

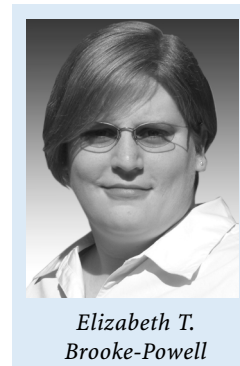
Use of Transcriptor Reverse Transcriptase in Microarray Analysis

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Researchers studying tissue and cellular differences in gene expression have applied microarray analysis to questions ranging from cancer research to plant development studies. Although the method is well established, many of the steps in the process continue to be optimised to increase overall sensitivity and reliability. Here, we monitor gene expression in the obligate intracellular pathogen *Toxoplasma gondii* in order to compare reverse transcriptase target labelling performance on a custom *T. gondii* 12,000 cDNA microarray. A histogram of signal intensities shows that the Transcriptor Reverse Transcriptase produced a major shift in the signal distribution to higher signal intensity classes compared with the most commonly used RNase H⁻ M-MuLV reverse transcriptase. This indicates the generation of a labelled target with an overall higher specific activity. The correlation of Cy5/Cy3 ratio data from four replicate microarrays demonstrate that target labelling with Transcriptor Reverse Transcriptase is highly reproducible and produces fewer “outlier” signals compared with the updated version of the RNase H⁻ M-MuLV reverse transcriptase. The known enzymatic properties of Transcriptor Reverse Transcriptase suggest that it will produce a labelled target that closely reflects the experimental input RNA.



Elizabeth T. Brooke-Powell

Introduction

Microarray analysis of gene expression is rapidly becoming a common tool for molecular biological investigations. The ability to simultaneously monitor tens of thousands of genes affords an unprecedented view of the transcriptional changes that underlie cellular processes. Microarrays are ideal for genetic investigations of intracellular pathogens such as *Toxoplasma gondii* because it is possible to simultaneously monitor gene expression in both host and pathogen [1]. However, the system must be optimised for pathogen gene expression since the *T. gondii* RNA can be more than an order of magnitude less concentrated compared with the host cell RNA. Over the past few years, technological advances have made the method much more reliable and robust.

Curiously, the standard protocols for generating the labelled cDNA target from the experimental RNA, arguably the most critical step in the procedure, remain virtually unchanged. The vast majority of investigators use either a direct incorporation of deoxynucleotides with covalently attached fluorophores or the incorporation of amino-allyl deoxynucleotides with subsequent fluorophore conjugation. In both methods, the efficiency of the target labelling reaction and quality of the labelled

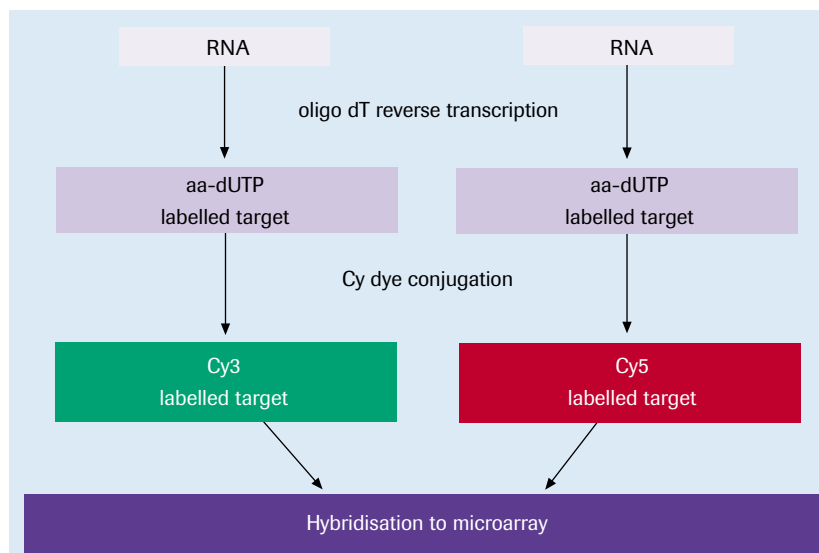
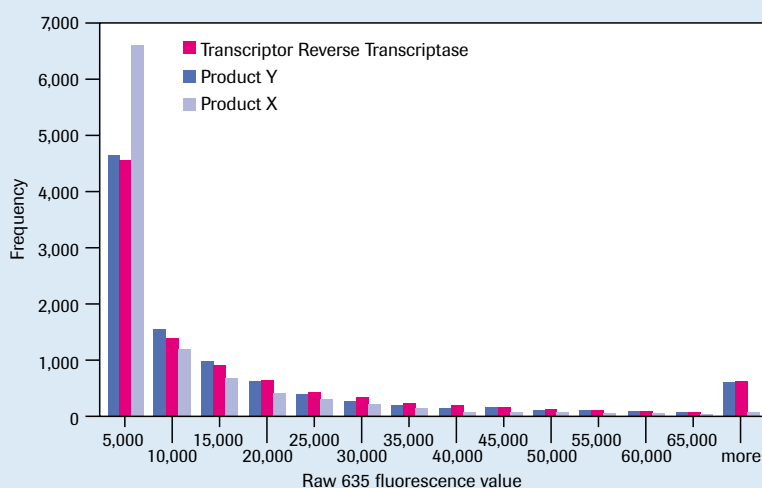


