Performance of the Oncomine Immune Response Research Assay—a highly sensitive and robust tool for immune response research

Introduction

Immunotherapy and the need for biomarkers

Immunotherapy has emerged as one of the most promising options for the treatment of an increasing number of cancers. While there have been successes with various malignancies, favorable response occurs only in a fraction of patients. Side effects of treatment can include severe adverse events such as inflammatory toxicity, underscoring the challenges that remain in optimizing the use of these powerful therapies.

An improved understanding of biomarkers associated with response to immunotherapy will help overcome some of these challenges. Optimizing such biomarkers could help to properly select patients for treatment, identify rational combination therapies, and define disease progression and resistance to treatment. In addition, biomarkers may help define the mechanism of action for different pharmaceutical agents and help with dose selection, as well as the sequence of administering drug combinations [1].

The cancer immune response is a multistep process involving interactions between the tumor's malignant cells and their microenvironment. While a few, single-analyte immunohistochemistry tests targeting specific, immune-response biomarkers have received FDA approval to date [2], the complexity of tumor biology and the immune response suggests that multipanel markers may be more informative for understanding predictors of response to immune-targeted therapy.

Precision immunotherapy driven by genomics can help select specific strategies matched to patient and tumor characteristics. The development of large-scale, rapid-throughput gene expression profiling of tumors has facilitated research into identification of predictive biomarkers and significant molecular pathways, and shows promise to enable patient selection and prediction of response [3].

The Oncomine Immune Response Research Assay

The Ion Torrent™ Oncomine™ Immune Response Research Assay is a targeted next-generation sequencing (NGS) gene expression assay, developed using the proven Ion AmpliSeq™ technology. It enables immunotherapy research with the quantitative evaluation of the expression of markers associated with different leukocyte subsets, antigen presentation, checkpoint pathways, and tumor progression. The assay sensitively measures the expression of genes involved in tumor–immune system interactions including low-expressing genes involved in inflammatory signaling.

This panel can help enable translational biomarker studies into the mechanism of action of pharmaceutical agents, response classifier discovery, and development toward evaluation of optimal combination drug therapy.

Selection of genes

The content for the gene panel was selected to assess the tumor microenvironment and was based on literature, noting potentially predictive markers for drug response. The genes represented were carefully and extensively curated from multiple sources, which included over 200 peer-reviewed articles, input from experts at the Japan National Cancer Center, pharmaceutical companies, public databases such as the Database for Annotation, Visualization, and Integrated Discovery (DAVID), and **clinicaltrials.gov** (the registry for clinical trials), as well as the Ion Torrent™ Oncomine™ Knowledgebase [4,5], one of the world's largest collections of curated oncology data. This resulted in comprehensive coverage of targets associated with key genes expressed in the tumor microenvironment, as well as biomarkers involved in the immune response.

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Genes represented

The 395 genes represented in the assay include drug targets, genes involved in checkpoint pathways, markers of different types of immune cells, signaling molecules that inform B and T cell activation, tumor characterization markers such as tumor antigens, markers of proliferation and apoptosis, and housekeeping genes for quality control.

The assay genes, spanning across 36 functional groups, fall into 6 major categories as listed in Table 1. These categories are lymphocyte regulation, cytokine signaling, lymphocyte markers, checkpoint pathway, tumor characterization, and housekeeping. A complete list of genes is available at **http://www.thermofisher.com/us/en/home/global/forms/gene-list-request-form.html**

Assay overview and requirements

The Oncomine Immune Response Research Assay, designed with proven Ion AmpliSeq technology, requires a minimum of 10 ng of total RNA isolated from formalin-fixed, paraffin-embedded (FFPE) tissue. Performace of the Invitrogen™ RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (RNA extraction only) has been verified for this assay. Using a greater amount of FFPE-derived RNA (up to 100 ng) has also been tested, and has resulted in good yield, especially with challenging, degraded samples.

Table 1. List of 395 genes included in the Oncomine Immune Response Research Assay—based on 6 major categories.

