Ovation® RNA Amplification, Fragmentation and Labeling on the Biomek® FX

Introduction

NuGEN's Ovation® family of products provides a rapid and simple means of efficiently amplifying RNA from an input sample size as low as 5 ng of total RNA. While this produces sufficient quantities of cDNA for expression arrays, performing the multiple enzymatic reactions and product purification manually on large batches of samples can be a tedious process. Handling errors occur more frequently in high volume sample processing and can be very costly to detect and correct. Automating this process is, therefore, of particular importance for improving throughput while ensuring consistency and reproducibility. We describe here a procedure to automate the Ovation Whole Blood Solution on the Biomek FX Laboratory Workstation and present test results demonstrating its effectiveness.

Materials and Methods

RNA Preparations

For this study, RNA samples were arrayed in a microtiter plate such that 48 wells contained 10 ng Universal Human Reference (UHR) total RNA (Stratagene, Cat. # 74000) and the other 48 wells contained 10 ng human brain total RNA (Ambion, Cat. #AM6050) for a total of 96 samples. In addition, the brain RNA was spiked with a 2×10^{-6} dilution of poly-A RNA controls (Affymetrix, Cat. # 900433). The RNA was arrayed in an alternating pattern to enable easy evaluation of potential cross-contamination. Refer to **Figure 1** for a diagram of experimental design.

Automated RNA Amplification and Labeling

Our approach to automated method development on the Biomek FX was to create modular procedures that require a moderate level of user intervention. This approach was designed to offer a flexible platform for those customers who have a need for custom configurations and the ability to process up to 96 samples at a time. Multiple modules allow for easy modification and the methods can be run either in continuous sequence or individually so as to free the instrument for other tasks. Procedures were carried out on a dual arm Biomek FX equipped with a 96-multichannel pipetting head with gripper on one arm and Span-8 'pod' on the other. The controller software was Biomek ver. 3.2, build 10.

Biomek methods were created that captured the major procedural steps in the Ovation System, following the manufacturer's instructions in the user guide:

- Primer annealing
- First strand synthesis
- Second strand synthesis
- Ribo-SPIA® linear amplification
- RNAClean® magnetic bead purification
- Normalization of cDNA concentrations
- Fragmentation
- Biotin-labeling

Manual intervention steps include the preparation of master mixes before each plate distribution method and the transfer of the reaction plate to and from a thermocycler for incubation. Reagents and reaction mixes must be kept on ice until ready to use. On the instrument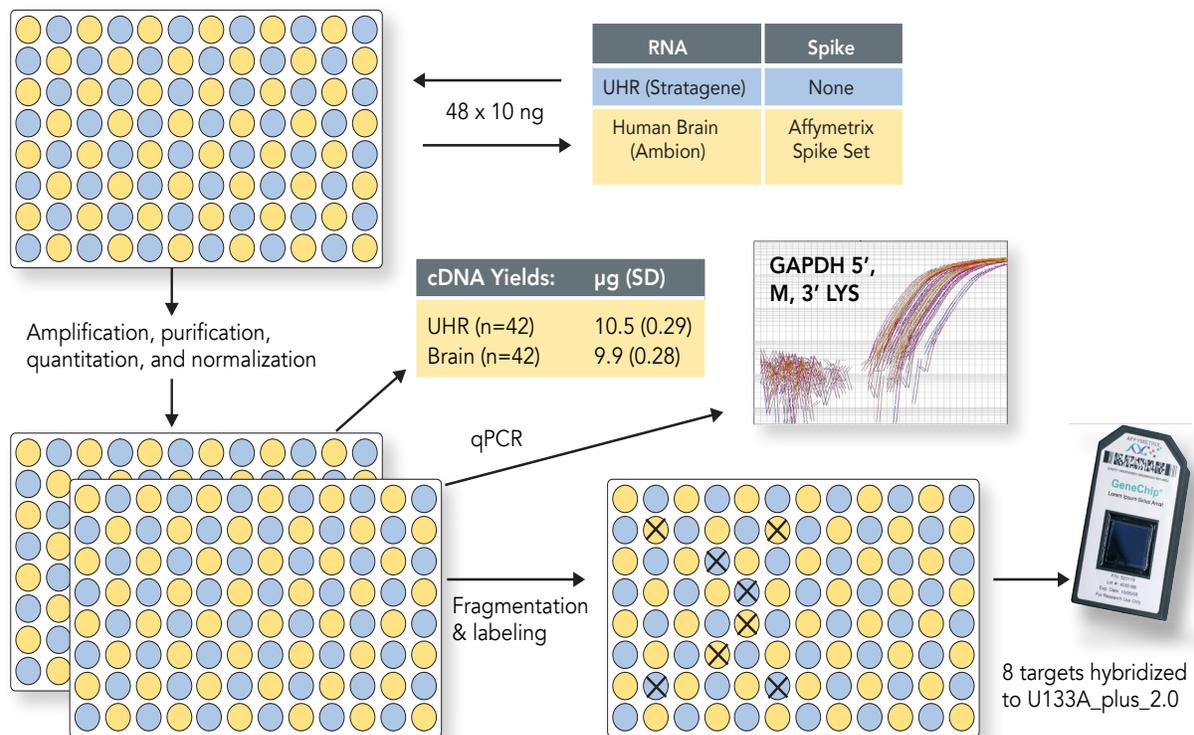 that was used to implement and test these meth-ods, three different deck configurations were required, see **Figure 2**. The Ovation RNA Amplification System V2 and the Ovation WB Reagent (NuGEN, Part Nos. 3100 and 1300, respectively) were used for the amplification reactions. The amplified product was bound to RNAClean beads (Agencourt, Cat. # 000494) and purified on a SPRIPlate® 96R ring magnet plate (Agencourt, Cat. # 000219). An aliquot of the eluted cDNA was removed for yield determination and a worklist was generated from the quantification results. The worklist is a .csv file containing volume and well information used to normalize the concentrations to 150 ng/µL. Fragmentation and labeling with Encore® Biotin Module (NuGEN, Part No. 4200) subsequently proceeded using the normalized cDNA products.