Figure 1: Microarray experimental design: A single *T. gondii* RNA preparation was used throughout. For each microarray the same reverse transcriptase was used for both labelling reactions. Dyes were independently conjugated prior to hybridisation to the microarray. Data from each microarray are derived from two independently labelled targets simultaneously hybridised to a single microarray. Four microarray experiments were performed for both Transcriptor Reverse Transcriptase and the updated RNase H⁻ M-MuLV (product Y, M-MuLV, moloney murine leukemia virus).

Figure 2:
Histogram of raw Cy5 per-spot fluorescence. The Cy5 signal for 9737 spots was quantified over each of the three microarrays. The distributions show that a higher proportion of the RNase H- M-MuLV reverse transcriptase (product X) signals are represented in the lower fluorescence classes compared with Transcriptor and the updated RNase H- M-MuLV reverse transcriptase (product Y). Conversely, the highest fluorescence class (saturation) shows a greater representation from Transcriptor Reverse Transcriptase and product Y.



cDNA product depend almost entirely on the reverse transcription enzyme used.

We employ an amino-allyl labelling reaction to generate and compare microarray data from different commercially available reverse transcriptases.

Materials and Methods

Parasite and RNA preparation

Parasites (RH strain) were cultured according to a standard protocol. NIH 3T3 cells were used as host cells, and the parasites were harvested by centrifugation just prior to monolayer lysis. The cells were suspended in TRI reagent (Sigma). RNA was extracted according to the manufacturer's protocol, except that the RNA was not ethanol precipitated following incubation with 2-propanol, but was added directly to an RNeasy Midi column (Qiagen). Binding of the sample was followed directly with the recommended wash and two extra washes. The RNA was eluted with a total of 450 μ l RNase-free water. This was followed by a standard ethanol precipitation. The RNA was vacuum dried and resuspended in RNase-free water to a final concentration of 1 μ g/ μ l.

Target labelling reaction

Labelling was performed using a modified version of the published protocol from the DeRisi laboratory (www.microarrays.org). The modifications included an increase in the ratio of amino-allyl-dUTP:dTTP to 6:4. The neutralisation was performed with Tris-HCl. All reverse transcription reactions were performed at 42°C using 15 μ g of total RNA previously extracted as described above. The total unit amount of Transcriptor Reverse Transcriptase was 20 units per reaction (10 units added, and 1 hour later another 10 units were added). The total

unit amount of product Y was 400 units per reaction (400 units added and left for 2 hours according to the manufacturer's recommended protocol). The labelled product was vacuum dried and finally resuspended in 34.5 μ l HGMP hybridisation buffer (40% formamide, 5x Denhardt's solution, 5x SSC, 0.05 M Tris-HCl pH 7.4, 1.0 mM sodium pyrophosphate, 0.1% SDS), 0.4 μ g yeast tRNA, 0.96 μ g poly-dA and 2.7% SDS (www.hgmp.mrc.ac.uk/Research/Microarray/index.jsp).

