

WT-OVATION™ ONE-DIRECT RNA AMPLIFICATION SYSTEM PERFORMANCE

BACKGROUND

Biological systems are typically composed of heterogeneous cell populations that collectively determine the physiological state of the organism or biome. Determining the functional contribution of distinct cells in such a complex matrix requires the granularity of a true single-cell interrogation methodology.

Traditional gene expression studies use RNA isolated from a large number of cells, and therefore inherently average effects across the population. Single-cell differences in gene expression levels are not observed. For example, it is critical to consider cell-to-cell differences in expressed genes when studying blood-borne tumor cells, activated leukocytes, and cells involved in early-stage development. Moreover, the inherent inefficiency of traditional RNA isolation and purification processes limits their usefulness when working with samples consisting of small numbers of cells.

The WT-Ovation™ One-Direct System is based on the proven Ribo-SPIA® amplification process. This system makes microarray analysis possible with material from just a few cells, or even one cell, while eliminating the requirement for RNA isolation and purification steps. Amplification from cell lysates without additional purification steps is a key technological breakthrough that eliminates the sample-consuming steps of RNA

isolation and purification and enables researchers to work with just a few isolated cells, allowing them to access their smallest, most challenging samples.

Some of the inherent challenges of working with such small samples are 1) total RNA content of most cells is not known with any degree of precision, and 2) global gene expression patterns and variability at the level of single or small numbers of cells have not been previously characterized. These factors make proper experimental design (running the appropriate number of biological replicates, employing sound statistical data analysis techniques, including appropriate controls, etc.) paramount to ensuring success.

The WT-Ovation One-Direct System allows amplification of total RNA from lysates derived from one or more cells, or purified RNA ranging from 10 pg to 500 pg. A typical yield is approximately 10 µg amplified cDNA from 50 pg of input control RNA. Following a single-day protocol, the amplified cDNA can be used directly as a template for qPCR analysis, or labeled for use in microarray experiments on a variety of array platforms.

MATERIALS AND METHODS

The acute monocytic leukemia cell line THP-1 (ref 1) was initially grown in RPMI 1640 media with 10% fetal calf serum (FCS) and 20 µM 2-mercaptoethanol. The

growth media was changed to 10% FCS plus 5 nM PMA for 48 hours, then the cells were starved in 0.5% FCS/5 nM PMA for an additional 18 hours. The cells were subsequently treated with lipopolysaccharide (LPS, 100 ng/mL) for 5 hours. Cells were harvested prior to LPS exposure (untreated) and also following LPS treatment (treated) then washed, pelleted and stored at -80 °C until lysis.

Cells were lysed by adding them to NuGEN Direct Lysis Buffer (provided in the WT-Ovation One-Direct kit) at a density of 20 cells/µL and vortexing for 1 minute. For each condition, 3 lysates were prepared in parallel, pooled, and stored at 4 °C prior to amplification. Lysates were further diluted in Direct Lysis Buffer to 2.5 cells/µL and 10 cells/µL.

The WT-Ovation One-Direct System was used to amplify 2 µL of each of the lysates (equivalent to 5 cells and 20 cells per amplification, respectively) in triplicate, as described in the WT-Ovation One-Direct System User Guide. The amplified cDNA was purified using MinElute Reaction Clean Up columns (Qiagen, Valencia, CA) and quantitated by UV spectrophotometry using a Nano-Drop UV/Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

Five micrograms of amplified cDNA were fragmented and labeled for microarray analysis using NuGEN's

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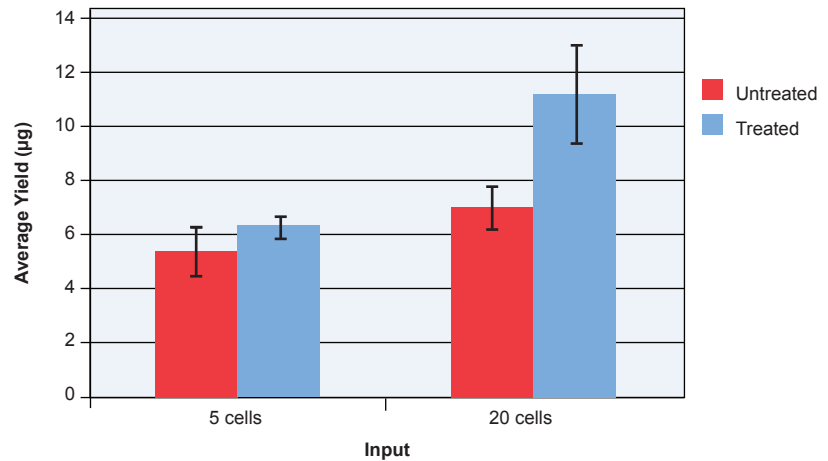


