

# Normalization of qPCR array data: a novel method based on procrustes superimposition

Ali Mohammadian<sup>1,\*</sup>, Mohammad Reza Nourani<sup>2</sup>, Seyed Javad Mowla<sup>3</sup>, Mahmood Tavallaee<sup>4</sup>, Yu Liang<sup>5</sup>

<sup>1</sup>Department of Biotechnology, College of Science, University of Tehran, Tehran, Iran.

<sup>2</sup>Chemical Injury Research Center, Baqiatallah Medical Sciences University, Tehran, Iran.

<sup>3</sup>Molecular Genetics Department, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran.

<sup>4</sup>Human Genetic Research Center, Baqiatallah Medical Sciences University, Tehran, Iran.

<sup>5</sup>Division of Molecular Medicine, Life Technologies, Foster City, CA 94404, USA.

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## Abstract

MicroRNAs (miRNAs) are short, endogenous non-coding RNAs that function as guide molecules to regulate transcription of their target messenger RNAs. Several methods including low-density qPCR arrays are being increasingly used to profile the expression of these molecules in a variety of different biological conditions. Reliable analysis of expression profiles demands removal of technical variations in data, which is achieved via applying normalization techniques. Most normalization techniques have been developed for mRNA microarrays and new and modified methods should be used for miRNA studies in general and RT-qPCR miRNA arrays in particular, because of low number of miRNAs. Here, we introduce a new method based on Procrustes superimposition of arrays to be normalized on a reference array. To assess the performance of our normalization method, we compared this method to the common miRNA normalization methods. Removal of technical variation was assessed by robust modeling of mean square error (MSE) in different subsets of real miRNA datasets before and after applying normalization. We show that our method outperforms the other normalization methods in concurrent reduction of technical variation and retention of biological variability.

**Keywords:** miRNA, Low-density qPCR array, Normalization, Procrustes superimposition

## Introduction

MicroRNAs (miRNAs) are small inhibitory endogenous RNAs. They regulate target mRNAs through repressing translation or promoting mRNA degradation(1, 2). These tiny molecules control a variety of cellular processes, and possess promising diagnostic and therapeutic potentials(3). Their small length and high inter-molecular similarities has not impeded the use of high-throughput profiling technologies to unravel their roles in a variety of biological milieus. Microarrays(4), low density qPCR arrays(5), bead based flow cytometry assay(6), and next generation sequencing techniques(7) have all been successfully applied to profile expression of these

molecules. Like other RNA species, qPCR based methods are superior to other methods in terms of accuracy and sensitivity for the quantification of miRNAs(8).

Even with reliable methods such as qPCR, technical variations (experimental error) negatively affect the reliability of data. To minimize non-biological sources of variation and retain true biological variations, normalization techniques are applied to raw data(9). Although many different housekeeping miRNAs have been suggested for the normalization of miRNA qPCR data, mean Cq values of samples have been shown to provide a superior normalization factor for qPCR array data than do housekeeping miRNAs(9, 10). In addition, cDNA array

normalization techniques have also been applied to normalize qPCR array data(11). Due to the low number of miRNAs, the assumptions underlying mRNA normalization methods can be easily violated. This may lead to introduction of inaccuracy in high-throughput miRNA data. Each miRNA may target some hundreds of mRNAs, and misclassifying a single miRNA as (non-) differentially expressed is equivalent to wrong classification of all its targets. Therefore, the removal of experimental error and retention of true biological signal are essential requirements in miRNA profiling studies. Further, to achieve a more precise analysis of differential gene analysis, proper removal of experimental error and retention of biological variation is necessary. Novel normalization methods are needed to increase the precision and accuracy of high-throughput miRNA qPCR array data.