Hybridisation

The microarrays used in this study are from a single printing run, custom made with approximately 12,000 sequenced expressed-sequence tags (ESTs) from a nonnormalised library. The ESTs were PCR amplified and the products used for printing were analysed for content and quality on agarose gels. Every microarray was visually inspected and samples were prescanned for printing defects. The microarrays were preblocked (10% BSA, 3x SSC) for 1 hour at 55°C. Slides were washed with Milli-Q water (Millipore), and dried by centrifugation. Fixation of cDNA products was achieved after boiling the product and submersing it in 95% ethanol. It was pre-hybridised (0.001% SDS, 10% BSA, 3x SSC) at 55°C for 25 minutes, washed twice in Milli-Q water and washed in 100% ethanol. Hybridisation was carried out in a humid ArrayIt hybridisation cassette using a lifter slip coverslip (Erie Scientific) overnight in a 42°C water bath. Post hybridisation, the slides were washed for 4 minutes in progressively reduced SDS concentrations (solution 1: 0.5x SSC, 0.1% SDS; solution 2: 0.5x SSC, 0.01% SDS; solution 3: 0.06x SSC). Finally, the slides were dried by centrifugation.

Spot finding and scanning

The slides were scanned using an Axon 4000A Scanner according to the manufacturer's instructions. The images

were spot-found using GenePix 4.1 software with a fixed spot diameter of 150 μ m. The median fluorescence value for the spot was used for final analysis without background correction or within chip normalisation.

Data analysis

Microsoft Excel was used to create the correlation curves and histogram data. The Cy5/Cy3 data were also exported from Microsoft Excel into GeneSpring 6.0 for final analysis of gene-specific effects.

Results and Discussion

Since a single microarray experiment consists of multiple steps, the evaluation of any particular step is assessed from the resulting data. Ideally, the labelled target should accurately reflect the input mRNA in both content and representation, have a high specific activity to maximise sensitivity and be very consistent between reactions using the same input mRNA. The first of these properties is inferred from prior enzymology. The second and third properties are experimentally evaluated using the scheme outlined in Figure 1. Briefly, a single RNA source is used throughout and each target labelling represents an independent reverse-transcriptase and dye-coupling reaction. Data from each microarray are derived from two independently labelled targets simultaneously hybridised to a single microarray.

Although the relative specific activities of a hybridised labelled target can be estimated by total mean fluorescence over an entire microarray signal set, the distribution of signal intensities on a per-spot (probe) basis is more informative. Figure 2 illustrates a histogram of three representative sets of 9,737 raw (nonnormalised) fluorescent signals from the Cy5 channel. Compared with Transcriptor Reverse Transcriptase there is a much greater abundance of signals in the 0–5,000 class and relative paucity of signals in the higher fluorescence classes for product X. This signal distribution indicates that Transcriptor Reverse Transcriptase labels cDNA more efficiently than product X. In contrast, Transcriptor Reverse Transcriptase and product Y show similar signal distributions where more than half of the signals were in classes greater than 5,000. Members of the highest signal class are signals at saturation, some of which are *T. gondii* gene transcripts known to be in very high abundance. The high number of signals in this class for Transcriptor Reverse Transcriptase indicates a relatively strong labelling reaction compared with the low signal numbers for product X. Since the optimal laser power and PMT levels were similar for all three microarray scans, image acquisition can not account for the difference in results.

Consistency of target labelling is critical for microarray analysis of gene expression to detect real transcriptional differences between experimental treatments. Since the same input RNA was used in all experiments, it was possible to test an enzyme's ability to generate reproducible results; microarrays using Transcriptor Reverse Transcriptase and product Y were replicated four times each. Ratio (Cy5/Cy3) data from each of the four replicates plotted against the mean show similar results, where the Transcriptor Reverse Transcriptase replicates show generally better correlation to the mean than product Y (Figure 3). This appears to be due to fewer "outlier" spots that depart substantially from the trend lines in the four Transcriptor Reverse Transcriptase replicates.