Function	Number of genes
Antigen presentation	3
Antigen processing	19
Innate immune response	11
Leukocyte inhibition	2
Leukocyte migration	5
Lymphocyte activation	2
Lymphocyte development	3
Lymphocyte infiltration	46
B cell receptor signaling	3
T cell receptor signaling	6
T cell regulation	9
TCR coexpression	19
Chemokine signaling	10
Cytokine signaling	15
Interferon signaling	8
Type I interferon signaling	8
Type II interferon signaling	23
Housekeeping	11

Function	Number of genes
B cell marker	11
Dendritic cell	7
Dendritic cell, macrophage	6
Helper T cells	8
Macrophage	5
Myeloid marker	7
Neutrophil	5
NK cell activation	8
NK cell marker	4
T cell differentiation	2
Checkpoint pathway	30
PD-1 signaling	9
Drug target	21
Adhesion, migration	14
Apoptosis	4
Proliferation	10
Tumor antigen	17
Tumor marker	27

Workflows

The assay allows sample multiplexing with flexibility to run up to 32 samples per run. Three different workflows (Figure 1) have been verified, which deliver results in 48 hours starting from the addition of total RNA.

Performance characteristics of the Oncomine Immune Response Research Assay

High sensitivity of the targeted assay

By querying a targeted set of genes that are involved in the immune response instead of the entire transcriptome, the ability to detect and accurately quantify low-expressing genes such as cytokines is significantly improved since a targeted assay allows for greater sensitivity through more reads per gene.

Figure 2 highlights this increased sensitivity in comparison with the Ion AmpliSeq™ Transcriptome Human Gene Expression Kit. In data generated on 8 non-small cell lung cancer (NSCLC) samples, the Oncomine Immune Response Research Assay shows 20- to 30-fold higher sensitivity compared with the transcriptome assay for genes involved in the immune response. For example, interferon-gamma (*IFNG*, the final gene shown on the graph) is an important biomarker of the immune response, and is reliably detected using the Oncomine Immune Response Research Assay, but not always with the Transcriptome Human Gene Expression Kit or other gene expression assays.

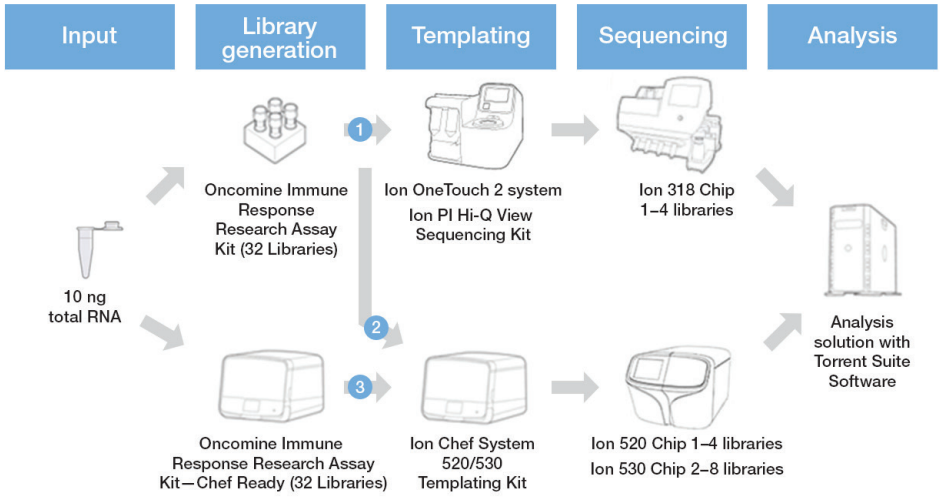

Figure 1. Oncomine Immune Response Research Assay steps: three different workflows with high sample-multiplexing capability.