Herein we propose a novel normalization method based on Procrustes superimposition, and assess its power to remove experimental error in miRNA qPCR data. Procrustes superimposition uses translation, rotation, and scaling of data to find the closest match between two configurations. Generalized Procrustes analysis (GPA) has been recently used for single and double channel arrays(12, 13). Here we introduce a novel method based on Procrustes superimposition (PS). Our proposed method is applicable to both single- and double channel array data. We assess the performance of PS normalization method against common normalization methods for miRNA qPCR arrays. Our analysis is based on the reduction of experimental error by using mean squared error (MSE) criterion(4) and retention of signal-to-noise ratio(14) in different subsets of a real miRNA dataset.

## **Materials and methods**

### **Datasets**

The miRNA expression profile of 40 human tissues was used as dataset A. This dataset contained four replicates per tissue(5). The experiments had been performed on TaqMan<sup>®</sup> microRNA platform to provide the miRNA signature of different tissues. To assess different normalization methods, three different subsets of this dataset were used in addition to dataset A, as

follows. Two tissues with the highest degree of differentially expressed miRNAs (peripheral blood mononuclear cells (PBMC) and placenta) were used as dataset B. More than 75% of miRNAs were differentially expressed between these tissues in raw data. The remaining 38 tissues from the original dataset were used as dataset C. Finally, four biologically-related tissues, left and right ventricles and left and right atria, which also have comparable miRNA expression patterns, were used as dataset D.

### **Common methods for mirna high-throughput data normalization**

The method proposed by Metsdagh (Mts), subtracts mean Cq value of a sample from Cq values of miRNAs in the sample to find the normalized Cq values(9). In the method proposed by Pradervand (Prd), the differences in background levels are optionally removed. Then, the dependence of standard deviation on mean of expression values in all samples is removed, and a set of low standard deviation miRNAs that have high expressions (the invariant set) is determined by a mixture model across all samples. Normalization coefficients are computed by a robust regression method (an M estimator with Huber influence function) and are used to scale the data(15). This method has been developed for miRNA microarrays, and to apply this method to qPCR array data, we replaced Cq values with 40-Cq, performed the normalization and reverted normalized data back (40- normalized Cq) (Personal communication with S. Pradervand). Quantile normalization first sorts Cq values in each sample. Then a baseline array is then created by taking the mean of Cq values in each rank across samples. In the next step, original values in each sample are replaced by values of the baseline array. Then each sample Cq values are sorted back to their original order. This normalization makes all samples share a single distribution of Cq values.

### **Assessing removal of error**

We used mean square error (MSE) criterion to assess removal of experimental error after different normalization techniques(4, 13). This criterion is widely used to compare the

performance of different normalization methods. As true (theoretical) Cq values are unknown in our datasets, we robustly modeled MSE. Briefly, miRNAs were ranked according to their average Cq values in duplicate samples and sorted into groups of thirty miRNAs (17). Differences in duplicate Cq values in each group ( $diff_{i,b}$ ,  $1 < i < 30$ , and  $1 < b < \text{number of groups}$ ) were obtained. Subsequently, median ( $\text{med}(diff_{i,b})$ ) and median absolute deviation of  $diff_{i,b}$  ( $\text{MAD}(diff_{i,b})$ , equation (a)) were calculated.

$$\text{MAD}(diff_{i,b}) = \text{med} |diff_{i,b} - \text{med}(diff_{i,b})| \quad (\mathbf{a})$$

In the next step,  $\text{med}(diff_{i,b})$  and  $\text{MAD}(diff_{i,b})$  were modeled as smooth functions of Cq values. Weighted smoothing spline (function “smooth.spline” from stats package, R language, equation b), and smoothing splines with the smoothing parameter selected by generalized maximum likelihood (function “ssanova0”, package gss, R language, equation c) were used to model  $\text{med}(diff_{i,b})$  and  $\text{MAD}(diff_{i,b})$  respectively. Weights used for “smooth.spline” were equal to the reciprocal of the squared MAD of difference for each group.

