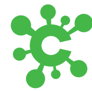

CELLECTA

Profiling of Immunity Biomarkers in Whole Blood

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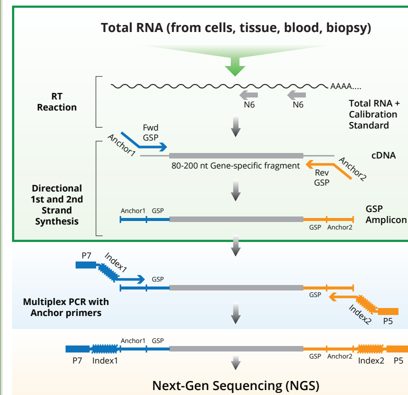
Abstract

Cancer immunotherapies are rapidly changing traditional treatment paradigms and expanding the therapeutic landscape for cancer patients. However, despite the current success of these therapies, significant number of patients do not respond to immunotherapy and responders often experience significant toxicities. Therefore, there is a growing need to identify predictive and prognostic biomarkers that enhance our understanding of dynamic immunity mechanisms involved in cancer development. The genome-wide expression profiling of whole blood is an attractive method for the discovery of biomarkers due to its non-invasiveness, possibility for retrospective simple clinical site processing, and rich biological content. To this end, we have developed GeneNet, a genome-wide targeted RNA expression assay that profiles 19,000 genes based on multiplex RT-PCR followed by NGS in whole blood samples stabilized in PAXgene. The GeneNet 19K assay provides comprehensive and unbiased profiling of immune cell activation and canonical immune pathway genes for both innate adaptive and humoral immune responses. In this study, we present the performance of the assay for immunophenotyping of immune cells in blood samples from TNBC patients and assess the immune response to complex immunomodulatory stimuli in ex vivo model system. Preliminary studies demonstrate the assay's unparalleled specificity and sensitivity resulting in better detection of low abundance mRNA transcripts as well as an improved cost-effectiveness for high-throughput clinical applications.

Features and Benefits

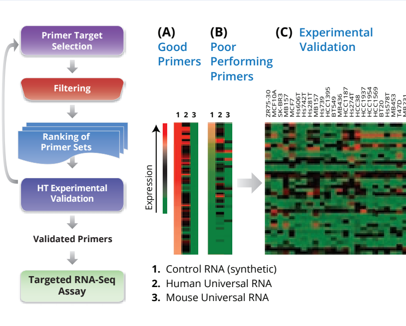
Key Features	Benefits
Multiplex RT-PCR	No rRNA or globin depletion required Use directly with total RNA
RNA Input	10 pg total RNA (single-cell) minimum 1 ng - 50 ng (optimal)
Sensitivity / Dynamic Range	Linear data in 10^5-fold dynamic range 100-fold more sensitive than RNA-Seq or GeneChip
Specificity	No background (sequencing data) Low cross-amplification of mouse cDNAs
Single test-tube Protocol	96 samples per day (2 hours hands-on time)
Result Validation	Conventional qRT-PCR using the same PCR primers

Driver-Map Expression Profiling Assay Workflow



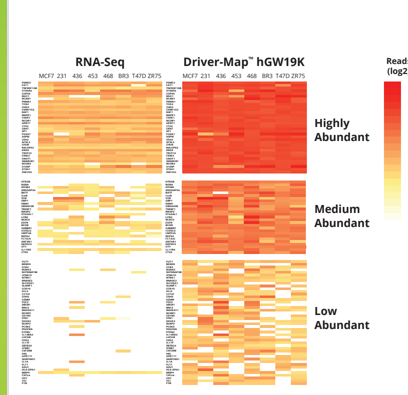
The Driver-Map workflow leverages the power of quantitative PCR with Next-Gen Sequencing (NGS). Experimentally validated primers amplify specific fixed-length regions of all protein-coding genes in a multiplex reaction. The number of reads of each of the resulting amplicons, as determined by NGS, provides a highly quantitative linear measurement of the abundance of each transcript across a range of 5 orders of magnitude. Defined amplicons also greatly facilitate alignment and downstream analysis.

Development of Driver-Map Functionally Validated RT-PCR Primers



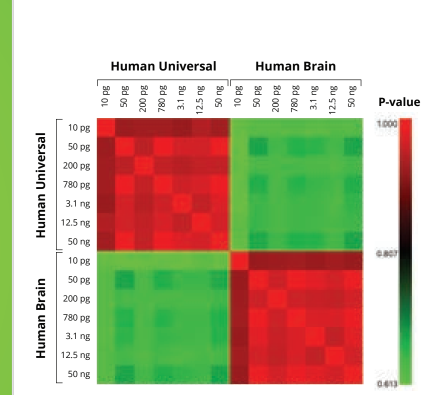
As shown in schematic on left, the final PCR primers targeting each protein-coding gene were selected experimentally for high on-target and low off-target activity from sets of 5-20 primers per each gene through multiple sequential rounds of multiplex RT-PCR/NGS experiments. A number of criteria, such as selecting low efficacy primers for highly abundant genes, enabled us to develop a set of genome-wide, PCR primers that reliably measure at least 95% of mRNAs in the 100,000-fold dynamic range (100-fold more than RNA-Seq or microarrays). Synthetic RNA control and Universal RNA demonstrate the results with well selected primers (A) versus poor performers (B). In addition, Mouse Universal control RNA enabled us to weed out primers with cross reactivity to murine homologs. Effective primers were then used with the Driver-Map approach to profile 24 cancer model cell lines (C).

