WHITEPAPER

Biomarker Translation from Global Expression Arrays to Focused Arrays on the Ziplex® System

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Abstract

Gene expression signatures are typically discovered with global expression arrays. To advance the signatures to clinically relevant assays the biomarkers are usually validated on a different analytical platform with independent sample sets. The validation platform should use different probes for well-defined targets, faithfully reproduce the results from the global expression platform, enables high throughput processing of large numbers of biological samples, and offer operating simplicity to facilitate routine testing in clinical research laboratories. We have tested a workstation with desirable attributes for clinical validation of expression-based biomarkers. The System automates hybridization with Flow-Thru Chip® technology and chemiluminescent detection to provide results in a relatively rapid turnaround time. With a subset of genes studied in the MAQC study, the results were equivalent to those from other gene expression platforms, although some discrepancies were observed between platforms. As a practical example of translation from a global expression platform to a validation platform, we have designed an array to quantify the expression of 93 genes relevant to ovarian cancer. Using sequence-matched probes which were screened for optimal performance with a set of normal and cancerous tissues the differential expression pattern was successfully translated to the new platform.

Introduction

We are planning clinical research and validation studies in ovarian cancer involving a large number of samples and the relative expression of focused sets of genes. We require a translational research platform that will reliably reproduce research results obtained on a global expression platform, and that is cost-effective, reproducible, and easy to use. To this end, we have evaluated the Ziplex® Automated Workstation (Axela Inc.), and the efficacy of translating an expression signature for ovarian cancer from the Affymetrix platform to Ziplex.

The Ziplex® Research System

The Ziplex Automated Workstation (Figure 1) is designed to consolidate array molecular binding, imaging, data quantification, and quality control into a single, small bench-top unit. The System uses flow-thru chip technology in which probes are immobilized within a highly porous silicon matrix providing a very high surface area for binding probes. During hybridization/protein binding, washing and detection, samples and reagents are continually perfused through the chip within a microplate well to provide

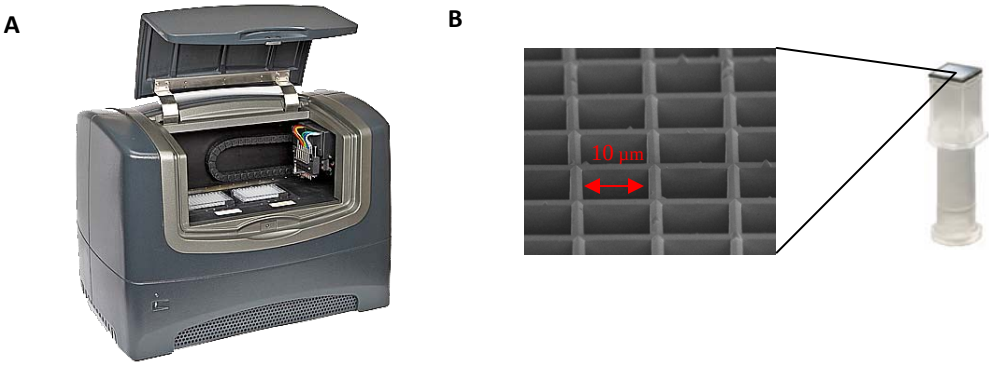


Figure 1

(A) The Ziplex Research System: a fully automated, bench-top instrument oligonucleotide and protein microarray analysis. (B) Scanning EM of the porous silicon substrate and an assembled TipChip

efficient mixing and transport of target molecules to the immobilized probes. Figure 1B shows an electron micrograph of the silicon chip which is fixed to a plastic tube to form a TipChip.

