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Microsampling for RNA biomarker profiling in blood

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Abstract

Multiomic analysis of microsamples of lancet-induced blood drops allows frequent capture and quantitation of numerous metabolites, lipids, cytokines, and proteins. Microsample-based transcriptome profiling would facilitate the use of RNA biomarkers in the diagnosis and treatment of immunotherapy patients, but a suitable method has not been available. We tested a targeted RNA-sequencing protocol (DriverMap™ EXP assay) for this purpose in human blood microsamples.

In the first study, we compared gene expression in normal blood collected through standard phlebotomy and 30 μL of blood absorbed onto a Mitra® microsampling device with an RNA-stabilization reagent (GenTegraRNA-NEO). Anticoagulated blood was incubated with endotoxin (18 hr) and RNA was extracted from stimulated and unstimulated blood samples followed by targeted PCR amplification of 274 immune/ inflammatory genes using the DriverMap™ protocol. We found robust detection of gene expression and correlation using the two methods in both unstimulated (r = 0.94) and endotoxin-stimulated blood. The differentially expressed genes (DEGs) identified in both standard and microsample methods showed high overlap with DEGs reported in public datasets in similar experiments. In the second study, we compared whole blood extracted from Tempus™ blood RNA tubes to Mitra microsamples before and after immunization with Pneumovax® vaccine. We used DriverMap™ EXP genome-wide 19K panel for targeted RNA-sequencing. Results show that ~90% of the top 10K genes overlap between Tempus and Mitra microsamples. Moreover, microsamples stored at 4°C for over one year showed similar expression profiles to whole blood samples. We conclude that RNA transcriptome profiling using targeted sequencing allows sensitive detection of gene expression levels in normal and activated blood samples. Because microsamples can be air-dried and mailed in for analysis, this approach has great promise for simple and repeated monitoring of RNA biomarkers in immunotherapy.

Introduction

Goal

- To evaluate RNA profiling in a small volume of dried blood collected from volumetric absorbtive microsampling technology using Mitra® devices treated with GenTegraRNA-NEO™ before blood collection
- To compare results from dried microsamplers to direct analysis of identical/larger volumes of whole blood
- To test the ability of dried microsamplers to quantify biomarker and activation genes in normal and stimulated blood

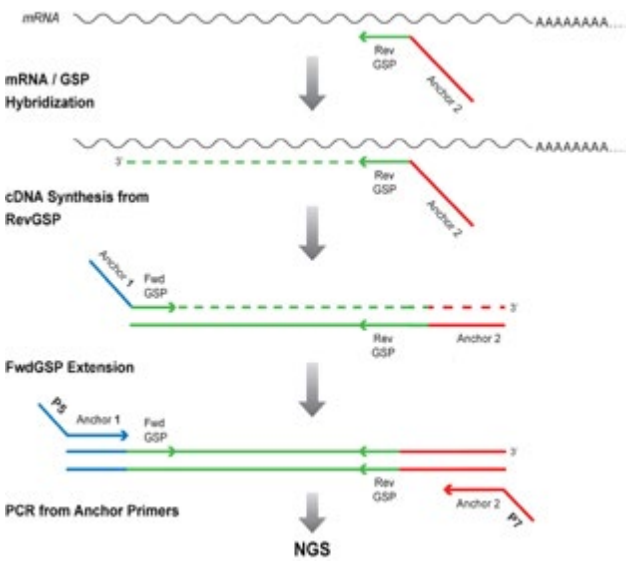
Strategy

- Compare gene expression in the same blood sample, assayed directly or from Mitra/GenTegra microsamplers
- Optimize Cellecta DriverMap™ targeted sequencing assay to work with Mitra/GenTegra microsampling technology

Method

Microsampling Technology

- Mitra devices collect 30 μL of blood by capillary action.
- The dried blood is stored and transported to the lab for further analysis.
- The microsamples are stable for at least a few months with minimum handling or processing.



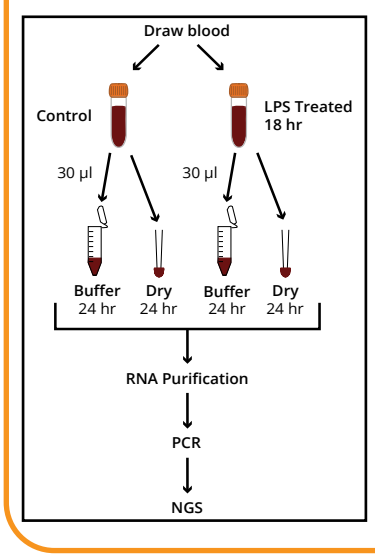
- Samples were analyzed using the DriverMap™ Multiplex RT-PCR-NGS assay for expression profiling of up to ~19,000 genes or T/B Immune Marker (~300 gene panel) in a single test-tube assay.
- Requires 100pg-10ng of total RNA from whole blood, single cell, or biopsy samples. Assay provides higher sensitivity for low expressed genes than RNA-Seq with 10-fold less sequencing depth required.

