Application of a Blood-based Dynamic Genome Signature: How MAS5/RMA/PLIER Normalization Batches Affect Measured Gene Expression Profiling Stability in Clinical Diagnosis

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Abstract

Objective: A number of studies compare the microarray normalization methods MAS5 (Microarray Suite Version 5), RMA (Robust Multi-array Average) and PLIER (Probe Logarithmic Error Intensity Estimate) with respect to the rate at which genes of interest are identified. Here we evaluate and compare the stability of the measured gene expression when identical or technical replicate arrays are analyzed in batches of differing sizes and composition. These variations in measured gene expression have implications for clinical applications, which have requirements that differ significantly from those of research applications.

Methods: We evaluated the samples from data set E-MTAB-1532, available on ArrayExpress, a public repository of microarray data using the MAS5, RMA, and PLIER methods. We then evaluated a sample run as triplicate arrays and compared results among the different normalization methods.

Results and conclusion: Our study found that for some applications MAS5 is superior to the other methods, although the MAQC (Micro Array Quality Control) project, which extensively evaluated the performance of the platforms, reached a different conclusion.

Keywords: Microarray normalization methods; Microarray suite version; Robust multi-array average; Probe logarithmic error intensity estimate; Microarray; Gene expression; Microarray quality control project

Introduction

More than two thousand years ago during the Age of Warring States in China, Sun Tzu, the Chinese philosopher and general, recognized the uncertainties of war and concluded that only methodical “Measurement, Estimation, Calculation and Balance of chance (probabilities)” can lead to victory. These steps are still necessary in the modern world of genomic molecular biology where quantities are minute and measurements bound by fundamental uncertainties.

In previous publications, we described the methods we employed for analyzing and identifying gene panels obtained from peripheral blood samples predictive of various diseases and medical conditions [1,2]. In this paper we will describe in more detail the normalization steps that we found helpful to increase the level of repeatability in our experiments. This increased repeatability should be useful in clinical applications where disease models represented by gene expression profiles need to be stable and consistent.

Much has been written on the relative merits of different normalization methods, but most studies to date approach the problem from the biological point of view. That is, most studies set out to assess how many genes can be identified as differentially expressed or to determine gene network(s) that can be identified from correlated gene expression profiles [3,4].

MicroArray/Sequencing Quality Control (MAQC) is an effort to develop, standardize and validate procedures for microarray analysis of gene expression [5,6]. However, the goals of the project are necessarily broad to suit a wide spectrum of research efforts on various tissue types.

Our specific research purpose in past publications has been to detect disease signatures in peripheral blood samples. To this end we needed to examine microarray data normalization from a different perspective: We need to identify those genes which can return reliable and consistent measurements in replicate experiments during system validation and which therefore warrant further analysis. If measured gene expression level changes are a result of normalization methods and/or specific analysis procedures, such added “noise” may mask an actual biological effect. By specific analysis procedure, we mean the exact number and composition of the set of microarrays that are analyzed together to perform normalization and not merely the mathematical method being used (i.e., PLIER or RMA). This analysis...
"context" effect is one of the major differences between MASS and RMA, which normalize each array by explicitly relying on the data from the other arrays normalized at the same time. Published papers usually note the normalization method that was used, but when RMA or PLIER are used, the context may not always be made clear. For example, the authors may not state the number of arrays used or whether disease states are balanced or adjusted to simulate real-life distribution.

In this study we use exact duplicate data files to compare the estimated measured gene expression levels obtained by the three normalization methods, MASS, RMA, and PLIER, offered in the Gene Expression Console software provided by Affymetrix. We compare these methods when samples are processed in different batch sizes and with a disease/control ratio of 50% (unbiased gene discovery scenario) and 1% (to simulate diseases with a low prevalence). We also compare the results from technical replicates (same blood draw, multiple arrays run on different days).

Materials and Methods

We used a 200-sample data set from E-MTAB-1532, available from the Array Express repository at http://www.ebi.ac.uk/arrayexpress/ experiments/E-MTAB-1532/.