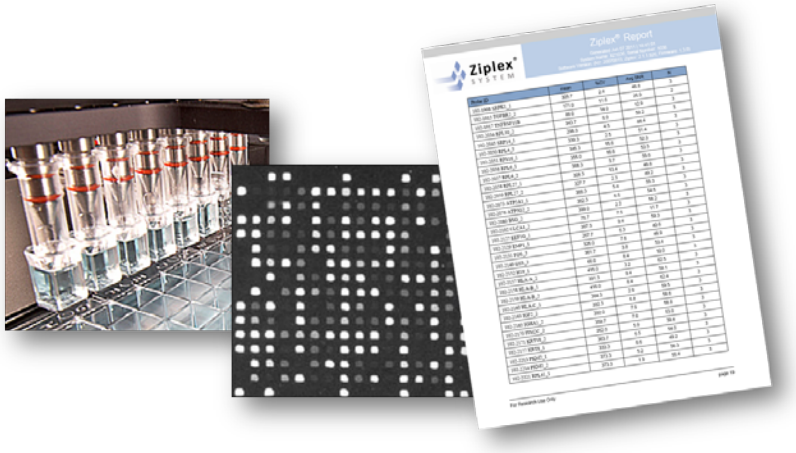
Sample Hybridization

Total RNA samples were quality controlled with the Agilent Bioanalyzer, amplified, and biotin-labeled by oligo-dT primed in vitro transcription. TipChip microarrays, samples, and reagents were loaded into specific microplate wells, and then hybridization, washing, chemiluminescent imaging and data reduction were performed automatically on the Ziplex workstation (Figure 2).

The Ziplex manifold picks up the TipChips and lowers them into specific wells where solutions are repeatedly aspirated and dispensed through the chips. Up to eight TipChips were hybridized and analyzed simultaneously in less than three hours. Tables of mean intensities and coefficients of variation of triplicate spots for each probe were outputted by the instrument and analyzed on an external computer.

Figure 2

Ziplex manifold and TipChips; chemiluminescent image; report from Ziplex .



MAQC Experiment

Following the MicroArray Quality Control (MAQC) protocol, mixtures of Universal Reference RNA (sample A) and Human Brain RNA (sample B) in 75:25 (sample C) and 25:75 (sample D) mass ratios were analyzed on a focused microarray comprising 52 genes represented in the MAQC study. The results were compared to the results for the same set of genes in the publicly available MAQC data.

The differential expression between samples C and D measured with Ziplex was concordant with other microarray platforms, although there were outlier genes in some comparisons (Figure 3). Median CVs (Table 1a) and the number of significantly differentially expressed genes between samples (Table 1b) observed with Ziplex were similar to the MAQC microarray platforms.

Figure 3

Concordance of differential expression between microarrays. AFX = Affymetrix, ILM = Illumina, AGI = Agilent

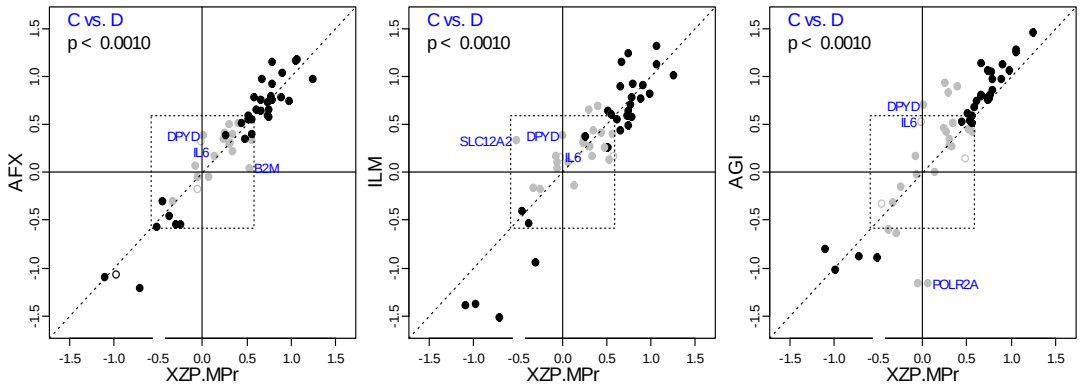


Table 1

A) Median CVs on microarrays
B) Number of differentially expressed genes.