Study 1: Microsampling & Whole Blood Activation

Microsampler Testing and Whole Blood Activation Study

We collected normal blood by phlebotomy in two vacutainer tubes (Qiagen®) with anticoagulant (heparin). One of the collected anticoagulated blood samples was incubated with endotoxin (100 ng/ml LPS) to stimulate gene activation and the other was used as a control.

- After treatment, three-30 μL technical replicate samples were isolated by two different methods from both the treated and untreated samples.
- One set (n = 3) of samples were pipetted from treated vs. untreated blood, added to the lysis buffer (Proteinase K, EDTA, NP-40, and high NaCl) and shaken at 60°C for 30 minutes.
- A second set (n =3) of samples from each tube of collected blood were isolated using Mitra devices to mimic a home-use scenario, and dried at RT for 24 h (as described by the Mitra protocol). The dried blood was then added to the lysis buffer as described.
- RNA was then purified from each sample and screened using a panel of 274 immune/ inflammatory genes that were amplified using targeted DriverMap™ gene-specific primers in a multiplex RT-PCR reaction, and then quantified by next-generation sequencing (NGS) following the standard Cellecta DriverMap™ protocol.
- After normalizing the targeted NGS results, we compared gene expression variation of treated and untreated samples for both the standard pipetted collection vs. Mitra/air-dried microsamples.



Data Processing Outline

- RNA-Seq data normalized to counts per million
  – Total counts per sample: range from 860k - 920k (very similar)
  – Mean count per sample: range 3150–3351
- For variability analysis
  – Exclude genes with very low counts (<10 in both WB Ctrl & LPS
  – 29 genes excluded
  – 245 genes analyzed further

Results

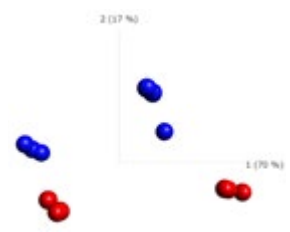


Fig 1: Principal Component Analysis (PCA) reveals a distinct separation between whole blood control vs LPS samples (blue) and dried blood control vs LPS samples (red).

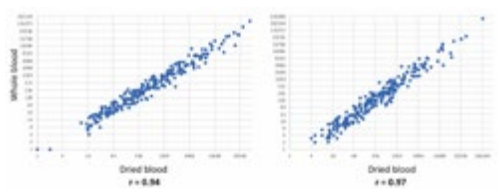


Fig 2: Comparison of whole blood (WB) vs dried blood (DB) gene counts reveals a significant correlation between the unstimulated control WB vs. DB and LPS-stimulated WB vs. DB samples

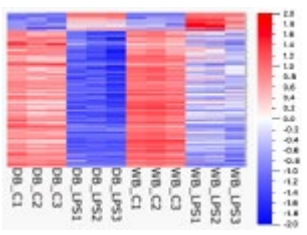


Fig 3: Differential gene expression analysis shows similar markers in unstimulated vs. stimulated samples of dried and whole blood samples. (210/274 genes, q<.05, FC 1.5+, log2 transformed, values >2 compressed)

Gene Counts	Whole Blood Control vs Dried Blood Control	# genes	Whole Blood LPS vs Dried Blood LPS	# genes
ALL	0.94	274	0.97	274
0-99	0.78	68	0.73	87
100-999	0.79	91	0.67	98
1000-111,000	0.94	86	0.97	59

Table 1: Correlation of Whole Blood vs Dried Blood Gene Counts. Correlation is good overall for high-expression genes (counts >1K). Room for improvement in low-expression genes.

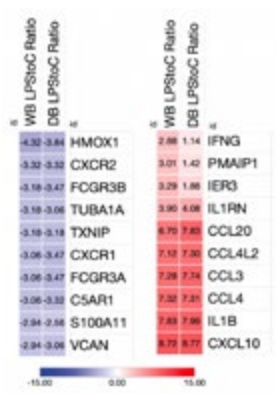


Fig 4: Top 10 Down-Regulated (Blue) and Up-Regulated (Red) genes in activated WB and DB. LPS to Ctrl Ratios are similar in WB and DB samples. (log 2 scale)

Study 2: Microsamplers & Immune Response

Microsamplers vs. Tempus Whole Blood Pre- and Post-Immunization

- Whole Blood samples were collected from a single donor pre- & post-immunizations and antibiotic (Rx) treatment in Mitra microsamplers and Tempus tubes.
- RNA was extracted from 30 μL Mitra Microsamples (n=4) and whole blood Tempus tubes (n=8) and DriverMap™ genome-wide expression 19K panel was used for targeted amplification and quantification by NGS as outlined above.