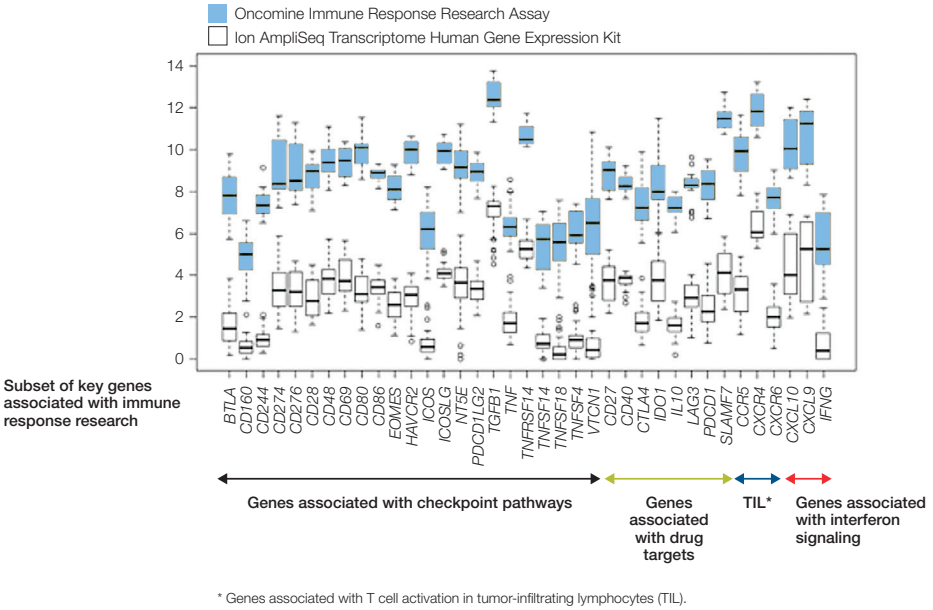

Figure 2. The box-and-whisker plot depicts the $\log_2$ ratios of the reads per million (RPM) for the genes shown on the x-axis in 8 NSCLC samples. The box represents the interquartile range, the line within the box represents the median value, whiskers represent the 10th and 90th percentile values, and outliers are represented by dots.

A study performed with 8 colorectal cancer samples (Figure 3) shows that both assays are highly comparable across a wide range of gene expression. However, when comparing the same genes across both assays, nearly 10% of the genes, robustly estimated using the Oncomine Immune Response Research Assay, were within the statistical noise level of low-expressed genes with the transcriptome assay, and therefore undetected. These low-expressing genes are shown within the red ovals in Figure 3.

Limit of blank and limit of detection

The limit of blank, which establishes the specificity of an assay, was performed with a sample containing no RNA, i.e., water only. Of the 395 amplicons, 382 were never detected, and the remaining 13 amplicons were detected in one or more experiments, but at very low levels, i.e., <5% of the levels in typical samples. In an additional experiment, two FFPE melanoma DNA samples were treated with DNase prior to processing with the standard protocol. In one of the samples, there was a zero count for all genes, and no more than 6 counts for any gene in the other samples, indicating the high specificity of the assay.

To assess the limit of detection for fold change, two cell lines with dissimilar gene expression profiles, the Invitrogen™ Promyelocytic Leukemia (HL-60) Total RNA (Cat. No. AM7836) and Human Lung Total RNA (Cat. No. AM7968) were selected for use in mixing experiments. Six dilutions were prepared (20:0, 10:10, 4:16, 2:18, 1:19, and 0:20) and each dilution was analyzed in triplicate. With 2 runs each, a total of 36 libraries were prepared and analyzed.

The assays showed ≥99% specificity even at the 2-fold dilution level. Sensitivity was also high, >90% for a 4-fold change and 76% for a 2-fold change.

