TECHNICAL REPORT

Performance verification of the automated NuGEN Ovation® Whole Blood Solution

Introduction

NuGEN's Ovation series of products is widely known and accepted as an efficient, reliable and rapid system for amplifying small quantities of RNA for global gene expression analysis. Using the Ovation Whole Blood Solution, as little as 20 ng of total RNA from whole blood clinical samples or 5 ng of high quality RNA from other sources can be converted into sufficient quantities of cDNA to enable subsequent analysis by microarrays or qPCR. The assay does not require a globin reduction step due to the proven higher specificity of cDNA targets compared to cRNA targets in microarray analysis.[1]

Although manual processing of large batches of samples is possible with the simple add-and-incubate Ovation protocols, it can be labor intensive and potentially prone to handling errors. Automating the entire process has the advantage of increasing throughput while minimizing variance, human errors and hands-on labor time.

The Ovation Whole Blood Solution has been successfully automated on liquid handling platforms such as the Biomek FX, the Biomek FX ArrayPlex (both Beckman Coulter), the Hamilton Starlet and the Affymetrix GeneChip® Array Station (GCAS). While the methods and results are similar across all platforms, in this report we describe the methods and results using the Affymetrix GCAS.

The protocol is intuitive and easy to operate (Figure 1, next page), offering flexible sample processing from as few as 8 samples up to a full plate of 96 samples. The entire process from total RNA to a hybridization cocktail that is ready for use on Affymetrix 3′ expression arrays takes about 9.5 hours. As an added benefit, there are several convenient stopping points in the assay to enable maximum workflow flexibility.

Assay performance and QC data for the automatically processed samples will be discussed in comparison to that of manually processed samples, including a close look at biological pathways.

Protocol

The automated Ovation Whole Blood Solution protocol on the GCAS consists of 11 sub-methods (**Table 1**). The estimated times shown assume 96 samples are being processed. The cDNA quantitation method and an automation deck layout change prior to the fragmentation and labeling methods require a brief user intervention during the protocol. When processing more than 48 samples, two additional user interventions are required to retrieve one PCR plate from the instrument and deliver it to an off-deck thermal cycler.

Assay Setup and Performance

MAQC-A (UHR, Stratagene) is a Universal Human Reference RNA, a mixture of RNAs from different human cell lines including a glioblastoma line. MAQC-B (Ambion) is a Human Brain Reference RNA and consists of RNA from human brain tissue obtained from multiple individuals.

Figure 2 represents an example plate layout that was used to assess the protocol's performance with MAQC-A and MAQC-B samples. **Table 2** shows the cDNA yield and standard deviation from a variety of RNA samples, including human, mouse and rat blood.

TABLE 1. Estimated processing times

Method	Estimated Time (min)
Primer Anneal	25
First Strand	90
Second Strand	60
SPIA amplification	60
Whole Blood reagent addition	70
cDNA purification	50
cDNA quantitation	30
Normalization	20
Fragmentation	50
Biotin labeling	90
Hyb cocktail formulation	20
Total time	**9.5 hours**

Total time shown for each sub-method of the automated Ovation Whole Blood Solution on GCAS when 96 samples are being processed. To facilitate error recovery, the method can be started or stopped at any submethod. For ease of workflow management, many users stop just after cDNA purification or quantitation and continue the process the next day.