The replicate data were processed in GeneSpring to detect particular spots that are consistently biased towards greater signal in either Transcriptor Reverse Transcriptase or product Y. Inspection of a few cDNA probes showed that the greatest signal bias towards Transcriptor Reverse Transcriptase appear to be in those probes that are substantially truncated, and those with a greater signal bias towards product Y are

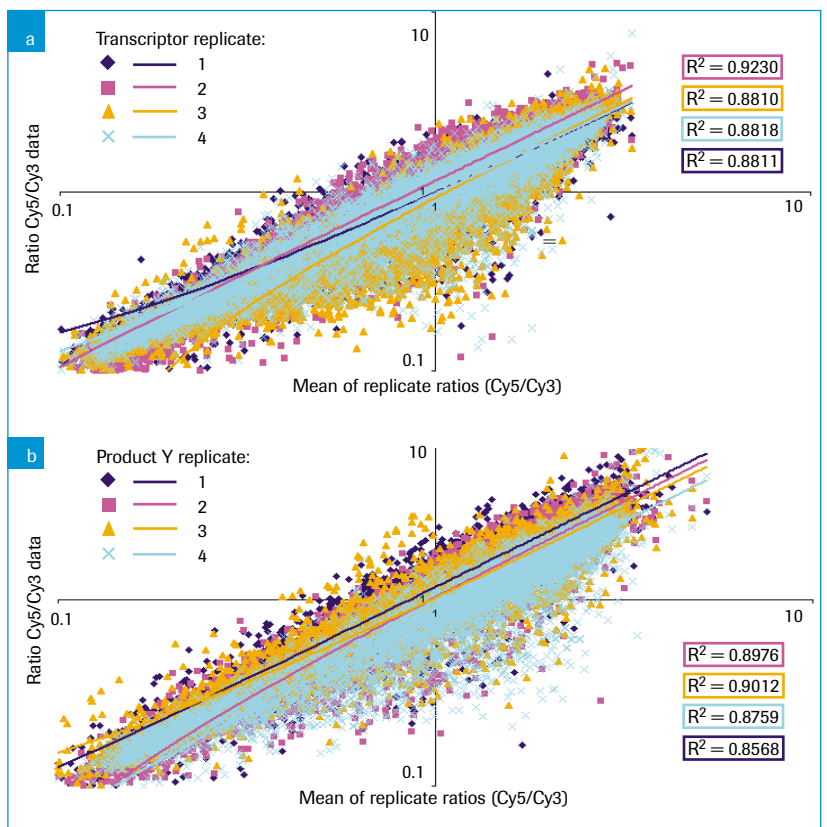


Figure 3 a, b: Correlation plots for Cy5/Cy3 signal ratio data, (a) four replicate Transcriptor Reverse Transcriptase microarrays and (b) four replicate microarrays of the updated RNase H⁻ M-MuLV reverse transcriptase (product Y), where each enzyme replicate set is correlated to their respective means.

truncated much closer to the 5' end. Although this anecdotal observation could be interpreted in several ways, it is at least consistent with the notion that the high processivity of Transcriptor Reverse Transcriptase may produce a better labelled target and more robust data in cases where the spot/probe cDNA is substantially truncated.

Conclusion

The histogram of signal intensity distributions show that Transcriptor Reverse Transcriptase can be used for high efficient target labelling. Since many investigators eliminate data with signal intensities below a particular threshold (e.g., 1,000–2,000), an increased specific activity of the labelled target may be critical for analysing genes with low expression.

In general, the data above suggest that Transcriptor Reverse Transcriptase produces a labelled target with results similar to those of the updated RNase H-M-MuLV reverse transcriptase. But Transcriptor Reverse Transcriptase appears to produce a more consistent labelled target, as the four replicates generally correlate better, with noticeably fewer "outlier" spots. The known

enzymatic properties of Transcriptor Reverse Transcriptase (high processivity and RNase H activity) should produce a labelled target that closely reflects the experimental input mRNA. This will be critical for using the latest generation of oligonucleotide microarrays, which include probes designed to the 3' end of a transcript to facilitate the analysis of mRNA splice variants, multiple poly-A+ addition sites, etc. ■

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Reference

1. Ajioka J et al. (2001), Exp. Rev. Mol. Med. 6 January, <http://www-ermm.cbcu.cam.ac.uk/o1o02204h.htm>

Product	Pack Size	Cat. No.
Transcriptor Reverse Transcriptase	250 U (25 reactions)	03 531 317 001 NEW!
	500 U (50 reactions)	03 531 295 001 NEW!
	2,000 U (200 reactions)	03 531 287 001 NEW!



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Increased Sensitivity in Microarray Analysis: Transcriptor Reverse Transcriptase in the "Eberwine" Target Preparation Workflow. (Donner et al.)

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