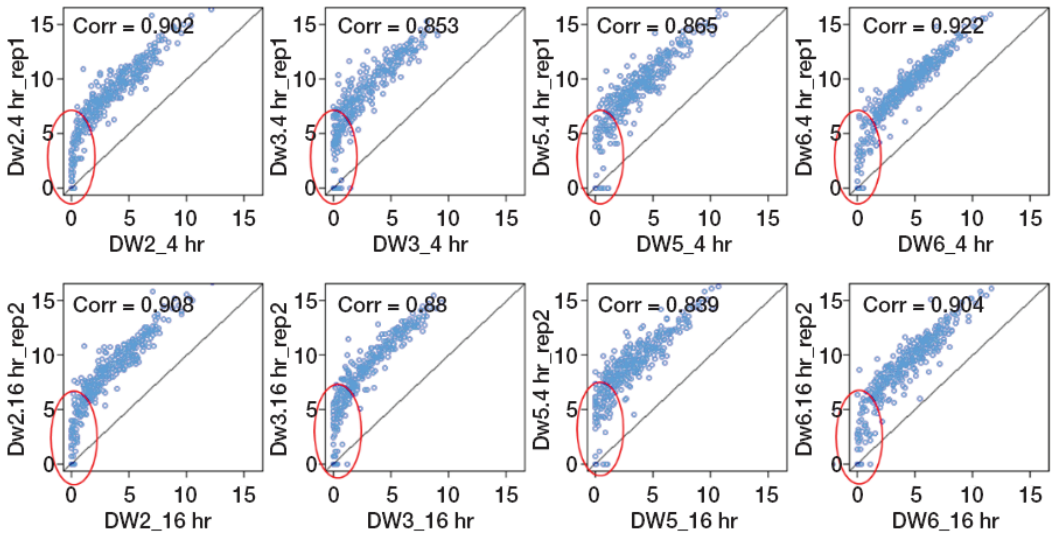

Figure 3. The scatterplots show the $\log_2$ ratios of the reads per million (RPM) for all genes detected with the Oncomine Immune Response Research Assay (y-axis) and the transcriptome assay (x-axis) in 8 colorectal cancer samples. The red ovals indicate the genes that are detected by the Oncomine Immune Response Research Assay but are within the statistical noise for the transcriptome assay. These data collectively demonstrate the high performance of the Oncomine Immune Response Research Assay.

Highly concordant results between FFPE and fresh-frozen (FF) tissue samples

The Oncomine Immune Response Research Assay performance was compared between FF and FFPE tissues. Three different NSCLC samples were compared for the two specimen types (Figure 4). Within each specimen type, the expression levels were highly concordant (Figure 4A), demonstrating the reproducibility of the assay. For the same sample, a comparison of FF vs. FFPE samples also shows high concordance (Figure 4B). These data show that the Oncomine Immune Response Research Assay data generated from FFPE tissue is also representative of data generated from FF tissue.

In contrast, two different biological samples revealed lower concordance in expression (Figure 4C), reflecting the expected biological variation between samples. The significantly lower technical variability provides confidence that the assay can be used to measure important biological differences.

Concordance with TaqMan assay

The concordance in the expression of 22 genes (*CD2*, *CD28*, *CD52*, *CDKN3, CTLA4*, *CXCL9*, *DDX58, FOXP3*, *GUSB*, *GZMA*, *ID2*, *IFNG*, *IL6*, *IL7R*, *KLRG1*, *LCN2*, *MLANA*, *PMEL*, *TBP*, *TFRC*, *TNF*, and *TNFRSF14*) was evaluated using both the Oncomine Immune Response Research Assay and the Applied Biosystems™ TaqMan® qPCR assay. The genes were selected to include high-, medium-, and low-expression using NGS. Total RNA samples from 8 NSCLC FFPE tissue samples and 2 control samples were used, from the human promyelocytic leukemia cell line HL-60 and normal human lung cell line as previously mentioned.

