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# Targeted RNA-seq of dried blood microsamples for convenient RNA biomarker monitoring

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## Abstract

Microsampling lancet-induced blood drops enables frequent and comprehensive analysis of various metabolites, lipids, cytokines, and proteins. This approach holds promise for monitoring immunotherapy patients using RNA biomarkers, but a suitable method for processing RNA has been lacking.
In this study, we employed a targeted RNA-sequencing protocol, the DriverMap™ EXP assay, to process 30 µl of dried blood. We compared the gene expression profile in traditionally collected blood samples with that of blood absorbed onto a Mitra® microsampling device containing an RNA-stabilization reagent. Following endotoxin incubation, RNA was extracted from stimulated and unstimulated blood samples. Targeted PCR amplification of 274 immune/inflammatory genes using the DriverMap™ targeted RNA-Seq protocol demonstrated robust detection and high correlation (r = 0.94) between the two methods in both unstimulated and endotoxin-stimulated blood. Moreover, differentially expressed genes (DEGs) identified in standard and microsampling methods exhibited substantial overlap with publicly available datasets from similar experiments. Furthermore, we compared whole blood extracted from Tempus™ blood RNA tubes to Mitra® microsamples pre- and post-immunization with the Pneumovax® vaccine using the DriverMap™ EXP genome-wide 19K panel for targeted RNA-sequencing. We observed approximately 90% overlap in the top 10K genes between Tempus™ and Mitra® microsamples. Notably, microsamples stored at 4°C for over a year exhibited similar expression profiles to more recently drawn whole blood samples.


## 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

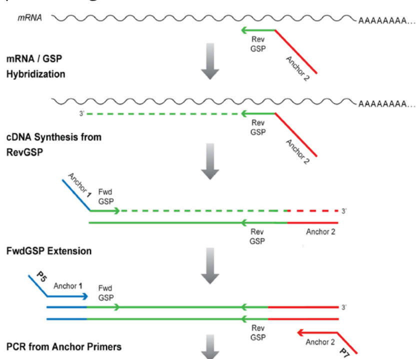
**Strategy**

- 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 obtained venous blood from two healthy donors and analyzed gene expression profiles in the same samples either using the 30 µL VAMS® tips of the microsamplers in a Mitra® device treated with GenTegraRNA-NEO™ or adding Tempus RNA reagent at the usual 2:1 ratio to 0.4 ml blood aliquots to stabilize and then isolate RNA using standard methodology (Qiagen).

- Replicate samples of blood were studied either immediately after collection (time zero) or after 24h of incubation with a panel of agents to compare the detection of differential gene expression after ex vivo whole blood activation (Fig. 1).

- For the microsamplers, after air-drying at room temperature for 24h, RNA was extracted by immersing the tips in small volumes of Cellecta's optimized hybridization buffer, shaking on a heating block for 1h, and isolation of RNA using magnetic beads, yielding approximately 50 ng RNA/tip.

- DriverMap™ target sequencing assay was then applied to each of the isolated RNA samples (Microsamples (MS) or Standard (Std)), followed by library preparation, sequencing, and bioinformatic analysis of the gene count data. Data was normaized to counts per million (CPM).

## Study 1: Microsampling & Whole Blood Activation (cont.)

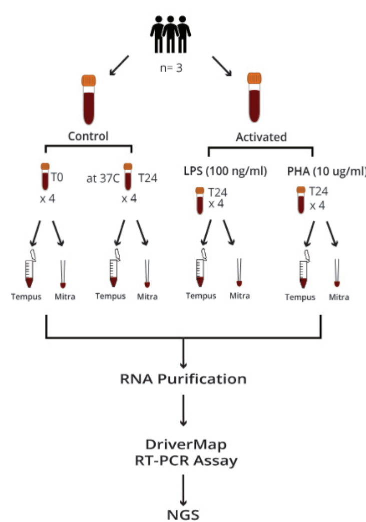
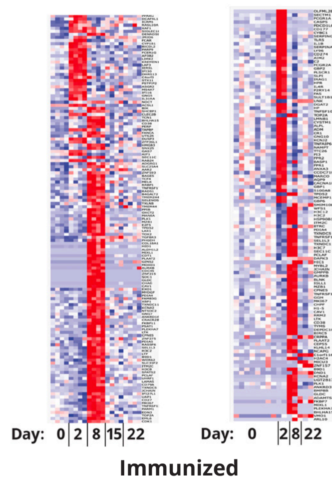



**Fig 1:** Design of Comparison Study

## 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.




**Immunized**

**Fig 2:** 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.

**Controls**

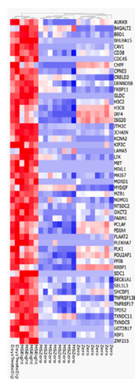


**Fig 3:** Heatmaps show the overlap of genes for Tempus and Microsamples whole blood before and at 8 days after immunization. 53 DEGs shared vs Controls. Tempus 148 DEGs and Microsamples 163 DEGs (q0.1).