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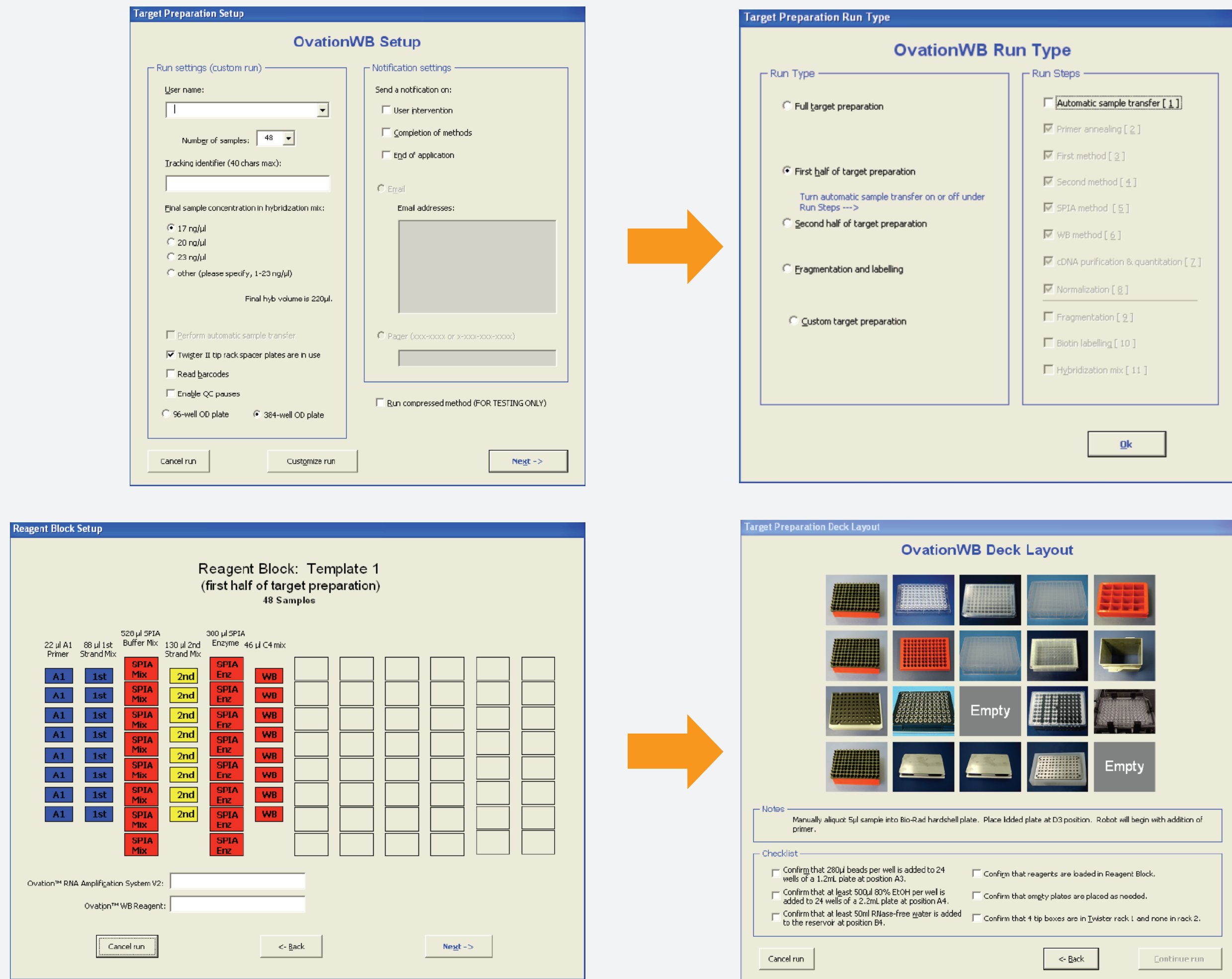
FIGURE 1. **Intuitive user interface**



The user interface of the automated NuGEN Ovation Whole Blood Protocol on GCAS guides the user through the setup of the run and minimizes the chance for errors.

For hybridizing the amplified cDNA to standard Affymetrix 3′ expression arrays, the hybridization cocktail is formulated in 220 µL total volume.

The setup interface allows the user to choose from three different concentrations, however, a concentration of 20 ng/µL is used as the default for blood samples and requires a minimum cDNA yield of 5.4 µg.

Array Performance

Ten samples each of MAQC-A and MAQC-B were hybridized to Affymetrix HG-U133A 2.0 arrays following the Encore® Biotin Module User Guide and

FIGURE 2. **Example of a plate with 96 samples**

	1	2	3	4	5	6	7	8	9	10	11	12
A	8.4	7.2	8.2	7.5	8.0	7.4	8.0	7.4	8.4	7.2	7.5	7.8
B	7.7	1.5	7.6	1.8	7.2	1.9	7.6	1.6	6.8	1.6	7.2	1.4
C	8.0	7.5	8.0	7.1	7.7	7.3	8.2	6.5	7.7	7.5	7.3	6.8
D	7.7	7.2	7.6	7.6	7.1	7.3	6.7	7.2	7.0	8.2	6.3	6.7
E	8.4	6.4	8.3	6.8	7.8	7.1	8.3	6.4	7.6	6.4	7.4	6.4
F	2.8	8.0	1.5	7.8	1.6	7.5	1.6	7.3	1.5	7.0	1.1	7.6
G	8.7	7.4	8.0	7.3	7.8	7.5	8.0	6.9	7.8	6.6	0.1	6.7
H	7.7	8.6	7.8	8.8	7.3	7.6	7.6	7.5	6.8	7.5	7.4	7.4

MAQC-A (green), MAQC-B (blue) and water controls (yellow). The values listed in each well are the total yield of amplified cDNA in micrograms. Note that position G11 is an outlier due to a defective pipette tip.

the Affymetrix GeneChip® Expression Analysis protocol. Basic data analysis was performed using Affymetrix Expression Console applying MAS5 or alternatively the RMA algorithm with default settings.