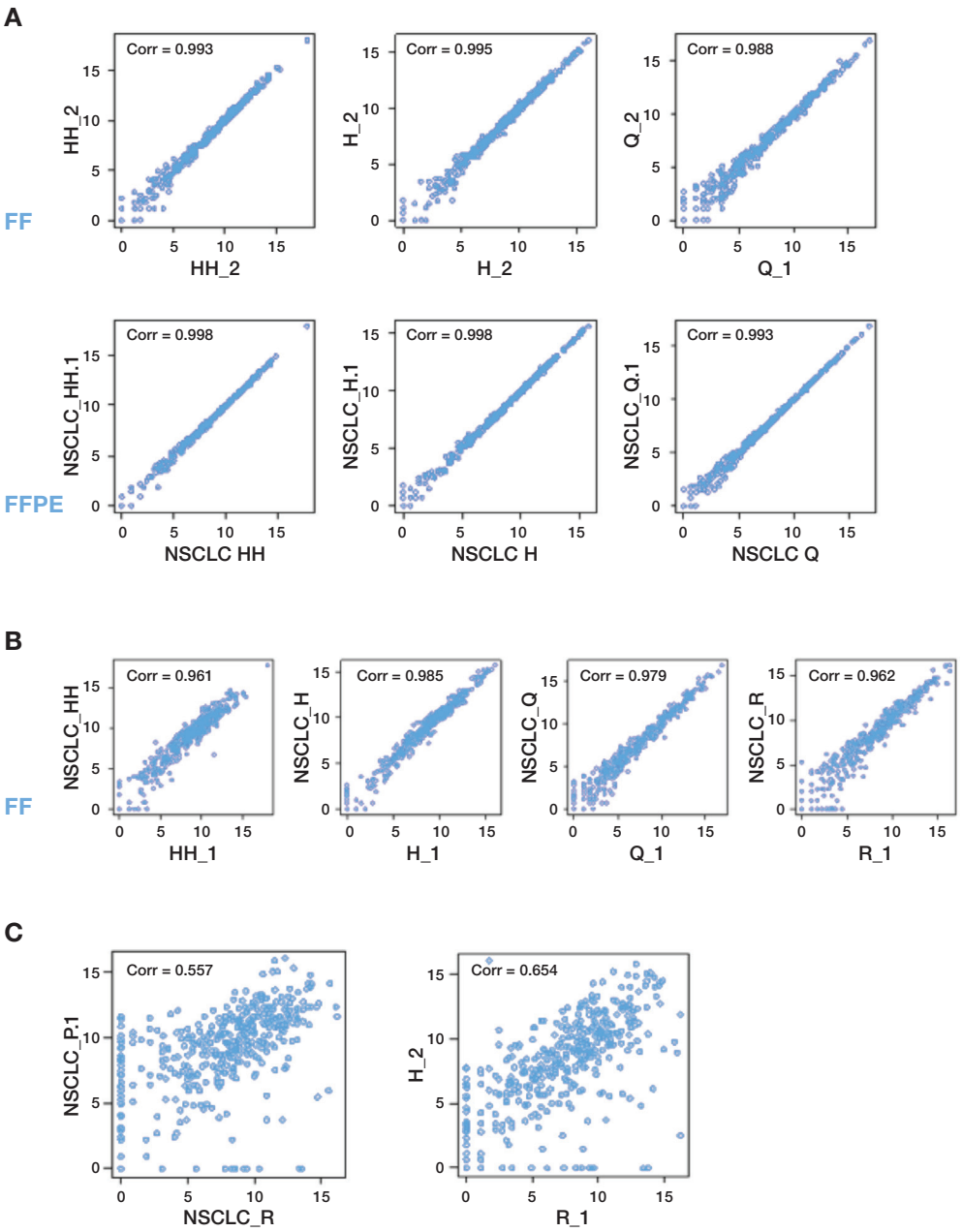

Figure 4. The scatterplots show the $\log_2$ ratios of the reads per million (RPM) for all genes detected with the Oncomine Immune Response Research Assay on NSCLC samples. Results from **(A)** technical replicates of 3 different samples, FF (top panel) or FFPE (lower panel); **(B)** FF sample (x-axis) vs. the corresponding FFPE sample (y-axis); and **(C)** two different FF (left) or FFPE (right) samples samples were compared. These data collectively demonstrate the high performance of the Oncomine Immune Response Research Assay.

The absolute expression values are highly concordant between the assays with high correlation coefficients (Figure 5A). Similarly, high concordance was observed in the expression of 50 genes expressed in the Universal Human Reference (UHR, Stratagene, USA) samples and the Invitrogen™ FirstChoice™ Human Brain Reference RNA (HBRR) samples (Figure 5B).