We also used three technical replicates from a 10-array experiment, run over 2 days, 3 weeks apart. (Private internal experiment and data)

We ran Affymetrix Expression Console with batches of arrays according to details described below. The normalization on each batch was performed using MASS, RMA and PLIER (Affymetrix Expression Console 1.4.1.46) in turn. We compared the results from the exact same sample data file across the different batches and normalization options. The evaluation of the stability of the measured gene expression is shown by MA plots. Tables were also generated listing count of probe sets within a range of variability tolerance with reference ± 1.1-fold and ± 1.3-fold limits.

Sample and Study Design

E-MTAB-1532 200-sample data set

From the 100 available blood samples taken from patients with colorectal (CRC) cancer in this publicly shared data set, we selected a single CRC subject, Sample (060), as our “reference sample” to be compared across batches and normalization methods (Table 1). We then repeated the analysis, with other subjects serving as "reference" for both CRC and controls, and obtained very similar results. (Data not shown)

| Colorectal Cancer | Control |
|------------------|---------|
| Batch            |         |
| 1                | 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 |
| 2                | 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 |
| 3                | 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 |
| 4                | 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 |
| Sample ID        | Sample(060) |
| N=20             | 10/10 |
| + 40 CRC & 40 Control N=100 | 1/19 |
| + 80 Control N=100 | 1/100 |

Table 1: E-MTAB-1532 sample selection for 7 batches.

**Batch 1: 20 sample balanced set**: The reference CRC sample was combined with another 9 CRC samples and 10 control samples for a “balanced” 20-sample set. This method of sampling attempts to remove experimental bias to achieve a balanced composition and simulates a small research batch.

**Batch 2: 20 sample unbalanced set**: In this batch, only the reference sample is a CRC subject, the other 19 samples are taken from the control group. This method of sampling simulates real-life conditions, in that CRC is a low prevalence disease and most of the subjects are likely to be non-CRC.

**Batch 3: 100 sample balanced set**: This method is similar to Batch 1 sampling, with additional samples added for a total of 50 CRC and 50 control subjects.

**Batch 4: 100 samples unbalanced set**: This method is similar to Batch 2 sampling, but with a composition that approaches real-life low-prevalence disease (CRC prevalence in the average population is <1%). Additional subjects are added for a total of 99 control subjects.

Technical replicates data set

This is a 10-array titration experiment using 4 samples: A, B, C, and D. Samples A and D were run in triplicate; samples B and C were run in duplicate (Table 2). Samples from other, unrelated projects were also run concurrently (as is often the case in a busy laboratory).
One blood draw from subject “A” was separated into three aliquots and hybridized to individual microarrays. The first two replicates, A1 and A2, were run on the same day together with 11 other arrays, while the third replicate, A3, was run three weeks later with three other arrays.

The data files were processed in three different batches:

**Batch 1R: All arrays run on day 1:** All 13 arrays that were run in the laboratory on the same day, including the two replicates, A1 and A2, were processed together.

**Batch 2R: All arrays run on day 2:** All 4 arrays including the third replicate, A3, were processed together. The other three arrays were also part of this titration experiment.

**Batch 3R: by project:** Only the 10 arrays which were part of this titration experiment were processed together. All other arrays unrelated to the titration experiment were excluded.

### Results

**E-MTAB-1532 batches**

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**Table 2:** Technical replicate sample batches.

| Batch | Sample | 10-array titration experiment | unrelated experiment |
|-------|--------|------------------------------|----------------------|
|       | A in triplicate | B in duplicate | C in duplicate | D in triplicate | single | batch size |
| Batch 1R | 1R | 1R | 1R | 1R | 1R | N=13 |
| Batch 2R | 3R | 3R | 3R | 3R | N=4 |
| Batch 3R | 3R | 3R | 3R | 3R | 3R | 3R | 3R | 3R | 3R | N=10 |
| Array | A1 | A2 | A3 | B1 | B2 | C1 | C2 | D1 | D2 | D3 | 7 others |

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**Figure 1:** MA plots for “reference CRC” Sample (060) in batches 1 to 4 (20-sample and 100-sample subsets). Note asymmetrical scatter pattern for PLIER results.