A

Sample	Median CVs			
	A	B	C	D
Ziplex	7.5	5.0	6.3	4.7
AFX	4.7	5.6	4.6	5.7
ILM	5.5	8.1	7.8	7.6
ABI	8.5	7	6.2	8.5
AGI	16.8	8.4	6.6	11.9
GEH	10.1	11	11.2	11.3
TAQ	2.8	3	2.6	3

B

p-value	A vs B		C vs D	
	0.001	0.0001	0.001	0.0001
Ziplex	43	40	35	26
AFX	47	44	42	34
ILM	41	37	31	20
ABI	40	34	30	18
AGI	32	12	32	22
GEH	37	24	17	6
TAQ	49	46	45	33

Ovarian Cancer Array Design

Oligonucleotide probes were designed to hybridize to the 3' UTR of transcripts corresponding to 93 genes selected based on previous studies of Affymetrix U133A expression microarray analyses of ovarian cancer vs. normal ovarian epithelium cells. Target sequences were reviewed by BLAST alignment to the RefSeq database to ensure accurate target identification of the probes. Three probes were designed for each target accession number, and the probes were screened by hybridizing a set of 11 samples from normal ovarian surface epithelial cells (NOSE) and 12 samples from malignant ovarian tumors (TOV). A single probe with optimal performance was chosen for each gene based on maximum signal intensity, minimum distance from the 3' end of the target sequence, correlation between different designs, and systematic differences between NOSE and TOV samples.

Correlations between different probes designs are shown in Figure 4. Good correlations between signal intensities of all three probes for a given target indicated that the probes were most likely hybridized to the same transcript, as seen for MSH6 and SELT. The slope of the regression line indicates the relative probe efficiency. Poor correlations or correlations in which the intercept of the regression line was significantly different from zero suggested that at least one of the probes hybridized to a different transcript or more than one transcript. For example, of the three probes for CDKN1A, only two correlated well where the regression line between their signal intensities passed close to the origin.

Inspection of the correlations indicated how well the probes discriminated between NOSE (blue) and TOV (red) samples. Two of the three probes for the gene CDKN1A discriminated between the sample types, whereas only one of the probes for the TIFF1 gene provided discrimination. All three of the probes for MSH6 provided some discrimination, unlike the probes for SELT.

Sample Quality Control

The tailored array contained seven pairs of probes positioned within about 300 bases and about 1200-1500 bases from the 3' ends of the targeted transcripts. The correlation between the 3'/5' intensity ratios of these pairs and the Bioanalyzer results (Figure 5) indicated that total RNA quality was reflected in the array results (or average cRNA length). However one TOV sample had a very poor Bioanalyzer RIN number (2.6, off-scale in Figure 5), but the geometric mean of the 3'/5' ratios was 7.4. This sample (#26) grouped with the other TOV samples in principle component analysis (Figure 6), indicating that meaningful results can sometimes be obtained from apparently poor quality RNA.

Figure 4

Correlations between different probe designs for the same gene.