qPCR and GeneChip® Array Hybridizations

qPCR assays were set up for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for 3 different amplicons located on the 5', middle, or 3' region of the gene (GAPE11, 12, 13). In addition, a SYBR Green assay was conducted for the LYS poly-A spike present only in the Ambion Brain RNA sample. For GAPDH, qPCR reactions were set up using approximately 6 ng of the amplified cDNA in TaqMan® Fast Universal PCR master mix with 500 nM each of the forward and reverse primers and 100 nM Exiqon® probes and probe mix following vendor's instructions in 20 µL final volume. The SYBR Green assays used SYBR Green Super Mix and 500 nM each of the forward and reverse primers.

Eight labeled cDNAs were selected as targets from multiple regions of the plate and then hybridized to

NuGEN®

A Tecan Group company

FIGURE 1. Experimental design

RNA	Spike
UHR (Stratagene)	None
Human Brain (Ambion)	Affymetrix Spike Set

48 x 10 ng

Amplification, purification, quantitation, and normalization

cDNA Yields:	µg (SD)
UHR (n=42)	10.5 (0.29)
Brain (n=42)	9.9 (0.28)

GAPDH 5', M, 3' LYS

qPCR

Fragmentation & labeling

8 targets hybridized to U133A_plus_2.0

GeneChip HGU133_ plus_2.0 arrays according to the Encore Biotin Module User Guide and the GeneChip manufacturer's instructions. The data were analyzed using the Affymetrix GeneChip Operating System and the Expression Console. In addition to standard array metrics, analyses were conducted as described by the Microarray Quality Control Project Consortium (*MAQC Consortium, Nature Biotechnology, Vol 24, Number 9, Sept 2006, pp1151 – 1161*). The MAQC project's reference data set provides a very useful point of comparison in order to assess the quality of the array data generated.

Results

cDNA yields: Amplified cDNA yields averaged 9.9 µg for brain and 10.5 µg for UHR. The amplifications were highly reproducible with a variance for both RNA types of less than 3 percent, shown in **Figure 1** inset.

qPCR: Figure 3 shows the cumulative results for qPCR from 84 samples. GAPDH levels (GAPE11, 12, 13) for each sample type were highly reproducible among replicates, while Cts could clearly distinguish the two different RNA types. No edge or row effects were detected. Assays for LYS from the Affymetrix poly-A control spikes showed a greater than 10-cycle difference between spiked and nonspiked

samples, wherein the latter values suggested undetectable levels. Therefore, no cross-contamination events could be discerned and the automated liquid handling steps safely segregated individual samples.