$$\text{med}(diff_{i,b}) = \eta(Cq_{med,b}) + \varepsilon_{1,b} \quad (\mathbf{b})$$

$$\text{MAD}(diff_{i,b}) = \xi(Cq_{med,b}) + \varepsilon_{2,b} \quad (\mathbf{c})$$

Here  $\xi$  and  $\eta$  represent the smoothed functions.  $\varepsilon_{1,b}$  and  $\varepsilon_{2,b}$  denote the errors of estimations (4). Now, MSE can be estimated as follows:

$$\text{MSE} = \left( \frac{\hat{\xi}(Cq_{med,i})}{0.6745} \right)^2 + \hat{\eta}(Cq_{med,i})^2 \quad (\mathbf{d})$$

Where the factor 0.6745 in the denominator is used as the mathematical expectation of MAD in normal distribution is  $0.6745 \sigma$ . Estimates in equation (d) were obtained for 100 different points. Fitted functions (equations (b) and (c)) were used to obtain estimated values for these points. These points were chosen to be between minimum and maximum Cq values of two replicates.

The R code for binning and calculating MSE may be requested from first author. The first term in equation (d) is the smoothed variance and the second one is the smoothed squared bias,

demonstrating precision and accuracy in differences of Cq values, respectively. Lower values of bias and variance and indicate a superior normalization method, and best normalization would be the one that leads to unbiased Cq values with the lowest MSE value. Bias and variance components were calculated for each bin in each tissue and average values of bias (Fig. 1), variance (not shown) and MSE (Fig. 2) in each dataset after applying different normalization methods was used to obtain a global view of the performance of normalization methods.

To assess whether normalization methods do not reduce biological variability along with reducing MSE, we obtained mean sum of squares (MSS) of each miRNA between tissues ( $MSS_b$ , equation e) and MSS of each miRNA within tissues ( $MSS_w$ , equation f) for each normalization technique(14).

$$MSS_b = \frac{R}{T-1} \sum_{t=1}^T (\bar{C}q_t - \bar{C}q)^2 \quad (\mathbf{e})$$

$$MSS_w = \frac{1}{N-T} \sum_{t=1}^T \sum_{r=1}^R (Cq_{t,r} - \bar{C}q_t)^2 \quad (\mathbf{f})$$

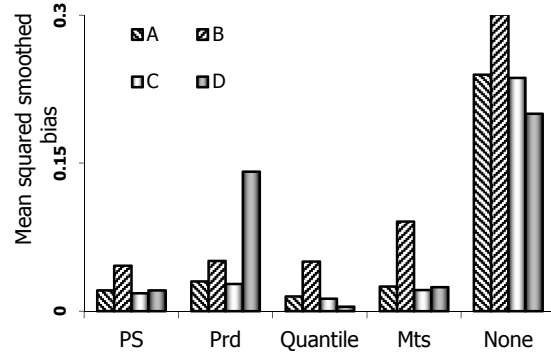


Figure 1. Global mean squared bias in difference of Cq values in replicates in raw data and after applying different normalization methods.

Where  $R$  is the number of replicates,  $T$  the number of groups (tissues),  $\bar{C}q$  the mean expression value for  $i^{\text{th}}$  miRNA across all experiments,  $\bar{C}q_t$  the mean expression value of  $i^{\text{th}}$  miRNA in tissue  $t$ ,  $Cq_{t,r}$  the expression in  $r^{\text{th}}$  replicate of tissue  $T$ . For each miRNA,  $MSS_b$  is a measure of variability between tissues, and  $MSS_w$  a measure of variability within tissues, and a good normalization method should reduce  $MSS_w$ .

Density curves of  $MSS_b$  and  $MSS_w$  curves of dataset A after different normalizations are shown in Figure 3.