Array QC data (**Table 3** and **Figure 3**) show a high degree of reproducibility within the sample groups. A high concordance with the QC data obtained from manual processing of the same samples has also been observed (data not shown), demonstrating that the automated protocol is at least as robust and reliable as manual processing.

Automated Versus Manual Processing

While cDNA yield and array QC metrics are good indicators for assay performance, the ultimate test is based on biology. Therefore, to compare the automated with the manual protocol, a closer analysis of the MAQC-A and MAQC-B samples was carried out.

Four replicates of each MAQC-A and MAQC-B samples were processed manually and hybridized to Affymetrix HG-U133 Plus 2.0 arrays. These data served as the basis for defining A-

TABLE 2. cDNA yield and standard deviation after SPIA amplification across different sample types

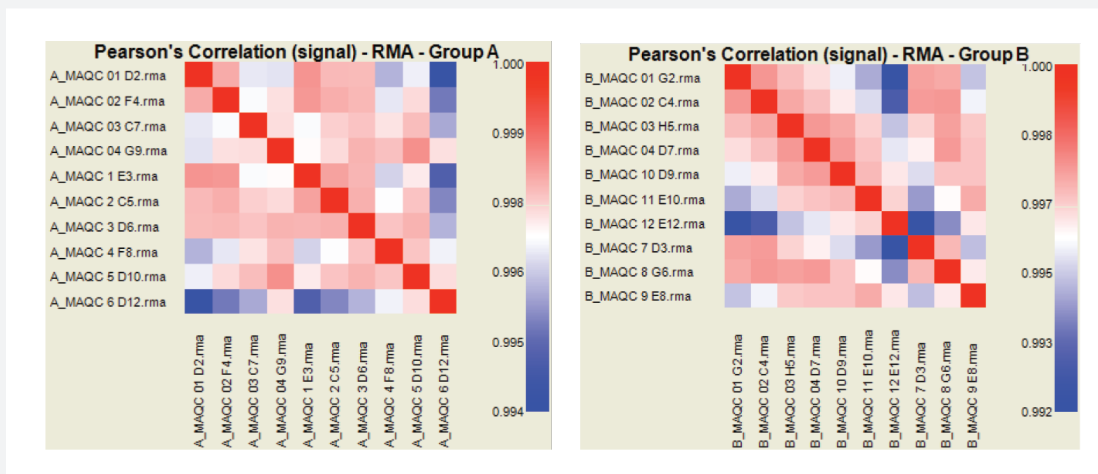
	Amplified cDNA Yield (µg)			
Input Total RNA (ng)	MAQC A	MAQC B	NTC*	Customer Sample
5	5.9 (0.2)	5.9 (0.2)	1.6 (0.4)	N/A
20	7.5 (0.4)	6.7 (0.4)	N/A	5.6 – 7.5 across different organisms
40	7.5 (0.1)	7.3 (0.2)	N/A	N/A

MAQC-A and MAQC-B samples are commercial RNA of excellent quality. The customer samples show a RIN >7 in the Agilent Bioanalyzer and are derived from different sources. Data for 5 and 40 ng of input RNA are based on results of four replicates. Data for 20 ng input material rely on 30 replicates per MAQC-A or MAQC–B sample. * NTC=No Template Control.

TABLE 3. MAS5 QC data on HG-U133 A 2.0 arrays, averaged over 10 samples each of MAQC-A and MAQC-B

	MAQC A	StDev	MAQC B	StDev
Background	39.3	3.3	38.4	1.5
Scaling Factor (at TGT = 500)	0.6	0.1	0.7	0.1
%P	80.3	0.8	78.4	0.5
3′/5′ ratio GAPDH	1.2	0.02	1.5	0.1

FIGURE 3. Concordance of signal values within the sample groups



Pearson's Correlation coefficient based on log2 signal from the RMA algorithm within sample groups A and B. Correlation for both sample types is clearly above 0.99.

and B-specific biological networks. In brief, Affymetrix Power Tools (APT 1.10.2) was used to generate signal estimates by the RMA algorithm and calls were generated by the MAS5 algorithm. Only probesets from the HG-U133 A 2.0 array were taken into account in this analysis.