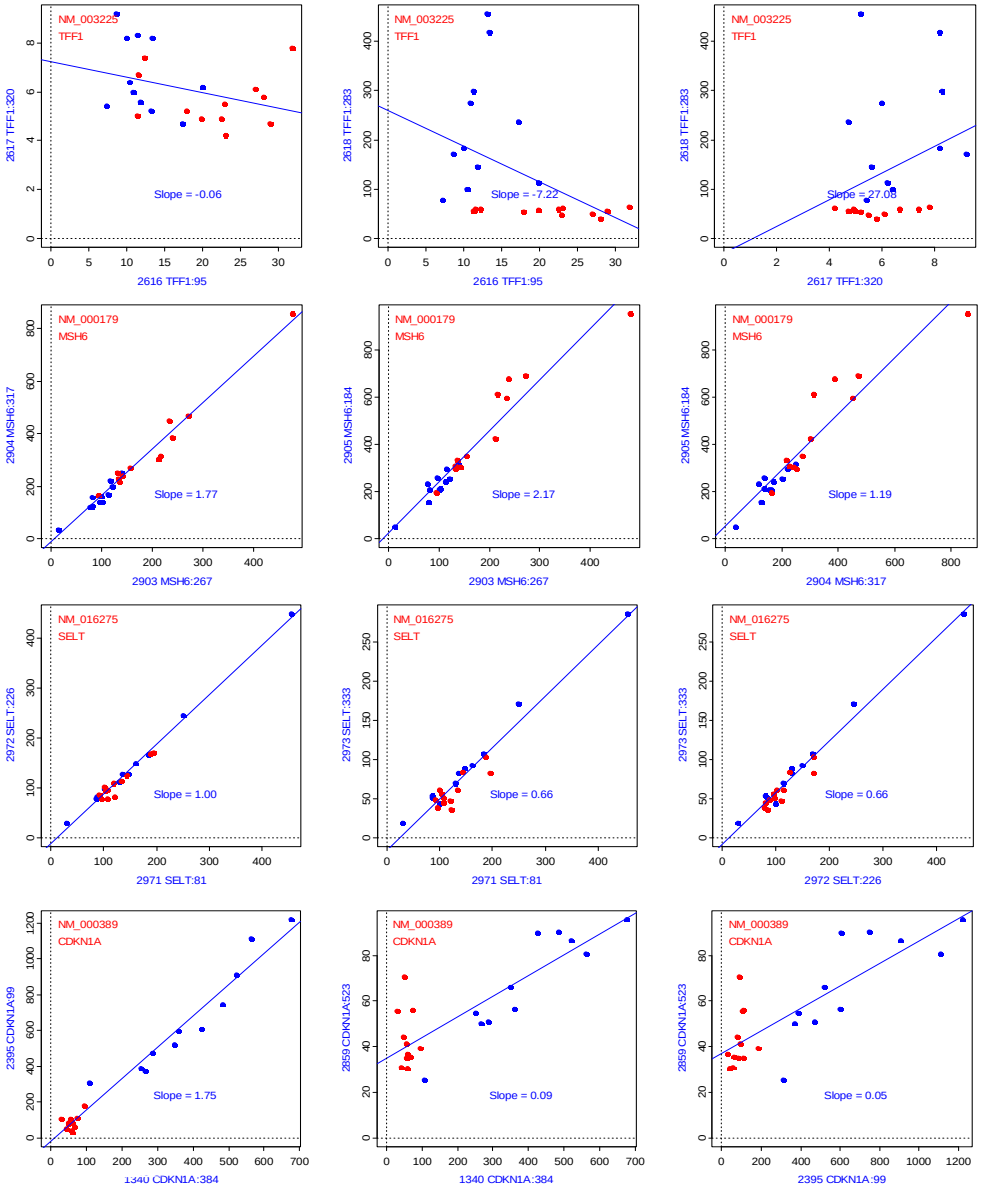


Figure 5

3'/5' ratios and Bioanalyzer results

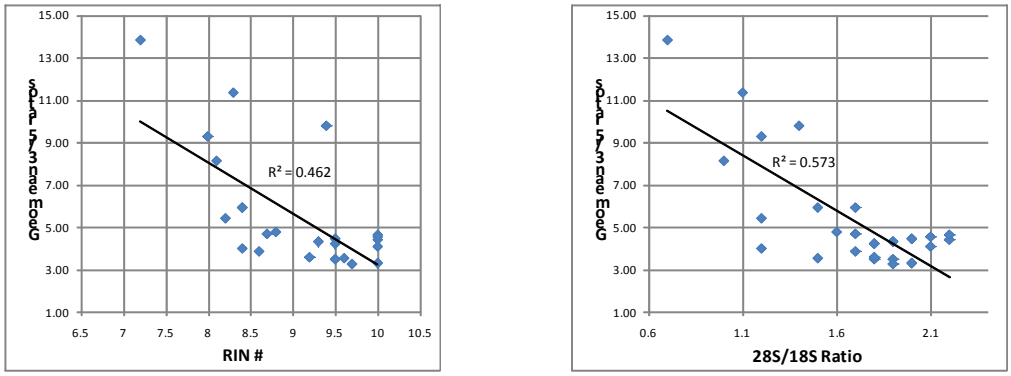
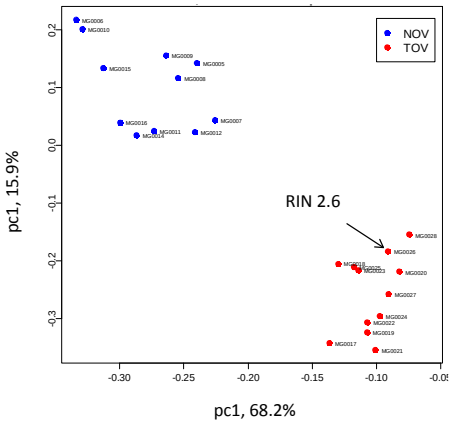


Figure 6

Principle component analysis of normal ovarian surface epithelial cells (NOSE) and malignant ovarian tumors (TOV).



