System Verification of Ziplex®, a multiplex gene expression system for translational research and clinical diagnostics

Englert DF[1], Wilson DJ[1], Dean D[1], Liederman J[1], Zafarana G[2], Albert M[2], Meng A[2], Bristow RG[2]

[1]Axela, Inc., Toronto, ON and [2]STTARR Innovation Centre, Departments of Radiation Oncology, University of Toronto and Radiation Medicine Program, Princess Margaret Hospital-University Health Network, Toronto, ON.

Abstract

Panels of genes differentially expressed between disease states or treatment regimes are under investigation by many laboratories with the expectation that these investigations will lead to the development of clinical tests. As research progresses from discovery to clinical implementation, confirmation of an expression profile with a different technology and/or different probes for the same nominal targets is an important step in defining a test for further development and clinical validation. Validation of a test requires the processing of a large number of samples with a reproducible and reliable system in multiple clinical diagnostic laboratories where ease-of-use and robust performance are paramount. We have developed an automated multiplex gene expression system, called Ziplex, intended for translational research and clinical diagnostics. We report here on an example of cross platform validation of a signature under development for clinical research and on system verification studies of system performance and robustness.

1) Concordance of differential expression between Ziplex and the NanoString nCounter™ system. Probes for 135 genes were assayed on both systems. There were two probes for each target on Ziplex, and one probe set for each target on the nCounter system. The data from both platforms were normalized to the geometric mean of the signals of the probes for the genes ACTB and GAPDH. There were robust signals for about 70-80% of the target genes on both systems. Neither system reported robust signals for about 3-5% of the genes, and only one system reported robust signals for 8-16% of the genes depending on the sample. The dynamic range measured from the samples in the study was similar for Ziplex and Nanostring (> 3,000-fold). Differential expression was concordant between the two systems in many cases - e.g., exposure of a cell line to irradiation resulted in increased expression of between 1.5- and 6-fold for seven genes on both systems. Cases of discordance were also observed - e.g., differential expression greater than two-fold on one system but not on the other in a knockdown experiment.

2) Verification of Ziplex system performance and robustness. Exogenous RNA was spiked into human total RNA and processed with standard procedures. The linear dynamic range observed from a dilution series of the spiked-in RNA was greater than 10^4. Two-fold differences in spike amount were resolved at the extremes of the dilution series. The median coefficient of variation across three instruments and multiple array lots was 12% for the raw data and 7% when the data were normalized. Selectivity which depends on hybridization temperature was not significantly affected when the instrument was operated at extremes of ambient temperature.

Conclusion: Expression signatures can be reproduced between Ziplex and the nCounter system, although cases of significant discordance were noted. Ziplex meets or exceeds the minimum performance and robustness requirements for which it was designed.

Introduction

We have developed the Ziplex Automated Workstation as a system for validation of gene expression profiles and for development of clinical diagnostic tests that can be distributed as *in vitro* diagnostics. Overall concordance between analytical platforms is essential for this development process, and good concordance between expression results from Affymetrix arrays and Ziplex has been demonstrated previously[1]. In addition to system validation, specific probes for target transcripts must be validated before incorporation into assays, since not all probes have adequate sensitivity and specificity for their intended targets. Different probe designs for the same target may be compared on Ziplex to determine whether they respond similarly to a set of relevant samples. A more powerful means of probe validation is to compare the response of probes on Ziplex to the response on an alternative analytical platform, especially if the alternative platform is based on a different technology.

An analytic system intended for clinical diagnostic use must consistently deliver good performance in a variety of operating environments. Verification against design requirements (including dynamic range, selectivity, repeatability and robustness over a broad temperature range) must be performed before commencement of clinical trials.

1Quinn, M. C., D. J. Wilson, F. Young, A. A. Dempsey, S. N. Arcand, A. H. Birch, P. M. Wojnarowicz, A. M. Mes-Masson, D. Provencher, D. Englert and P. N. Tonin (2009). "The chemiluminescence based Ziplex automated workstation focus array reproduces ovarian cancer Affymetrix GeneChip expression profiles." J Transl Med 7(1): 55.

Objectives

1) To validate the performance of Ziplex probes by comparing gene expression results acquired with Ziplex and the NanoString nCounter™ system, so that a single effective Ziplex probe could be chosen for each gene.

2) To verify the performance of the Ziplex instrument against design specifications in preparation for clinical trials.

Sample Processing/Data Analysis

Total RNA extracted from various cell lines exposed to different treatments was amplified and labeled by reverse transcription and *in vitro* amplification for hybridization on two separate TipChip arrays on Ziplex (Figure 1). Two different probes were printed on the TipChips for each target gene. The linearized data in the Ziplex output was used in the analysis (see Figure 5). Total RNA from the same samples was processed on the Nanostring nCounter system by hybridization of capture and reported probe pairs, affinity purification of the resulting complexes, and counting of signal probes hybridized to individual captured mRNA molecules[2]. One nCounter probe pair was used for each target gene.

For cross platform comparisons the results from both systems were normalized to the geometric mean of two "housekeeping" genes ACTB and GAPDH. Probes were considered detectable on Ziplex if the net signal was greater than 12, which is three times the median standard deviation of the background pixel intensities. Probes were considered detectable on the Nanostring system if the number of events was at least 25, which is the number of events that would result in a CV of 20% ($N^{0.5}/N$) assuming that the stochastic noise was the only source of noise.