Definition of MAQC-B Specific Signals

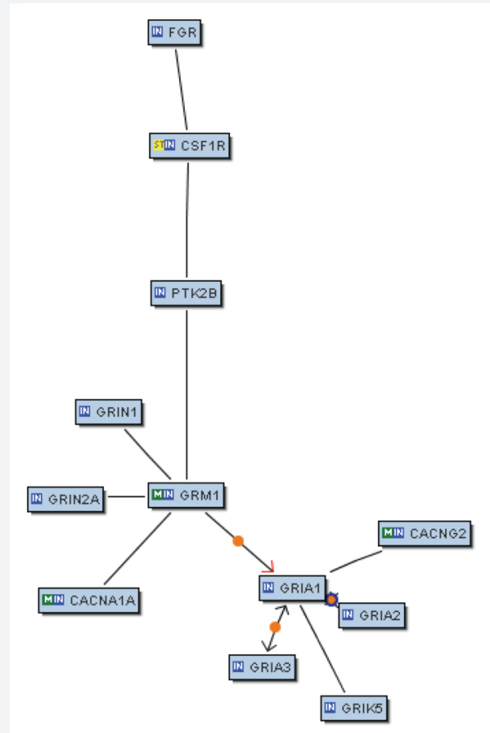
First, probesets at a log2 expression level below 6 in MAQC-B data files were excluded from the analysis. Furthermore, only probesets called "P" across all MAQC-B replicates and "A" in all MAQC-A replicates were retained. Next, Bibliosphere software (Version 7.21; Genomatix Software GmbH, Germany) was used to filter on probesets specific for the UniGene annotation "brain". In total, 148 probesets passed this procedure. Bibliosphere was used again to draw a network of probesets that are known from literature to be linked at a signal transduction level. Twelve probesets remained (**Figure 4**).

Definition of MAQC-A Specific Signals

Probesets below a log2 expression level of 6 in MAQC-A files were removed from the analysis. Of the remaining probesets, only those called "P" across all MAQC-A replicates were retained. Next, Bibliosphere software was used to filter on probesets specific for the UniGene annotation "brain" and the MeSH annotation "glioblastoma".

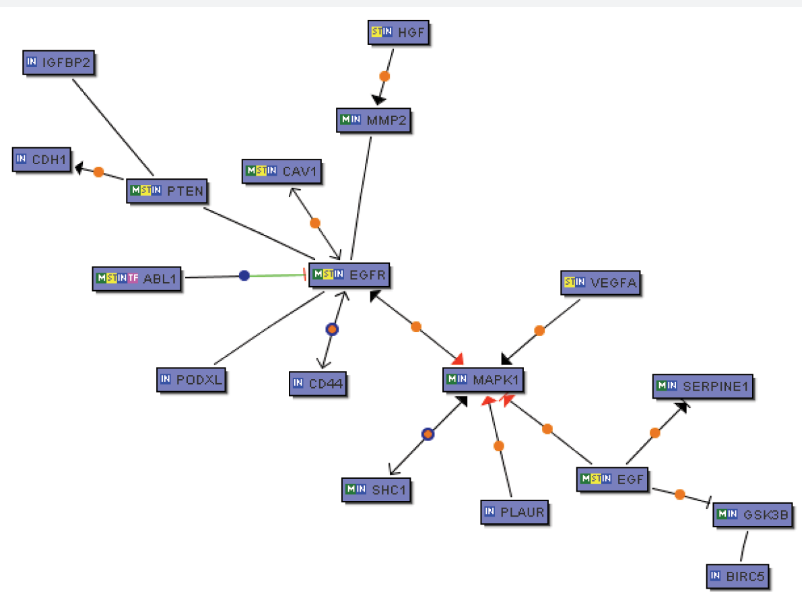
This filter provided 463 probesets. From this subset of probesets, only those that were at least 2-fold up-regulated in MAQC-A (relative to MAQC-B) and that belonged to a functional network based on signal transduction (ST) were retained. Eighteen probesets met all of these criteria and form a functional network in the Bibliosphere software (**Figure 5**).