## Results and Discussion

We first describe our own developed normalization method. Then we turn to analysis of reduction of errors and

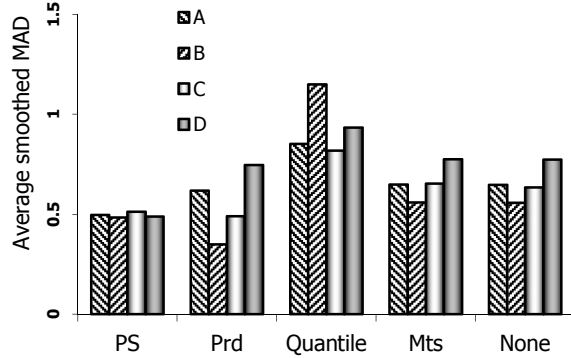


Figure 2. Average smoothed MAD in different datasets and after different normalizations.

### Novel normalization method

Consider an  $m \times s$  expression matrix containing  $m$  miRNAs in  $s$  samples, so that the  $j^{\text{th}}$  column,  $0 \leq j \leq s$ , in this matrix represents the Cq values of miRNAs present in the  $j^{\text{th}}$  sample in the study. Cq values of each column are sorted to create a sorted expression matrix ( $\mathbf{S}$ ). An  $m$ -dimensional “baseline vector” is created from  $\mathbf{S}$  as follows. The  $i^{\text{th}}$  value of this vector is equal to the mean (or

alternatively median) value of  $i^{\text{th}}$  row of  $\mathbf{S}$ , with  $0 \leq i \leq m$ . In the next step,  $\mathbf{S}$  is split to  $s$  column vectors. Then all sample vectors in addition to the baseline vector are merged with a new  $m$ -dimensional vector to create  $s+1$  matrices of  $m \times 2$ . The added column lists the ranks of Cq values in each sample. Now, a PS, a PS without rotation, or a partial PS can superimpose each of sample matrices on the reference matrix. The PS normalization method was implemented in R (v2.15.3, [www.r-project.org](http://www.r-project.org)), a statistical scripting language, and is available on request.

### Reduction of bias, variance and MSE

For datasets A-D, mean squared smoothed bias values before and after normalization are shown in Figure 1. Smaller values of squared bias denote increased accuracy in data. Normalization methods tested here increased the accuracy in difference of Cq values of replicate samples, i.e. shifted the center of distribution of these values toward zero. Quantile normalization led to the largest decrease in bias, and PS followed quantile normalization. The performance of different normalization methods in reduction of variance component of MSE is depicted in Figure 2. Compared to squared bias term, the variance term of MSE is not much affected by applying normalization methods, and the general trend in reduction of MSE closely follows the trend for bias. Quantile normalization is an exception to this observation, as it increases variance. The increase in variance in data means broader distribution, and set of Cq values with lower precision. PS normalization, on the other hand,