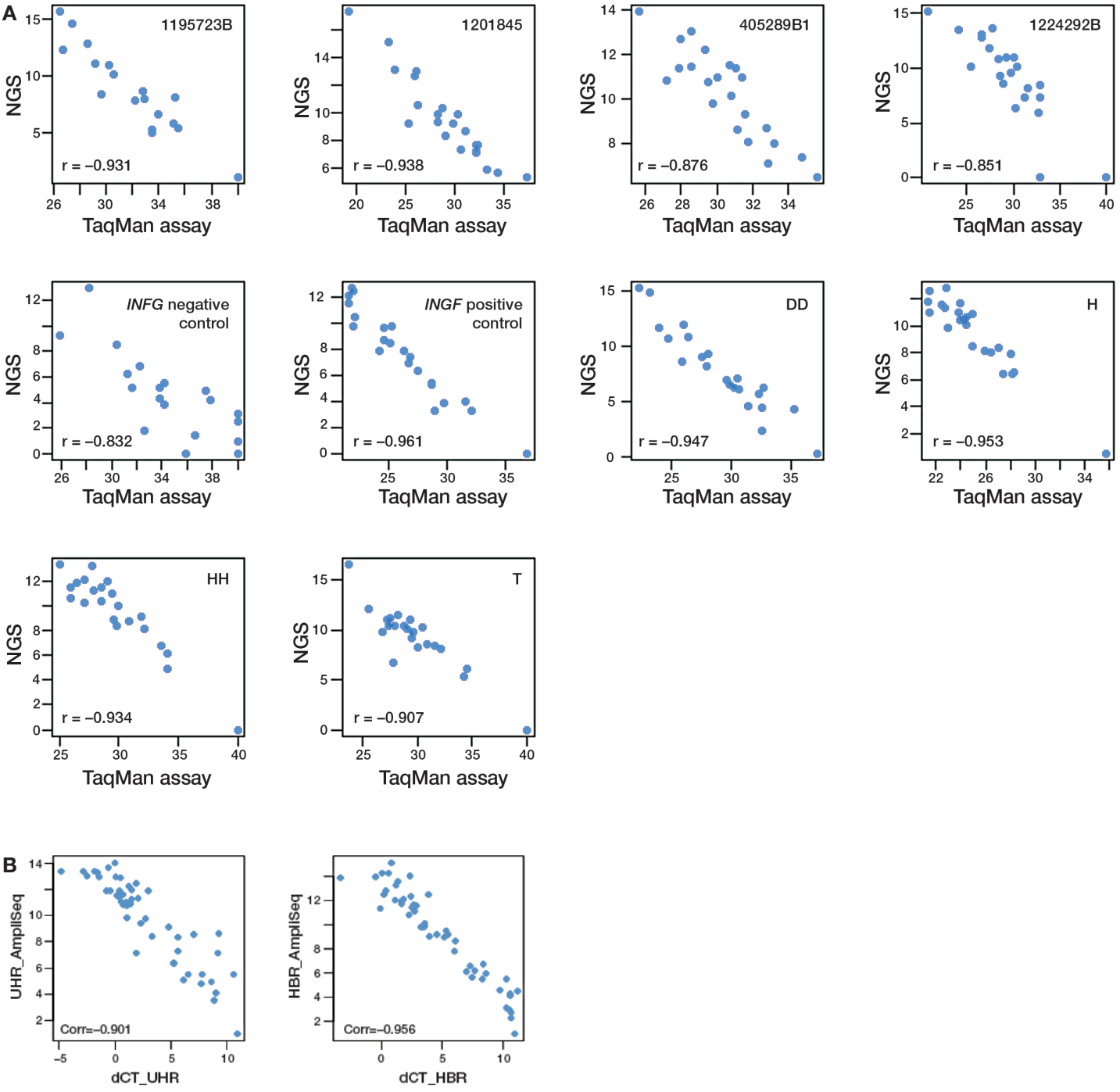

Figure 5. The scatterplots show the log_2 ratio of the reads per million (RPM) with the Oncomine Immune Response Research Assay on the y-axis and the corresponding C_t values from the TaqMan assay on the x-axis. (A) The expression of 22 genes in 8 NSCLC samples and 2 cell lines is shown. **(B)** The expression of 50 genes using two RNA reference samples, UHR and HBR, is shown.

Comparison of results between the Oncomine Immune Response Research Assay and NanoString nCounter PanCancer Immune Profiling assay

A set of samples from different tissues of origin was run using the Oncomine Immune Response Research Assay as well as a gene expression assay from another supplier, the NanoString™ nCounter™ PanCancer Immune Profiling assay, to assess the similarities and differences between the two technologies.

Genes represented

The genes common to both assays represent key indicators of the immune response. In the Oncomine Immune Response Research Assay, there is an increased focus on tumor antigens and tumor characterization genes. The genes exclusive to the Oncomine Immune Response Research Assay represent tumor-infiltrating lymphocyte markers important for response stratification, and T cell receptor coexpressing genes critical for biomarker discovery. The genes unique to the nCounter assay are either from pathways covered on the Oncomine Immune Response Research Assay through different gene targets or represent genes involved in more general pathways such as cell cycle regulation or transporter functions.

Samples and processing

Ten research samples (breast cancer, head and neck cancer, melanoma, NSCLC, and renal cell carcinoma samples) were run using both assays. The Oncomine Immune Response Research Assay was performed at the Thermo Fisher Scientific research laboratory on 530 chips sequenced on the Ion S5™ XL System at 8 samples/chip multiplexing, starting with 10 ng total RNA. The nCounter assay and data processing were performed by a third-party service provider, starting with 100 ng total RNA.

Reproducibility and concordance

When the same sample was run in duplicate, both assays showed high reproducibility with correlation coefficients (r) > 0.9. Moderate concordance was observed between the assays (r = 0.6 to 0.75), although as previously shown (Figure 5), the Oncomine Immune Response Research Assay shows high concordance (r > –0.9) with the TaqMan assay, considered the gold standard for gene expression.