2 Geiss, G. K., R. E. Bumgarner, B. Birditt, T. Dahl, N. Dowidar, D. L. Dunaway, H. P. Fell, S. Ferree, R. D. George, T. Grogan, J. J. James, M. Maysuria, J. D. Mitton, P. Oliveri, J. L. Osborn, T. Peng, A. L. Ratcliffe, P. J. Webster, E. H. Davidson, L. Hood and K. Dimitrov (2008). "Direct multiplexed measurement of gene expression with color-coded probe pairs." Nat Biotechnol 26(3): 317-25.

Figure 1 – Ziplex Automated Workstation with TipChips and reagent consumables.

Detection of Target Transcripts

As is typical of cross-platform comparisons, there was much scatter in the correlation of signal intensities indicating variable probe sensitivities (Figure 2). Transcripts were detectable with about 75% of the probes on both Ziplex and Nanostring, and about 4% of the transcripts were detectable on neither system. About 10% of the transcripts were detectable with one system, but not the other. The dynamic range of the two systems were similar.

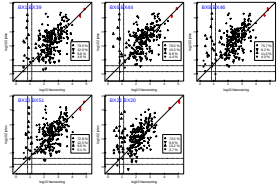

Figure 2 – Correlation of normalized signal intensities from Ziplex and Nanostring. Expression values for each gene were averaged from duplicate samples run on each system. The housekeeping genes used for normalization are indicated in red. The levels of the maximum and mean negative control probes on each system are indicated with dotted lines. The legends indicate the percent of probes detected on both systems (•), only on Nanostring (▽), only on Ziplex (Δ) and on neither system (o).

Probe Validation

Many duplicate Ziplex probe designs for the same targets correlated well with each other (e.g., genes L, B and K in Figure 3) indicating that the probes were probably hybridizing to intended target mRNAs. Other probes for the same targets correlated poorly (e.g., genes G, A, and J), indicating that at least one of the probes was hybridizing to a target other than the intended target.

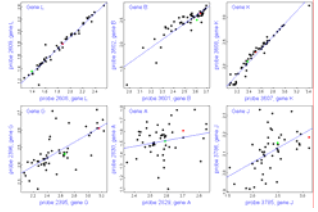

Figure 3 – Correlation of intensities of two Ziplex probes designed for the same gene. Samples from various cell lines representative of expression profiles expected in future studies were hybridized to the Ziplex arrays.

Differential expression between samples measured with Ziplex and the Nanostring system showed that a number of genes were differentially expressed (e.g., samples BX6 and BX1 in Figure 4). As expected from the correlations in Figure 3, only one of the Ziplex probes was concordant with Nanostring for some genes (e.g., gene G and J). Some measured expression differences were small. However, the good correlation between duplicate Ziplex probes (genes B and K in Figure 3) and the concordance with Nanostring (genes B and K Figure 4) suggests that expression differences of less than two-fold may be meaningful.

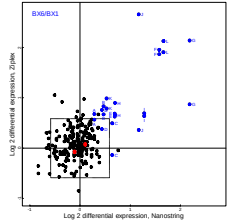

Figure 4 – Comparison of differential expression measured between two samples (coded BX6 and BX1) with Ziplex and Nanostring. The box indicates differential expression of 50%. Results from probes that are up-regulated in the BX6 sample are labeled in blue. Results from Ziplex probes for the same gene are labeled with the same letter. In some cases (e.g., F and L) both Ziplex probes are concordant with Nanostring. In other cases (e.g., G and J) only one of the Ziplex probes is concordant with Nanostring.

Ziplex Reproducibility

Ziplex was operated in an environmental chamber at temperatures above and below typical laboratory temperatures. For arrays hybridized at ambient temperatures of 16ºC, ~21ºC and 30ºC the median %CV was 7% when the data were normalized and 12% when the data were not normalized. Pairwise correlations between arrays hybridized at extremes of ambient temperature (Table 1) indicated that results were consistent over the ambient temperatures over which the instrument is specified to operate. The upper limit of the 95% confidence interval of the ratios of mismatched to perfect match probes (which are used to monitor hybridization selectivity) were within performance specifications over the tested ambient temperature range.

Comparison	Average R^2
RT-16	0.9873
RT-30	0.9848
16-30	0.9669

Table 1 – Average R^2 of correlations between arrays hybridized at different ambient temperatures.

Ziplex Linear Dynamic Range

The linear dynamic range of Ziplex was about four orders of magnitude (Figure 5). The response of Ziplex is somewhat non-linear, but a simple transformation incorporated in the Ziplex software linearizes the data. The linearized output was used in this analysis.

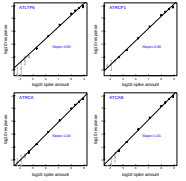

Figure 5 – Ziplex response to various amount of exogenous transcripts spiked into human total RNA. If the 95th confidence intervals of responses of two a spike amounts overlapped or the residual from the linear fit was greater than 0.15, the spike amount is plotted in gray.

Conclusions

- Comparison of probe designs on Ziplex and with Nanostring results identified probes that are good candidate for quantification of expression differences for further studies.
- Differential expression reported by Ziplex was generally concordant with Nanostring.
- Ziplex met design specifications over an ambient temperature range of 16 to 30 ºC.
- The linear dynamic range of Ziplex was determined to be four orders of magnitude of transcript abundance.
- Ziplex is a robust instrument platform to which expression profiles can be transferred from research platforms for development and validation of clinical tests.