Driver-Map Sensitivity vs. RNA-Seq



NGS read levels detected RNA-Seq and Driver-Map for selected high-abundant (10K-100K copies per sample), medium-abundant (1,000-10,000 copies per sample), and low-abundant transcripts (100-1,000 copies per sample) in 50ng of total RNA from seven common cancer cell lines.

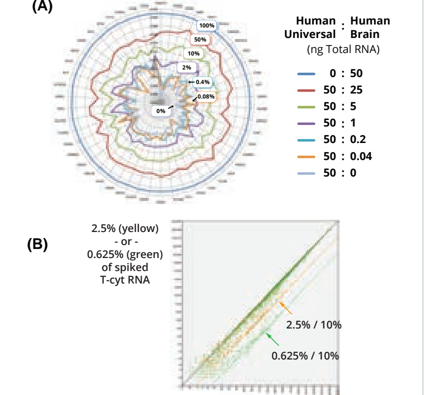
RNA Input and Reproducibility



The correlation (R-squared values) of detected genes between human universal RNA and total brain RNA remains highly consistent regardless across amounts ranging from 10 pg to 50 ng of starting total RNA.

Broad Quantitative Linear Range

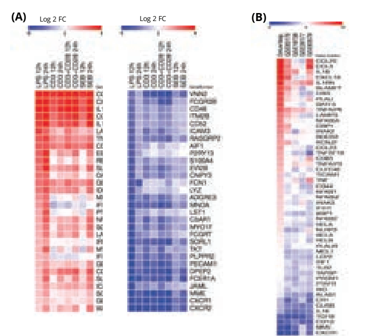
(A)



10% of spiked T-cyt RNA

Panel A: Human Universal RNA and Brain Admixture. Levels of 50 brain-specific transcripts are accurately measured from a 2:1 to 1:1250-fold dilution of brain RNA into universal RNA demonstrating a linear range of almost 3 orders of magnitude.
Panel B: Scatterplot analysis (each dot represents the number of NGS reads for a gene target) of duplicate runs of a model system with T-cyt RNA spiked at 0.625%, 2.5%, and 10% into 100ng of MDA231 RNA. Even at the lowest spike-in level, read levels for each of the detected gene remains highly reproducible.

Model System: Activation of Whole Blood

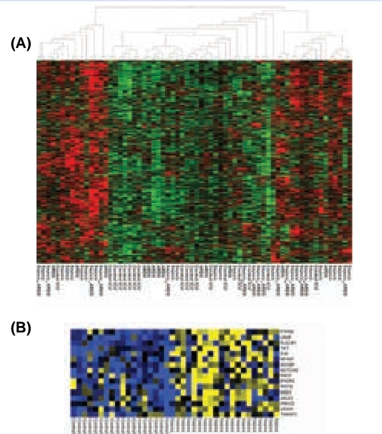


Panel A Discovery of novel biomarkers directly in whole blood (TruCulture®) without fractionation. DriverMap allows sensitive detection of biomarkers in rare cells without rRNA or globin RNA depletion. Test tubes were activated by LPS, CD3, CD3-CD28 or Enterotoxin SEB for 12 hrs or 24 hrs and changes in RNA (PAXgene®) expression level were analyzed by DriverMap 8K assay.

Panel B Comparison of DriverMap and Public Datasets of LPS-stimulated samples shows good correlation but higher sensitivity in detection of differentially-expressed genes.

Public Datasets:
GSE85176_NanoString GX Human Immunology v2
GSE19738_Agilent Whole Genome Microarray G4112A
GSE36177_Illumina HumanHT-12 v3 Expression BeadChip
GSE83578_Affymetrix Human Genome U219 Array

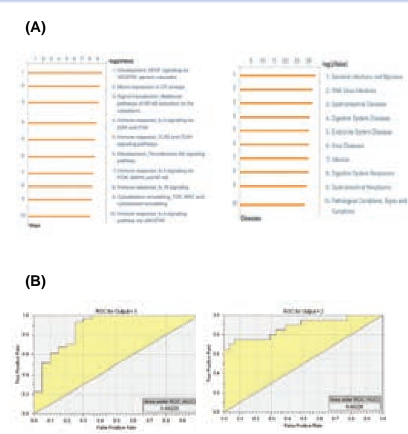
Hierarchical Cluster Analysis of Control vs Sepsis Patients



Panel A Hierarchical Cluster Analysis of Control vs Sepsis Patients. Driver-Map assay used for whole blood expression profiling of patients in intensive care unit (ICU) without additional complications (Control), with Acute Respiratory Distress Syndrome (ARDS), Sepsis-ARDS, or Sepsis indications.