Figure 1 – Amplification Yield vs Input. Yield increases with increasing cell input. However, amounts sufficient for microarray analysis are generated at the lowest cell input.

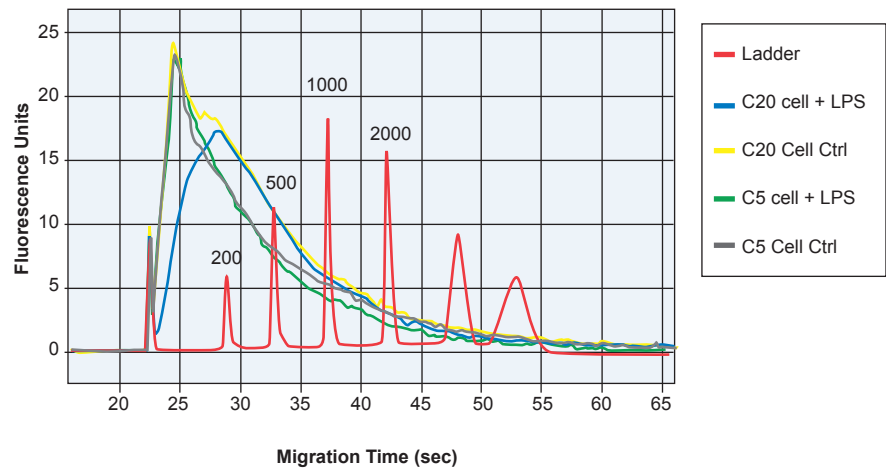


Figure 2 – Representative Bioanalyzer Traces of Amplified Samples. Amplified product from 20 cells +/- LPS treatment (dark blue and yellow, respectively) and from 5 cells +/- LPS treatment (green and gray, respectively) were run on RNA Nano 6000 chips. Ctrl represents untreated cells. Results from RNA standards are shown as a red line. Products amplified from 5 cells are typically shorter than those amplified from higher cell inputs.

FL-Ovation cDNA Biotin Module V2 (Cat #4200) according to the product User Guide. The labeled cDNA was hybridized to Affymetrix GeneChip® Human Gene 1.0 ST arrays. The arrays were hybridized at a target concentration of 33 ng/µL of labeled cDNA in order to enhance detection sensitivity due to the low amount of starting material. This represents an increase over the nominal recommended input range (23–27 ng/µL). Note that while increasing the target concentration in the hybridization mix can be a useful strategy to

increase sensitivity when working with the smallest samples, amplification yield limitations may restrict its use with certain array formats. Hybridization, staining and washing were performed as described in the FL-Ovation cDNA Biotin Module V2 User Guide.

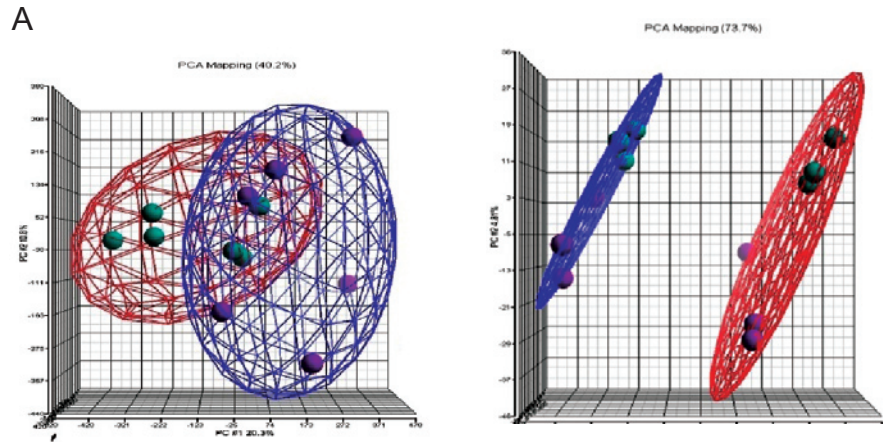
Figure 1 shows that amplification yields correlate with input cell quantities and vary somewhat for LPS treated cells versus untreated cells. Sufficient material was generated from each set of samples for analysis on Affymetrix GeneChip