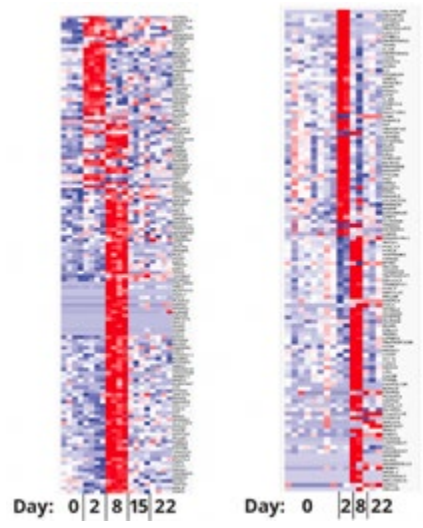


Fig 5: Heatmaps show the immunization response time course from a single individual, 30 μL samples onto Mitra microsamples (left) and Tempus tubes (right) before or after immunization (Day 0, 2, 8, 15, 22). Both samples show peak response at day 8 (onset of adaptive immunity) and drift towards baseline at days 15-22.

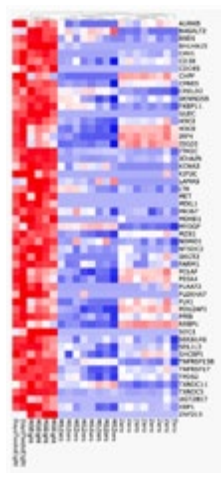


Fig 6: Heatmaps show the overlap of genes for Tempus and Microsamples whole blood before and at 8 days after immunization. 53 DEGs shared vs Controls. Tempus 148 DEGs and Microsamples 163 DEGs (q0.1).

Innate Immune Response Prominent on Day 2 after Immunization

Day 2 Top 10 Pathways Tempus Samples

Pathway	adj.P.Val
Pathway.REACTOME_INTERFERON_GAMMA_SIGNALING	6.92E-05
Pathway.REACTOME_CYTOKINE_SIGNALING_IN_IMMUNE_SYSTEM	6.92E-05
Pathway.REACTOME_INTERFERON_SIGNALING	6.96E-05
Pathway.REACTOME_INTERFERON_ALPHA_BETA_SIGNALING	0.00013283
Pathway.BIOCARTA_TOB1_PATHWAY	0.00013283
Pathway.REACTOME_TRANSLATION	0.00016438
Pathway.REACTOME_REGULATION_OF_IFNG_SIGNALING	0.00135919
Pathway.REACTOME_TOLL_RECEPTOR_CASCADES	0.00146458
Pathway.KEGG_COMPLEMENT_AND_COAGULATION_CASCADES	0.00147189
Pathway.KEGG_NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY	0.00161291

Day 2 Top 10 Pathways Mitra Microsamples

Pathway	adj.P.Val
Pathway.REACTOME_REGULATION_OF_IFNG_SIGNALING	2.09E-09
Pathway.REACTOME_INTERFERON_GAMMA_SIGNALING	1.27E-08
Pathway.REACTOME_INTERFERON_SIGNALING	2.95E-08
Pathway.REACTOME_INTERFERON_ALPHA_BETA_SIGNALING	3.71E-07
Pathway.REACTOME_IL_6_SIGNALING	2.19E-06
Pathway.PID_IL2_1PATHWAY	2.97E-06
Pathway.KEGG_PRION_DISEASES	3.43E-06
Pathway.KEGG_COMPLEMENT_AND_COAGULATION_CASCADES	4.15E-06
Pathway.BIOCARTA_CLASSIC_PATHWAY	5.91E-06
Pathway.REACTOME_CYTOKINE_SIGNALING_IN_IMMUNE_SYSTEM	1.57E-05

Fig 7: Pathway enrichment analysis shows the similarity between Tempus and Mitra Microsample Results. The results show that innate immune response is prominent on Day 2 after immunization. (Analyzed using Panomir R package)

Conclusion

- Overall, using the dried blood microsamplers with DriverMap™ Targeted RNA-Seq Expression Profiling Technology 19K panel or DriverMap™ T/B Immune Marker Panel is effective in detecting up- and down-regulated genes in microsamples.
- There is excellent concordance between direct assays of blood at time 24h vs. assays of dried blood microsamples. (r > 0.94)
- There is a good concordance of our results with public datasets such as GSE10309 Human Whole Blood LPS 4h RNASeq.
- Results from DriverMap™ 19K panel show that ~90% of the top 10K genes overlap between Tempus and Mitra microsamples.
- Microsamples stored at 4°C for over one year showed similar expression profiles to whole blood samples.
- Analysis of 30 μL of blood using Mitra microsamplers provides overall satisfactory performance and produces transcriptome profiles comparable to those from larger volumes of blood in Tempus tubes.