Dynamic range

The dynamic range is estimated as the difference between high and low quantiles of achievable expression values.

A primary feature of NGS is that nonexpressed genes produce zero signal and are centered around zero, allowing expressed genes to be robustly detected. This is a key advantage of the Oncomine Immune Response Research Assay. In Figure 6, which shows the complete distribution of gene expression for both methods (Oncomine Immune Response Research Assay: blue, nCounter assay: black), each line represents the range of gene distribution in a single run. For both methods, most of the samples express 80% of genes on the panel.

Visually, it is apparent that the Oncomine Immune Response Research Assay exhibits a strong bimodal distribution with a clear separation of expressed (right peak) from nonexpressed genes (left peak), and has a higher dynamic range. In contrast, with the nCounter assay, there is no clear separation of the two peaks, suggesting a compression of dynamic range and more difficulty in clearly distinguishing expressed from nonexpressed genes.

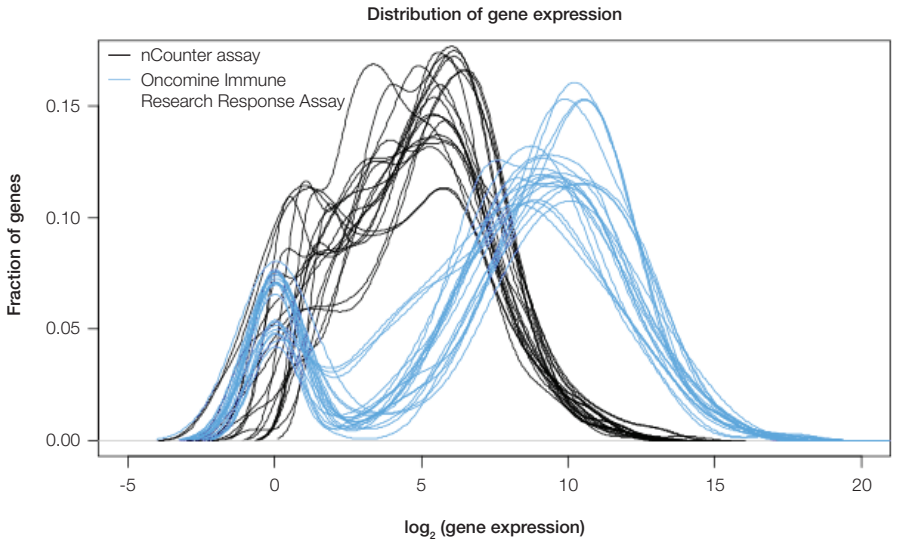

Figure 6. The y-axis shows the fraction of genes expressed. The x-axis shows the $\log_2$ ratios of the reads per million (RPM) for the Oncomine Immune Response Research Assay (blue lines) or the normalized expression for nCounter assay (black lines).

Sensitivity

The expression of all genes was compared between the two assays across 21 research samples: 4 breast cancer, 8 head and neck cancer, 5 melanoma, 3 renal cell carcinoma (RCC), and 1 NSCLC samples. As shown in Figure 7, the Oncomine Immune Response Research Assay offers 20–40% higher gene expression compared to the nCounter assay.

The expression levels for several genes involved in critical immune response functions were compared between the Oncomine Immune Response Research Assay and the nCounter assay across all 10 samples. The Oncomine Immune Response Research Assay shows a wider dynamic range of expression (Figure 8).

Conclusion

The Oncomine Immune Response Research Assay enables the interrogation of genes that are critical for research involving the immune response. Using input material as little as 10 ng of total RNA from FFPE tissue, robust and reproducible results are produced. The high dynamic range of the Oncomine Immune Response Research Assay and the ability to clearly differentiate expressed genes from nonexpressed genes, coupled with its high sensitivity in the detection of low-expressing genes, make the Oncomine Immune Response Research Assay an invaluable tool for biomarker discovery.