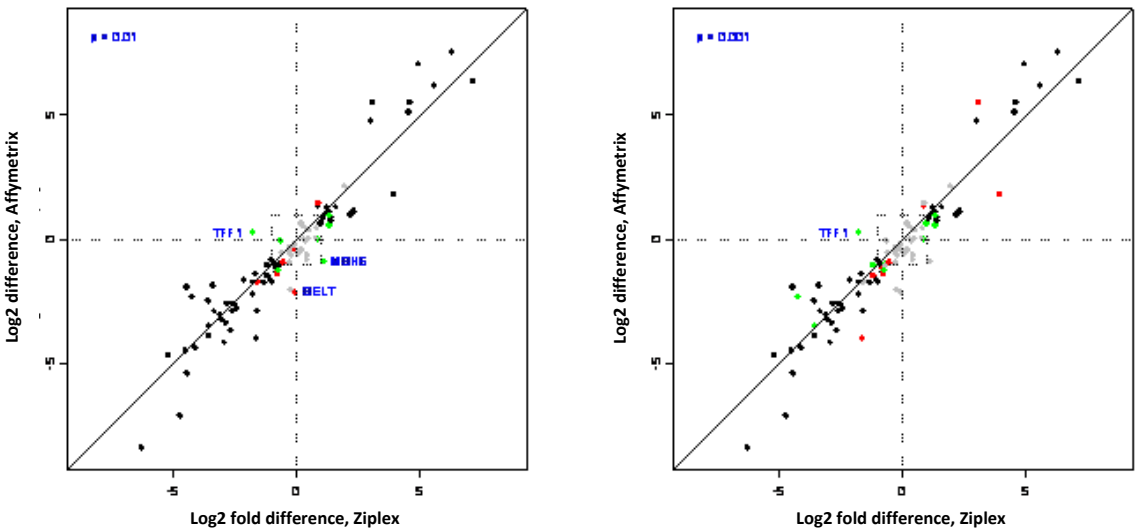
Concordance between Platforms

Figure 7 compares the fold differences between the mean log-transformed signal intensities of NOSE and TOV samples for the 93 genes measured with Ziplex and with Affymetrix arrays. A t-test was performed on

the log-transformed data, and those genes that were found to be significantly different between the two sample types ($p < 0.01$ or $p < 0.001$) are plotted in black. Gray indicates that a gene was not found to be significantly different on either platform; green and red indicate that a gene was significantly different only with Ziplex or Affymetrix arrays, respectively. Non-concordant genes with expression differences > 2 are indicated with blue labels. The three probe designs for these genes are compared in Figure 4.

Figure 7

Concordance of mean expression differences between NOSE and TOV samples on Ziplex and Affymetrix arrays for two p-value cut-offs.



Conclusions

- Expression differences measured with Ziplex are generally concordant with those measured with global expression arrays.
- Translation of expression signatures between platforms is straight-forward and reliable when target sequences are accurately identified and probes are empirically screened for optimal performance.

- Quantification of gene expression equivalent to that of discovery platforms can be realized with speed and automation using flow-thru chip technology.

- Discrimination between sample types sometimes can be achieved even with total RNA samples with apparently poor RNA integrity.

About Axela, Inc.

Axela's platforms provide powerful new approaches to multiplexed protein and nucleic acid analysis designed to greatly simplify biomarker testing in clinical research and diagnostics. Axela's commercial research products significantly improve the amount and quality of information derived from traditional assays. This approach shortens time to result and provides access to unique categories of markers that form a pipeline of future diagnostic offerings.

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