In Figure 1a, we compared the results for the reference Sample (060) when analysed in Batches 1 and 2 (20-sample set). The differences between batches are attributable only to the composition of the other 19 samples in the batch and its effect on the method of analysis. The scatter from the PLIER analysis shows an asymmetrical pattern (red data points) limited to about 1.3-fold in one direction and exceeding 2-fold in the other direction (1 unit on the Log2 scale). The RMA analysis produced a smaller, symmetrical scatter pattern which is...
The results from the technical replicates show similar trends. However, because the arrays are replicates, MAS5 results now show some scatter, which is wider than RMA at low intensity. PLIER still exhibits asymmetrical and large scatter at the low end, tapering to less scatter than RMA at the high end. Remarkably, while MAS5 underperformed both RMA and PLIER for most of the range, there are more probe sets with variability of less than 1.1-fold using MASS analysis (Figure 2c).

Typically, each probe set in the Affymetrix GeneChip arrays is complementary to a specific transcript within the target gene. The Perfect Match (PM) probe is composed of 11 to 16 base sequences exactly complementary to the target transcript and will therefore bind perfectly. The mismatched (MM) probe has the same sequence of bases, except that the middle base is intentionally substituted with the complementary base of the PM to measure non-specific binding (because mismatch should disturb binding to the specified transcript). Each probe pair in a probe set is considered as having a potential “vote” in determining whether the measured transcript signal is specific (PM>MM) and labelled “Present” or non-specific (MM=PM) and labelled “Absent”. The “Present” call feature of MASS effectively filters out less reliable, non-specific results with wide scatter. This Present/Absent call function uses the perfect-match and mis-match probe differential to estimate the reliability of the signal, reliability which is ignored by RMA and PLIER normalization.

In this study we found MAS5 to be the most stable normalization method for profiling gene expression in peripheral blood samples, as this method can reliably report gene expression levels that vary between technical replicates by less than 1.1-fold. As variations in gene expression differences obtained from tissue biopsies are expected to be 2-fold or greater, PLIER and RMA do perform better, as was reported by the MAQC consortium. However, both PLIER and RMA analyses can benefit from using the “Present” call feature of the MASS method of analysis, in which probe sets with ambiguous hybridization will be automatically filtered out, resulting in reduced scatter and improved detection of actual biological effects.

It may be observed here that for practical diagnosis in clinical studies, patient presentation and diagnoses are unexpected and uncontrolled. For mRNA gene signature studies, the individual patients’ gene profile measurements have been taken under the defined chronology of clinical laboratories. That is, patients seeking a clinical test for the disease of interest arrive randomly in no particular order, together with patients with requests for other diagnoses. In research studies by contrast, subjects are processed sequentially and all subjects have the same disease of interest. These differences create a subtle batch effect in the research situation that is different from the real-life situation. Thus the average of the research batch can be quite different from the average of a clinical batch. This batch effect is often overlooked, but it is central to the issue discussed here on two levels. This raises certain important questions: First, in the clinical situation do we batch similar tests together? or as the patients come to the lab? Second, do we batch in the same proportion of disease/control as in real-life or in 50/50 proportion as during research.

Because RMA is a global normalization method, which uses the signals from all the arrays in a batch, the measured gene expression for any single array will not be perfectly stable and will vary with the batch components. For best repeatability, experimental notes should include the number and identity of all companion arrays processed in the same batch. There is a trade-off, however, between more stable results with a larger number of arrays and the longer computer processing time this
would require. It may even be good practice to set aside a specific batch of arrays that can be used to act as a "constant background". Again, the extra processing time required may be a factor that reduces the practicality of this approach.

We also noted an asymmetrical scatter pattern in the PLIER results when evaluating "balanced" sets of equal number of CRC and control subjects as against "realistic" sets with a single CRC and many control subjects, as would be the case for low-prevalence diseases.

MAS5 circumvents these issues and achieves the most reproducible results while allowing maximum flexibility in the batching of arrays. The downside of MAS5 is that some genes may be overlooked because their signals are not sufficiently stable, and analysis in this case may benefit from RMA and PLIER methods. However, for detecting disease signatures from peripheral blood samples stability and reproducibility are more important priorities, and MAS5 would therefore be the preferred normalization method. Considering that disease onset usually involves many different signaling pathways and numerous genes, a failure in data mining to identify some contributory but unstable genes is not crucial to disease diagnosis.