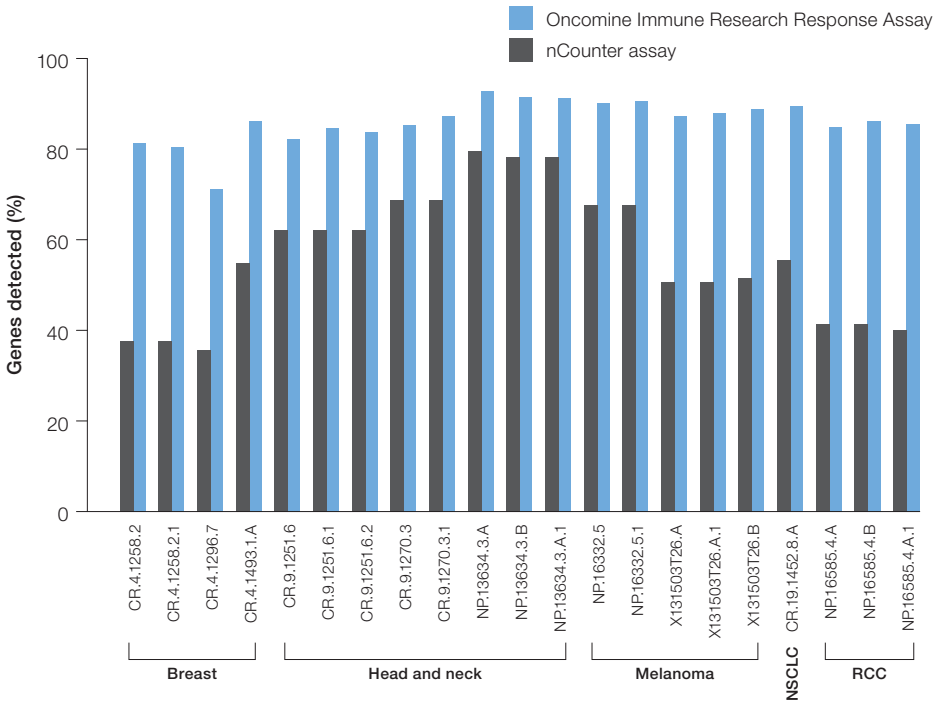

Figure 7. The y-axis shows the percent of low-expressing genes detected with either the Oncomine Immune Response Research Assay (blue) or the nCounter assay (black). The x-axis indicates the samples used in the comparison.

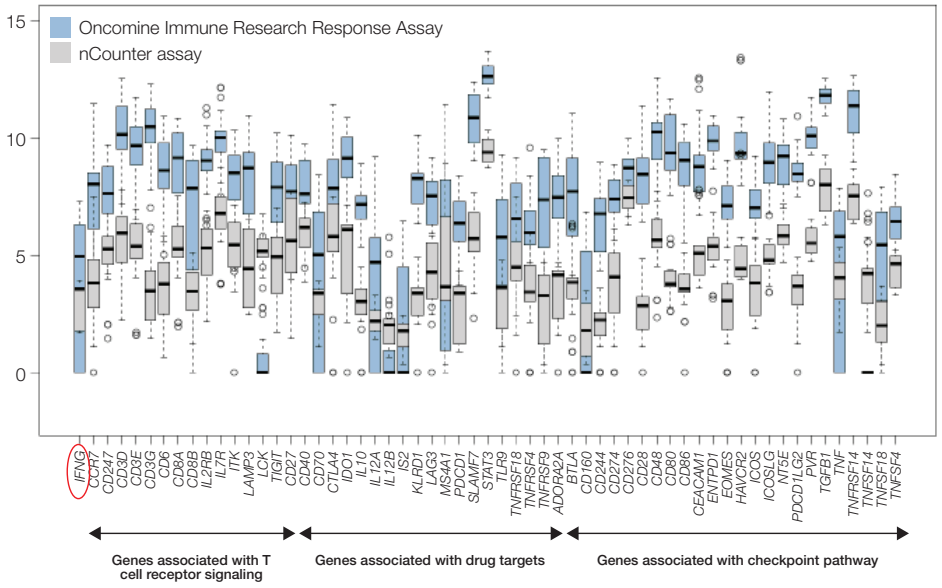

Figure 8. The box-and-whisker plot depicts the $\log_2$ ratio of the reads per million (RPM) for the Oncomine Immune Response Research Assay and the normalized expression for the nCounter assay for the genes shown on the x-axis. The box represents the interquartile range, the line within the box represents the median value, whiskers represent the 10th and 90th percentile values, and outliers are represented by dots.

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References

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Ordering information

Product	Cat. No.
Oncomine Immune Response Research Assay	A32881
Oncomine Immune Response Research Assay- Automated	A32928

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