RNA transcriptome profiling in microsamples of blood

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Abstract

Multi-omic 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 for this purpose in human blood microsamples. We tested normal blood collected either by phlebotomy into standard vacutainer tubes or by absorption of 30 uL of blood onto a Mitra device pre-treated with an RNA-stabilization reagent. We tested the detection of gene expression changes by incubating anticoagulated blood for 18 hours with endotoxin followed by isolation of identical volumes of blood using a standard method (Qiagen kit) or from Mitra devices air-dried for 24 h to mimic a home-use scenario. RNA was extracted from the microsamplers. After RNA purification, a panel of 274 immune/inflammatory genes was quantified by NGS after PCR using targeted primers following the Cellecta DriverMap protocol, which avoids the counting of abundant rRNA and mitochondrial sequences. The targeted RNA-sequencing results were normalized to counts per million and used to compare results in standard vs microsamples. 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. 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 blood collected from dried microsampling technology using Mitra/Gentegra
- 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

Microsamplers Testing and Whole Blood Activation

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

After treatment, three 30ul 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.

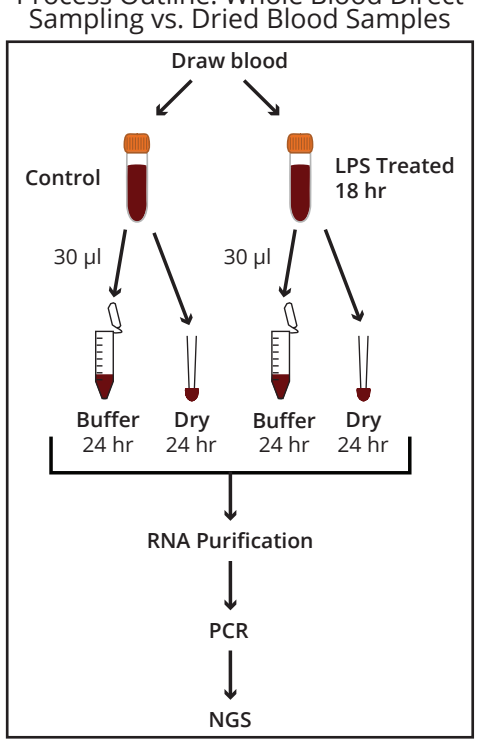
- A second set (n =3) of samples from each tube of collected blood were isolated using Mitra devices to mimic a home-use scenario, and dried at RT for 24 h (as described by the Mitra protocol). The dried blood was then added to the lysis buffer as described.

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.

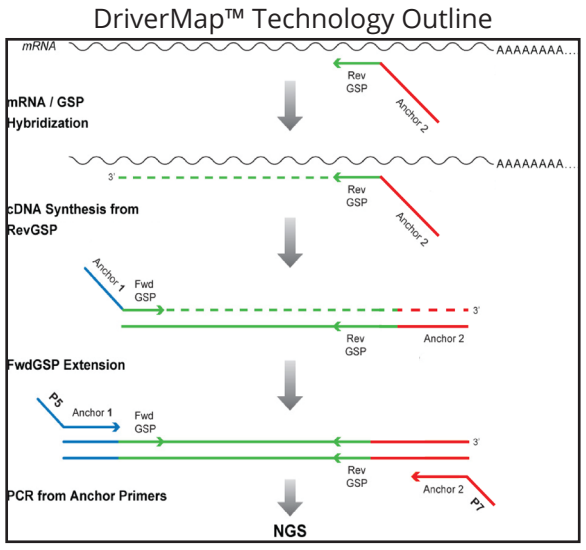
After normalizing the targeted NGS results, we compared gene expression variation of treated and untreated samples for both the standard pipetted collection vs. Mitra/air dried microsamples.

Method (cont.)

Process Outline: Whole Blood Direct Sampling vs. Dried Blood Samples



DriverMap™ Technology Outline



- Multiplex RT-PCR-NGS assay for expression profiling of up to ~19,000 genes in a single test-tube assay
- Requires 100pg-10ng of total RNA from whole blood, single cell, or biopsy samples
- 1st hybridization step starts directly from whole blood lysate
- Allows combination of multiple RNA samples for follow-up RT-PCR steps

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

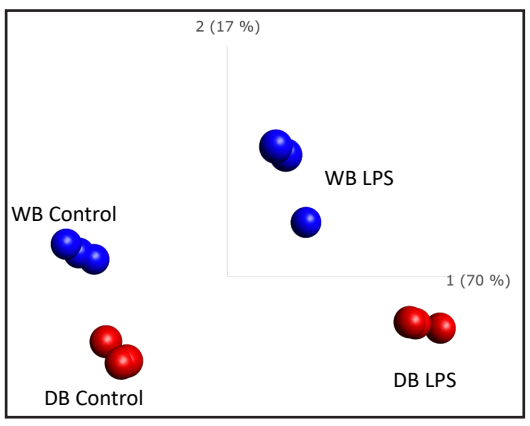


Fig 1: Principal Component Analysis (PCA) reveals a distinct separation between whole blood control vs LPS samples (blue) and dried blood control vs LPS samples (red).