Gene 1.0 ST arrays, as described in Materials and Methods. Treated cells generally gave higher amplification yields, most likely because cells treated with LPS grow more robustly than untreated cells.

Figure 2 shows bioanalyzer traces of the amplified products for typical 5 cell and 20 cell amplification reactions. Fragment sizes tend to be smaller when starting with lower amounts of input RNA.

Following hybridization to GeneChip Gene 1.0 ST arrays,

Figure 3 – A. Principal Components Analysis (PCA) of LPS-Treated and Untreated THP-1 Cells. In the left panel all probe sets are used for the analysis. In the right panel, only 6,462 probe sets exhibiting differential gene expression ($p \leq 0.01$) are used in the analysis. Following data filtration (right panel), treated samples (red ellipse) clearly separate from the untreated group (blue ellipse) in the PCA. Appropriate statistical filtering of the data is required to eliminate system noise when working with such low cell inputs.



B

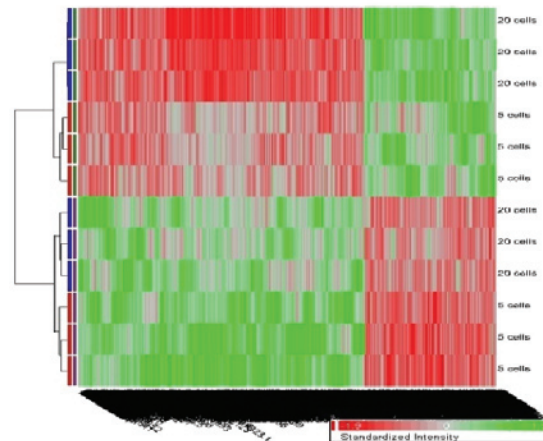


Figure 3 – B. Heat Map Showing Hierarchical Clustering of the 6,462 Differentially Expressed Probe Sets. Note that samples cluster primarily by treatment type and sub-cluster by cell input within the treatment type groups.

the data were analyzed for differential expression resulting from the treatment with LPS. Principle Component Analysis (PCA) is a statistical analysis method that allows similarities and differences between groups of samples to be visualized in a graphical display. A PCA analysis of the unfiltered data shows no statistical difference in expression patterns (Figure 3A, left). This observation is likely due to the low number of replicates run, as well as the biological variation expected when sampling low numbers of cells.

The data were then analyzed by one-way ANOVA (treated versus untreated groups) and the resulting gene list was filtered for those probe sets with a p value cutoff of ≤ 0.01 . The resulting 6,462 probe sets allowed complete separation of the samples by PCA based on the treatment group (Figure 3A, right). Hierarchical clustering of these probe sets indicates a discrete pattern of differential expression, in which the samples cluster primarily by treatment group and secondarily by cell input within each treatment group (Figure 3B).

LPS stimulation has been previously shown to up-regulate genes in the inflammation and immunity-related pathways, as well as the toll-like receptor signaling pathway (Ref 2). The list of differentially expressed genes resulting from the filtering of the ANOVA data contains many genes previously found to be involved in response to LPS stimulation, including the inflammation and immunity-related genes CCL4, CCL3, and THBS1, as well as the toll-like receptor signaling pathway members TLR2, TLR6, IRAK1, NFKB, and CD80.

In conclusion, gene expression analysis of extremely limited biological samples (one or just a few cells) has been a tremendous challenge for researchers. The WT-Ovation One-Direct System provides a robust method for working with even the smallest samples, with a simple, sensitive, robust and reliable amplification protocol. The ability to amplify from cell lysate without the need for further RNA purification opens the door to working with the most challenging samples, enabling the exploration of true, single-cell biology.

REFERENCES

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