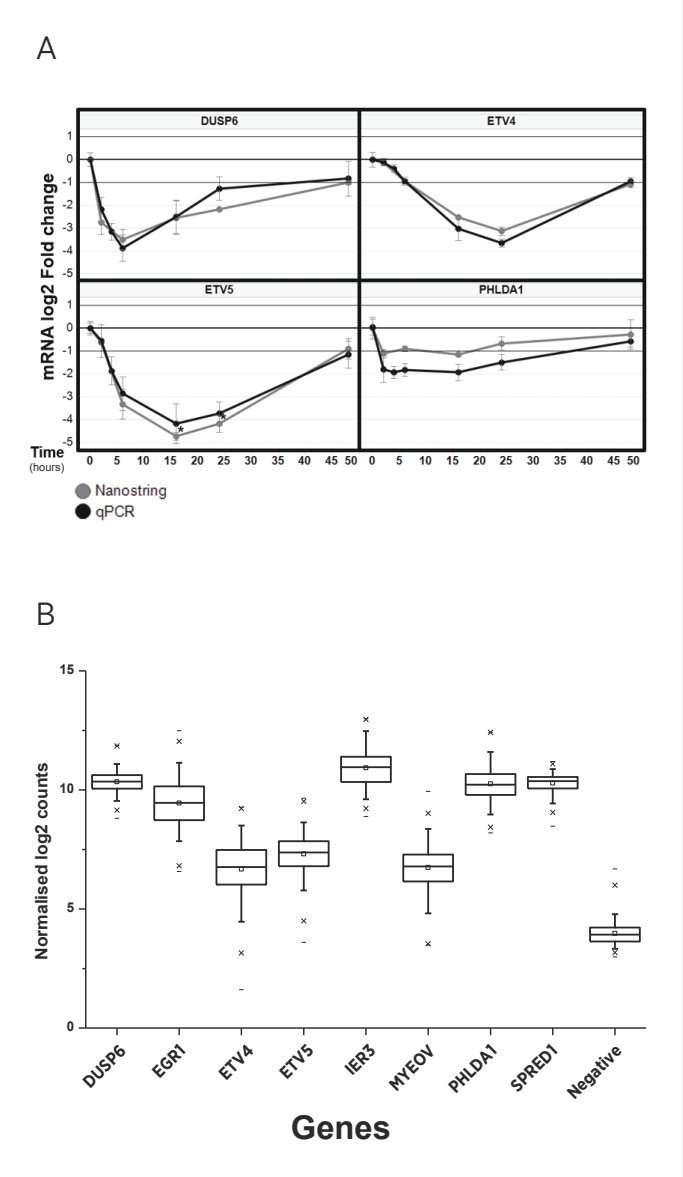

FIGURE 5: Transfer of AZD4547 transcript biomarkers to NanoString platform and detection in FFPE clinical tissue. (a) Correlation of NanoString and qPCR gene expression in xenograft model RNA from SNU16 xenograft. Samples were run on NanoString, data were normalized to vehicle control group and compared to Fluidigm qPCR data. ETV5 expression at 16 and 24 hours were below the limit of detection and highlighted with a star (*). (b) Baseline expression of dynamic genes in gastric cancer samples. RNA from 195 FFPE Vietnamese gastric cancer patients were analysed by NanoString. The range of expression of each dynamic gene is shown. Negative represents the limit of detection for each sample.

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