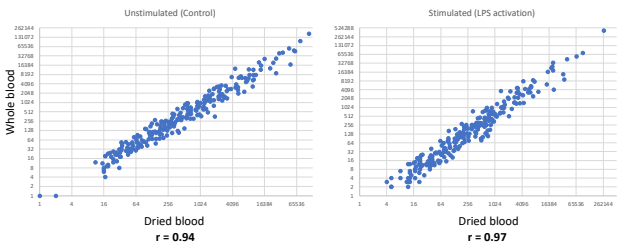


Fig 2: Correlation of WB vs DB gene counts reveals a significant correlation between the unstimulated control WB vs. DB and LPS-stimulated WB vs. DB samples.

Results (cont.)

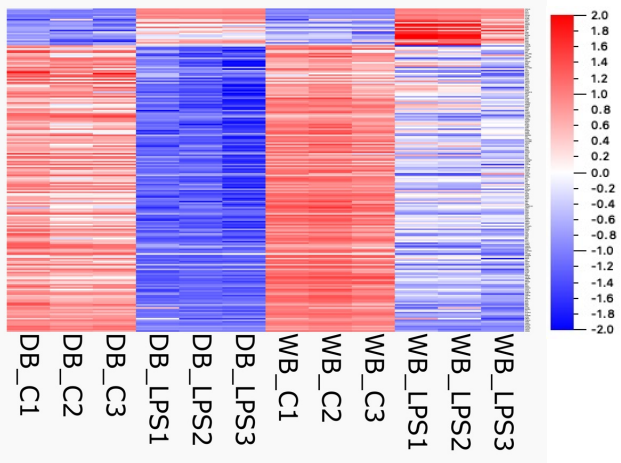


Fig 3: Differential gene expression analysis shows similar markers in unstimulated vs. stimulated samples of dried and whole blood samples. (210/274 genes, q<.05, FC 1.5+, log2 transformed, values >2 compressed)

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.

WB LPStoC Ratio	DB LPStoC Ratio	id	WB LPStoC Ratio	DB LPStoC Ratio	id
-4.32	-3.84	HMOX1	2.88	1.14	IFNG
-3.32	-3.32	CXCR2	3.01	1.42	PMAIP1
-3.18	-3.47	FCGR3B	3.29	1.88	IER3
-3.18	-3.06	TUBA1A	3.90	4.08	IL1RN
-3.18	-3.18	TXNIP	6.70	7.83	CCL20
-3.06	-3.47	CXCR1	7.12	7.30	CCL4L2
-3.06	-3.47	FCGR3A	7.28	7.74	CCL3
-3.06	-3.32	C5AR1	7.32	7.31	CCL4
-2.94	-2.56	S100A11	7.83	7.99	IL1B
-2.94	-3.06	VCAN	8.72	8.77	CXCL10

-15.00 0.00 15.00

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)

Conclusion

- Overall, using the dried blood microsamplers with DriverMap™ Expression Profiling Technology 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)
- Technical Challenges
  - Lower counts in dried blood samples as compared to whole blood samples
  - Most counts are used by a small minority of highly expressed genes

Comparing Results with Public Datasets

- There is a good concordance of our results with public datasets
  - GSE10309 Human Whole Blood LPS 4h RNASeq
    - 66 up-regulated genes (adj p < 0.5)
    - In WB 34 up-regulated genes, 30 of 34 overlap with 66 in GSE10309
  - In DB 27 up-regulated genes, 24 of 27 overlap with 66 in GSE10309
    - Similar trend with down-regulated genes
- Limitations
  - Incomplete concordance among public datasets of up/down genes
  - Differing protocols, statistical criteria, gene lists (microarray studies)

Next Steps

- Improve the sensitivity of low-expressed genes
  - Adjust primers
  - Use double-tip cassette on fingertip samples to increase RNA yield
- Expand the gene panel to a genome-wide gene panel (19k genes) using DriverMap™ Expression Profiling Technology