GeneChip Arrays: Results from array hybridizations are summarized in **Table 1**, showing high signal correlations within each RNA type. Likewise, other metrics were very consistent between arrays while maintaining low background values and 3'/5' ratios. The high level of Ovation amplification sensitivity is demonstrated in **Table 2**, by the fact that up to 79 percent of the 12,091 genes in the MAQC common gene set were detected. Differential

gene abundance correlation analysis was performed on 571 genes that were called present in both Brain and UHR data sets, and intersected the 906 TaqMan MAQC assays.

Results, in **Figure 4,** show a high correlation coefficient of 0.95 between TaqMan and Ovation confirming that Ovation System generated biologically accurate results, validated by qPCR.

Conclusions

Automated methods created on the Biomek FX have been shown to effectively carry out the liquid handling steps required to perform the Ovation WB RNA amplification and labeling. These methods successfully processed 96 RNA samples and generated cDNA that was analyzed on Affymetrix GeneChip arrays and in qPCR assays, yielding biologically accurate data. Automating Ovation enabled the production of high quality cDNA in a consistent and reproducible manner with no evidence of cross-contamination caused by liquid handling errors.

Acknowledgments

Originally published in March 2007.

FIGURE 2.   Biomek FX deck configurations for carrying out Ovation WB Automation methods
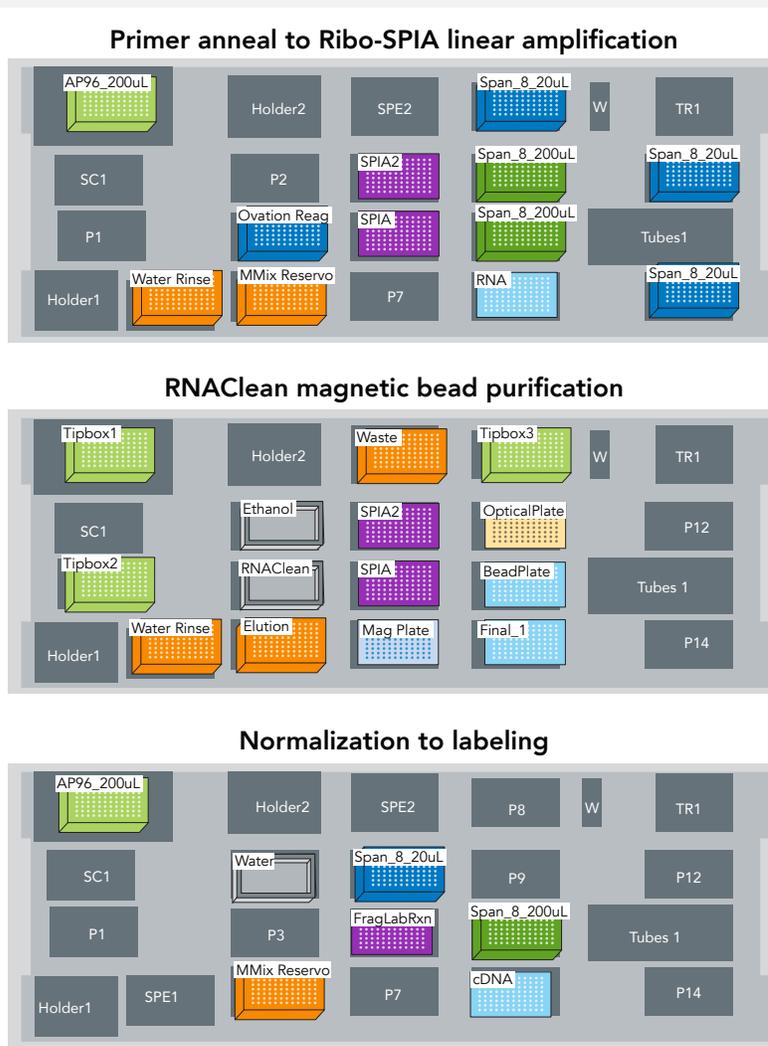
Three different deck layouts were required to accommodate the various labware necessary to perform the methods. Although a stacker carousel was available to reconfigure the deck between methods, we chose not to use it in order to create a generic layout that could be customized for different instrument configurations.

The first deck layout was used for the amplification reactions, including RNA/primer annealing, first strand amplification, second strand amplification, and Ribo-SPIA® linear amplification. The second layout was used exclusively for the magnetic bead purification and elution of the SPIA®-generated cDNA. The last layout was used for normalizing the cDNA concentrations and then setting up the fragmentation and biotin-labeling to prepare samples for array hybridization.