FIGURE 4. **Brain-specific network of genes as depicted by Genomatix' Bibliosphere 7.21 software**



Genes are linked on the signal transduction (ST) level

FIGURE 5. **Network of genes specific for glioblastoma**



Genes are linked on the signal transduction (ST) level

Comparison of Samples Processed Manually Versus Automatically

Four replicates of both MAQC-A and MAQC-B samples prepared by GCAS were hybridized to Affymetrix HG-U133 A 2.0 arrays. On the basis of the above described biological networks, the data from the GCAS-processed samples were compared to the data from the manually processed samples. As shown in **Tables 4** and **5**, data from the manually processed samples and automatically processed samples are highly comparable. Figures 6 and 7 further demonstrate the comparable results for a single gene selected as a representative for each network. Although there may be slight differences in the signal values (**Figure 6**), the degree of regulation is similar.

Conclusion

The NuGEN Ovation Whole Blood Solution, automated on the Biomek FX, Biomek FX ArrayPlex, Hamilton Starlet and the Affymetrix GCAS, enables users to efficiently run large numbers of samples while minimizing the risk of user-introduced errors and greatly reducing labor time. This report describes the methods used to process samples on the GCAS. After initial automation deck setup and preparation of master mixes, only a single user intervention is required to complete the protocol through formulation of the hybridization cocktail.

The user interface is intuitive and guides the operator through the setup process, serving as a reference for correct deck layout and master mix preparation. The entire protocol takes about 9.5 hours to complete with a full plate of 96 samples and can be stopped at several points throughout the assay, providing maximum flexibility. Subsequent hybridization and handling of the Affymetrix expression arrays uses the default Affymetrix protocol.

When compared to manually processed samples, automatically processed samples show a high

TABLE 4. log2 expression values, t-test p-values and signal log ratios of probesets from the glioblastoma network

	Manual				GCAS				
Probeset	Avg log2 A	Avg log2 B	T-test	SLR	Avg log2 A	Avg log2 B	T-test	SLR	Gene
202718_at	10.35	6.61	4.75E-10	3.74	11.72	8.19	8.59E-10	3.52	IGFBP2
201121_s_at	11.08	6.83	3.87E-10	4.25	11.77	7.72	2.86E-11	4.04	CDH1
204053_x_at	10.70	9.14	3.20E-09	1.55	11.55	10.65	3.34E-06	0.90	PTEN
210997_at	6.3	4.35	4.22E-05	1.95	7.22	5.23	3.90E-04	1.98	HGF
201069_at	10.28	6.73	3.34E-09	3.56	10.65	6.93	6.53E-09	3.72	MMP2
203065_s_at	11.11	7.06	2.40E-08	4.06	12.83	9.39	1.27E-09	3.43	CAV1
202123_s_at	9.71	8.64	8.04E-08	1.07	10.79	9.94	8.40E-07	0.85	ALB1
201984_s_at	8.31	6.93	4.58E-07	1.38	9.35	8.34	7.18E-05	1.01	EGFR
212014_x_at	10.77	7.76	3.83E-09	3.01	11.85	8.94	9.06E-10	2.91	CD44
201578_at	12.65	11.41	4.40E-08	1.24	13.40	12.28	3.55E-09	1.12	PODXL
208351_s_at	7.21	6.14	5.10E-05	1.07	8.50	7.52	4.89E-04	0.99	MAPK1
201469_s_at	9.00	7.07	4.39E-08	1.93	8.95	8.17	1.96E-07	1.68	SHC1
210513_s_at	7.56	5.84	3.58E-06	1.72	9.08	7.53	2.55E-06	1.55	VEGFA
206254_at	7.57	5.93	1.68E-08	1.64	8.16	6.65	1.23E-04	1.51	EGF
211924_s_at	8.24	6.29	2.94E-07	1.95	9.45	7.66	5.56E-07	1.79	PLAUR
202628_s_at	10.32	5.82	1.35E-10	4.50	11.73	7.22	9.73E-09	4.52	SERPINE1
209945_s_at	9.71	8.42	9.39E-09	1.29	10.66	10.37	2.44E-02	0.29	GSK3B
210334_x_at	9.07	4.95	2.41E-08	4.12	10.12	5.56	1.83E-08	4.55	BIRC5

TABLE 5. log2 expression values, t-test p-values and signal log ratios of probesets from the brain-specific network