To compensate for inter-array and reagent lot variabilities we analysed the data as specific gene-pairs or combinations of specific gene-pairs. The desired gene-pair is defined as a "primary" gene with high correlation to the feature of interest or disease state combined with a companion "suppressor" gene with very low correlation to the feature but high correlation to the "primary" gene with the pair achieving an increased correlation [7,8]. We believe this kind of gene pairing achieves a useful degree of self-normalization that overcomes such unavoidable manufacturing and experimental variabilities. Because there is a very large number of possible pairings and an even larger number of pair combinations, these specific gene pairs need to be evaluated using the method we presented in our previous paper [2].

In order to verify the success of this technique of gene pairing, we conducted several experiments. As described in our previous paper [2], we performed a series of technical replicates using 4 replicates across 7 different batches of microarrays. We also combined microarray data from samples using EDTA (BD Vacutainer) and PAXgene (PreAnalytiX) collection tubes. These two types of tubes employ very different stabilization chemistry and the resultant gene expression profiles are vastly different because of the high globin mRNA content from PAXgene tubes. The combination of MAS5 and gene-pair analysis was able to identify a collection tube-agnostic gene panel for hepatocellular carcinoma that successfully predicted with high accuracy, achieving an AUROC of 0.96 in an independent test set with a mixture of HCC, non-HCC cancer, chronic hepatitis-B, and symptom-free "normal" subjects (Figures 3 and 4).

Figure 3: Prediction scores using a panel of 6 gene-pairs (LogReg_6Pairs) identified from MAS5 normalized microarray data. Note the shift in the scores for the test set predictions using the Weka Raw17Gene panel which reduces the distance between the cancer and the control groups while the LogReg_6Pairs panel is able to maintain the separation in the test set.

Figure 4: Family membership prediction. Three gene panels were identified that were able to predict membership in one of three families. 100 subjects who don’t belong any of these three families correctly predict as “negative” to all three.
The choice or selection of microarray normalization technique becomes important in the integration of molecular genetic diagnostics into clinical medical practice. The interrogation of the blood [2] and bodily-fluid-borne [9,10] cellular and non-cellular (i.e., exosomal and non-vesicular plasma) components may allow for timely interventional targeting not only of occult neoplasms but also of diseases with inflammatory, infectious and degenerative etiologies. The mRNA, lncRNA and miRNA transcriptome profiles in some disease entities have already been addressed in the literature, including: gliomas [11-14], head and neck cancer [15], lung cancer [16], breast cancer [17], renal carcinoma [18], hepatocellular carcinoma [19], prostate [20] and colorectal cancer [21] as well as rheumatoid and osteoarthritis [22], schizophrenia [23] and Alzheimer's dementia [24].

Currently pathological examination is based on tissue biopsy and research has also explored methods that focus on detection of circulating abnormal cells (i.e., circulating tumor cells) and genomic fragments (i.e., circulating tumor DNA, exosomes). By contrast, in our work we measure the dynamic mRNA gene expression of blood cells, which by reflecting interactions between circulating blood cells and the body's cells, tissues and organs, may mirror the current state of a body's health or disease. Recently, mRNA gene expression changes have also been shown to correlate with the traditional Chinese medicine classification of Yin/Yang deficient or balanced states [25].

In conclusion, mRNA gene expression profiles from whole peripheral blood samples tend to track the activity of the immune system and therefore reflect the general health of a subject. This will be a changing, dynamic situation better captured with genomics technology than with the static DNA sequence-based profiles which predict propensity to develop any particular disease. By the very nature of this ever-changing landscape, mRNA gene profiling can be useful in the detection of the early stages of a developing condition. Gene profiling may also prove to be useful to monitor treatment response and to assess the progress of a disease. However, the very nature of this changing profile necessitates a high-precision, high-stability measurement system. Part of these requirements can be met with the adoption of MAS5 normalization rather than the more commonly accepted RMA and PLIER methods.
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Conflict of Interest

No conflict of interest is declared by any author.

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