FIGURE 3. **Cumulative results for qPCR from 84 samples**
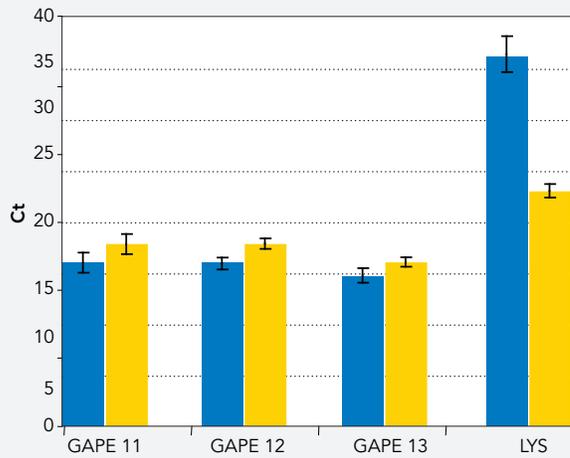
RNA	Spike
UHR (Stratagene)	None
Human Brain (Ambion)	Affymetrix Spike Set

- Highly reproducible amplification
- No detectable cross-containation
- No detectable edge or row effects

Ct values were consistent between replicates for each RNA type, and no edge or row effects nor any cross-contamination was detectable, demonstrating that automated liquid handling steps safely segregated individual samples.

TABLE 1. **Summary of U133_Plus_2.0 Array metrics**

| Array #	RNA	Input (ng)	SF	BG avg	%P	3'/5' ratio B-actin	3'/5' ratio GAPDH	Signal R^2 Avg
B2	UHR	10	1.51	33.52	72.28	5.49	1.34	**0.99**
B6	UHR	10	1.42	31.80	72.95	5.73	1.38	
E5	UHR	10	1.76	31.78	71.37	6.07	1.42	
F4	UHR	10	1.78	33.26	70.33	6.60	1.47	
C4	Brain+spike	10	1.61	31.89	69.27	10.01	1.71	**0.98**
D5	Brain+spike	10	1.78	31.13	68.81	10.79	1.68	
G2	Brain+spike	10	1.91	30.83	68.63	11.79	1.76	
G6	Brain+spike	10	1.46	33.24	68.41	10.82	1.72	

Summary shows high signal correlations within each RNA type. Likewise, other metrics were very consistent between arrays while maintaining low background values and 3'/5' ratios.

TABLE 2. Percentage of MAQC common gene set (12,091) detected on GeneChip U133_plus_2.0 array

Sample	UHR (MAQC-A)	Brain (MAQC-B)
RNA input (ng)	10	10
% Detected*	79%	78%

The Ovation System's high level of sensitivity is demonstrated by the high percentage of 'present' genes that were identified within the MAQC common set of 12,091 genes. *Called Present in ≥ 3 of 4 replicate arrays.

FIGURE 4. Differential gene abundance correlation analysis.

Correlation of array and qPCR differential gene expression

N = 571*
R = 0.95

Ovation on U133_plus_2.0
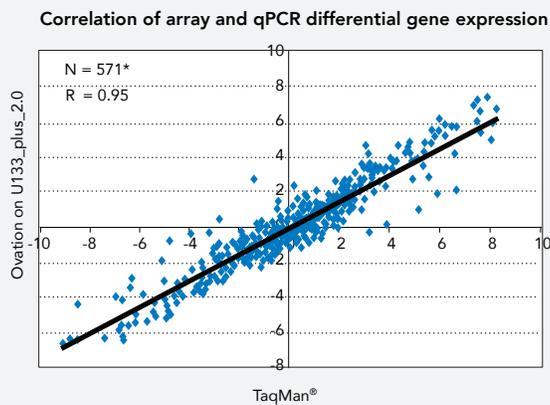
TaqMan®

Analysis was performed on 571 genes detected in both UHR and Brain on GeneChip Arrays (out of 906 MAQC TaqMan assays). The graph plots the Brain/UHR log ratio from GeneChip array as a function of the Brain/UHR log ratio from qPCR. The resulting high correlation confirms that Ovation System generated biologically accurate results.

NuGEN®
A Tecan Group company

For research use only. Not for use in diagnostic procedures.

M01052 v3.1