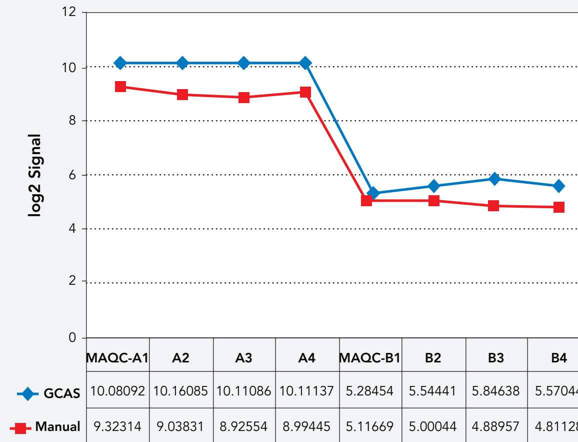
	Manual				GCAS				
Probeset	Avg log2 A	Avg log2 B	T-test	SLR	Avg log2 A	Avg log2 B	T-test	SLR	Gene
203104_at	4.41	8.33	8.85E-08	3.92	5.28	9.08	9.57E-07	3.81	FGR
208438_s_at	3.79	7.58	3.69E-08	3.78	4.29	7.64	1.60E-07	3.35	CSF1R
205915_x_at	4.17	6.70	1.65E-08	2.53	4.83	8.03	6.91E-07	3.19	PTK2B
206534_at	4.17	7.53	2.53E-09	3.36	5.87	9.36	12.54E-07	3.49	GRIN1
207299_s_at	3.85	11.09	4.01E-10	7.24	4.09	11.47	1.23E-10	7.38	GRIN2
203111_s_at	5.54	6.99	5.40E-06	1.45	6.95	8.65	9.07E-06	1.70	GRM1
210770_s_at	5.03	7.49	4.70E-07	2.46	5.69	8.20	4.06E-07	2.51	CACNA1A
214495_at	4.26	9.13	3.59E-12	4.87	5.12	9.73	3.36E-06	4.61	CACNG2
211520_s_at	3.48	8.23	1.21E-07	4.75	4.57	9.96	8.15E-10	5.39	GRIA1
205358_at	3.50	10.77	3.61E-13	7.27	4.25	12.69	3.30E-10	8.44	GRIA2
206730_at	5.20	9.08	1.36E-07	3.88	6.28	11.19	3.92E-07	4.91	GRIA3
217509_x_at	6.30	7.90	3.23E-05	1.60	7.00	8.03	7.82E-05	1.03	GRIK5

degree of concordance in array QC. Additionally, a close look at biological networks shows the two preparation methods produce similar results, suggesting that although absolute signal values may vary slightly between targets from each of the preparation methods, the biological findings remain the same.

References

1 Replacing cRNA targets with cDNA reduces microarray cross-hybridization. Aaron C Eklund, Leah R Turner, Pengchin Chen, Roderick V Jensen, Gianfranco deFeo, Anne R Kopf-Sill and Zoltan Szallasi (2006). Correspondence in *Nature Biotechnology* 24 (9) 1071–3. 2006.

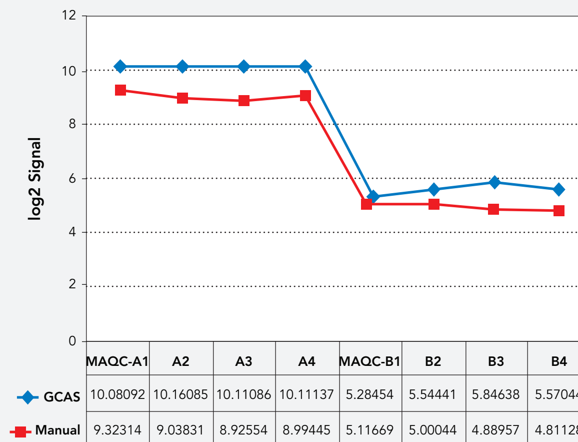
FIGURE 6. **Log2 signal plot of MAQC-A specific gene BIRC5**



	MAQC-A1	A2	A3	A4	MAQC-B1	B2	B3	B4
GCAS	10.08092	10.16085	10.11086	10.11137	5.28454	5.54441	5.84638	5.57044
Manual	9.32314	9.03831	8.92554	8.99445	5.11669	5.00044	4.88957	4.81128

While GCAS processing shows slightly higher signal values, the degree of up-regulation is similar.

FIGURE 7. **Log2 signal plot of MAQC-B specific gene GRIN2**



	MAQC-A1	A2	A3	A4	MAQC-B1	B2	B3	B4
GCAS	10.08092	10.16085	10.11086	10.11137	5.28454	5.54441	5.84638	5.57044
Manual	9.32314	9.03831	8.92554	8.99445	5.11669	5.00044	4.88957	4.81128

Values from GCAS processing are consistent with those obtained from manual processing.

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