Panel B Top Up-regulated Genes: Control vs Sepsis Patients.

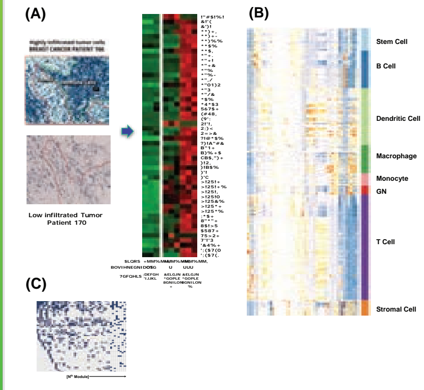
Pathways, Networks and Diseases Activated in Sepsis Conditions



Panel A Pathways and Diseases Activated in Sepsis Conditions

Panel B ROC AUC: Control vs Sepsis Patients

Driver-Map Profiling of Tumor Samples

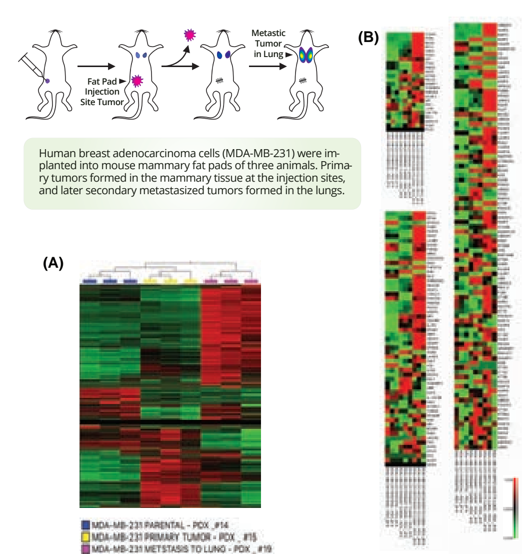


Panel A shows an example H&E stained triple-negative breast cancer tumor section with significant immune cell infiltration.

Panel B shows genome-wide Driver-Map analysis of tumor samples with varying degrees of immune infiltration. The presence and degree of infiltration of various cellular sub-types was determined based on indicative gene expression signatures present in the Driver-Map data. Red denotes control non-malignant breast tissue. Blue denotes TNBC patient with high immune cell infiltration.

Panel C shows enriched ssGSEA modules & gene conservation across gene signatures.

Profiles of Xenograft Breast Cancer Tumors



Human breast adenocarcinoma cells (MDA-MB-231) were implanted into mouse mammary fat pads of three animals. Primary tumors formed in the mammary tissue at the injection sites, and later secondary metastasized tumors formed in the lungs.

Expression Profiling in PDX Models

Driver-Map Advantage:

- Directly use RNA isolated from bulk tumor sample – no human cell purification
- Specific amplification of human mRNAs in the background of mouse transcripts

Driver-Map expression profiles of primary and metastasized xenograft mammary tumors. Human breast adenocarcinoma cells (MDA-MB-231) were implanted into mouse mammary fat pads of three animals. After several weeks, primary tumors formed in the mammary tissue at the injection sites, and secondary metastasized tumors formed in the lungs. Panel A shows the full Driver-Map profiles of the preimplanted cells, implanted primary tumors, and lung tissue containing the metastasized tumors from the animals. Analysis of the data show clear activation of signature pathways in lung tissue containing the metastatic tumors but not in the primary tumors. Panel B shows profiles for three of these pathways relevant to metastasis: cell migration, adhesion, and proteolysis active.

Conclusions

We demonstrate how multiplex PCR with well-designed targeted primers can be used in combination with NGS for robust, accurate, and reproducible genome-wide expression analysis. With a much simpler protocol, the Driver-Map Profiling Assay provides two orders of magnitude more dynamic range, 100-fold better sensitivity, and a broader linear range than RNA-Seq. Unlike RNA-Seq, the Driver-Map assay can be run with total RNA and, for blood samples, does not require globin depletion. We validated the Driver-Map assay for biomarker discovery in blood by using a model system of activation of whole blood in sepsis. We found that Driver-Map allows sensitive detection of biomarkers in rare cells without rRNA or globin RNA depletion. Comparison of Driver-Map and public datasets of LPS-stimulated samples shows very good correlation and much higher sensitivity in detection of differentially-expressed genes.

In addition, high sensitivity and specificity of the Driver-Map assay are important for its unique applications which include: (i) discovery of novel biomarkers directly in whole blood; (ii) analysis of cellular composition in complex tissue such as tumor microenvironment; and (iii) profiling of tumorigenesis mechanisms in PDX mice models. For the profiling of tumor cellular composition Driver-Map allows one to infer tumor infiltrating immune cell types and immune/stromal/cancer cell composition. The assay can be used to profile drug targets in tumors and to discover prognostic/predictive biomarkers. For the profiling of tumorigenesis mechanisms in PDX mice models, Driver-Map assay allows one to directly use RNA isolated from bulk tumor sample without human cell purification due to specific amplification of only human mRNAs in the background of mouse transcripts.

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