### 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 4:** 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)

## Results

**Comparison #1: Time zero**

- The number of genes detected in unstimulated whole blood at time zero show similar results for total genes detected using the two methods (~11K, with a slightly greater number of genes detected using the treated tips in the Mitra® devices) **(Fig.2).**
- This number is consistent with the number of protein-coding genes detected in normal blood in numerous RNA-Seq studies. If we filter the data to remove genes with low/marginal counts and examine the number of genes with CPM > 1 or > 5 (not shown), the number of genes detected decreases but remains similar for both methodologies.
- Finally, we analyzed the small number of genes unique to one method or the other (~1.5%) that have a very low median CPM count, where known variability may push a gene to the other side of the > 1CPM filter.
- In summary, the comparison showed excellent results with both methods for measuring gene expression in normal whole blood.

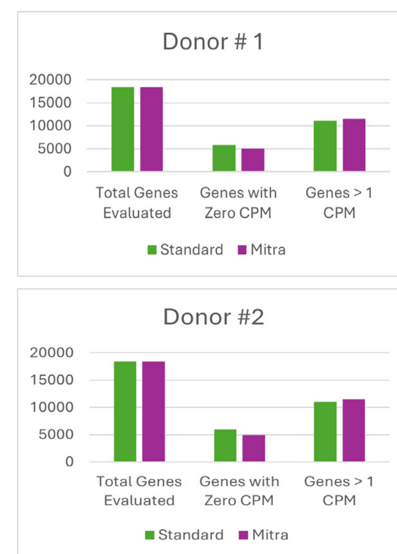



**Fig 5** Genes Detected at Time Zero across two donors show similar resu5ts for total genes detected using the two methods (Standard tubes and Mitra® tips).

**Comparison # 2: Ex vivo activation for 24h**

- To evaluate the ability to detect differential gene expression in blood activated by inflammatory agents, we used a standard ex vivo whole blood stimulation protocol (Fig. 1).
- After 24h, replicate samples (n=4/group) of untreated blood or blood activated by the addition of stimulation agents (LPS 100 ng/ml, PHA 10 ug/ml, or anti-CD3&CD8 1 ug/ml) were sampled using the Mitra® VAMS® tips and the remainder of the blood (0.4ml) processed by addition of Tempus reagent and isolation of RNA as detailed above.
- We counted differentially expressed genes (DEGs) using criteria of a fold-change of > 2 and q < .01. The results show very similar numbers of up-and down-regulated genes in response to endotoxin (LPS) and phytohemagglutinin (PHA) in both normal donor samples (**Fig. 3,** similar results in donor #2 not shown).
- Comparison of the up-regulated genes shows high overlap in the same individual using MS vs Std methods and overlap of activated DEGs between the two individuals (not shown).
- Finally, to test the suitability of the Mitra® VAMS® tips for multi-omic analyses, we sampled the LPS-activated and control blood replicates using regular Mitra® VAMS® tips and evaluated cytokine protein levels using a multiplex immunoassay. As illustrated in **Fig. 4**, the protein results match the RNA results.

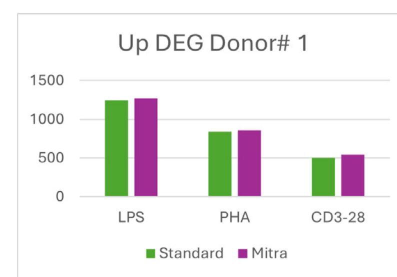



**Fig 6:** Number of Up-regulated genes at 24 h after activation in response to LPS and PHA show similar results for using both methods (Standard tubes and Mitra® tips).

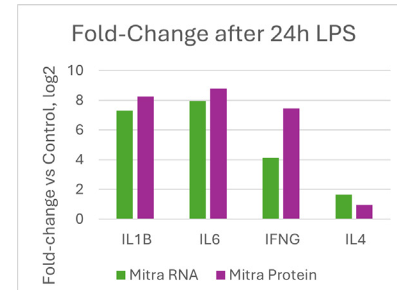



**Fig 7:** Comparison of fold-change detected by Mitra® at RNA or protein levels show that the microsamples can be used to detect both RNA and protein target analyses using customized immunoassay panels.

## Summary

- 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 both time zero and after 24h ex vivo activation and parallel assays of dried blood microsamples. (r > 0.94)

- Analysis of 30 µL of blood using Mitra microsamplers provides excellent performance and produces transcriptome profiles comparable to those from larger volumes of blood processed using Tempus tubes.