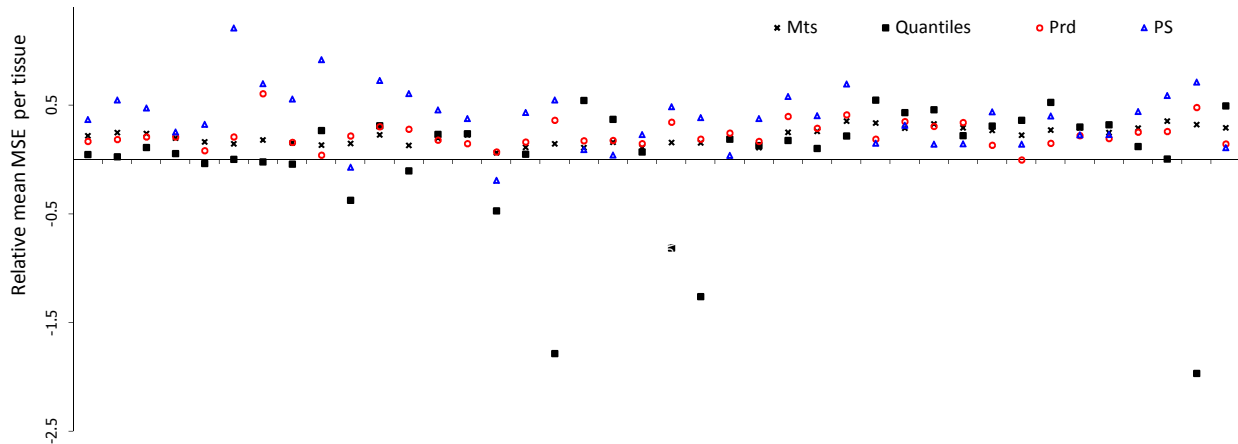


Figure 3. Reduction of MSE per tissue in dataset A after different normalizations. MSE value in raw data is subtracted from MSE values for each tissue.

decreased variance.

The efficacy of different methods in reducing MSE

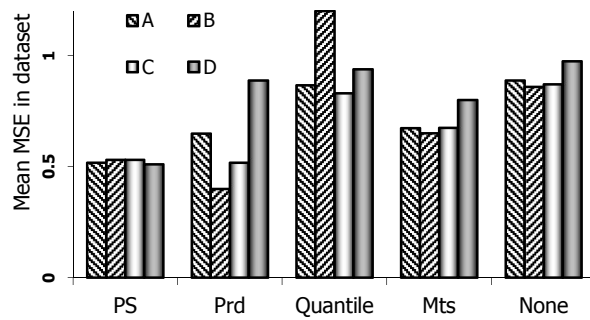


Figure 4. Global estimated MSE values in different datasets and after different normalizations.

is shown in Figure 4. The best method is PS, except for Prd in subset B. PS is often the most efficient method in reducing MSE in replicate experiments. Estimated MSE, estimates the difference between true and obtained values. Generally, when the bias is lower than original dataset, lower values of MSE indicate less error in data and better normalization. Although quantile normalization led to the least biased data, the lowering caused increased variance up to the point that MSE is increased. Disregarding quantile normalization because of increase in MSE, PS is the method with the least bias and lowest MSE, and is regarded the best method tested here.

Mean MSE value for each of the original 40 tissue after normalizing dataset A is depicted in Figure 3. PS also performs acceptably in most tissues and frequently leads to the least MSE. It should be noted that expression data are used for differential gene expression identification and subsequent downstream analyses. Introduction of error in any Cq value of any one tissue, negatively affect the differential expression analysis for that specific miRNA between the tissue and all other tissues.

### Retention of signal-to-noise ratio

Density curves of  $MSS_b$  and  $MSS_w$  for dataset A after different normalizations are shown in Figure 3.  $MSS_b$ , which indicates the variability of miRNAs between different tissues, is not reduced after PS normalization, while  $MSS_w$ , the variability of miRNAs within replicate samples, is

lowered by PS normalization, as the peak can be seen to shift to lower values (Fig. 3). The effect of normalization on the ratio of  $MSS_b$  to  $MSS_w$  should ideally leads to identification of only the true differentially regulated miRNAs, the knowledge of which is not accessible in this dataset. As we are testing whether biological variability is maintained after normalizations, we expect that  $MSS_b$  does not decrease for miRNAs. It can be concluded that our method does not reduce MSE by simply removing the difference between all pairs of arrays, and can be applied to miRNA qPCR data.

An acceptable normalization method is expected to perform acceptably on data with different structures. It should lead to removal of technical variation, maintain the differences between different biological groups and reduce the differences within each biological group in different types of expression data. Subsets of data we chose to normalize were different in nature: dataset D contained highly similar tissues with similar expression profiles whereas dataset B tissues showed differential expression in majority of genes. Dataset B contained only two tissues in contrast to 40 tissues of dataset A, and dataset C contained more homogenous collection of tissues in comparison of dataset A.

Here we proposed a method that uses Procrustes superimposition to remove experimental error in high-throughput qPCR data. Our method successfully reduces technical variation and other non-biological sources of variation in datasets with either highly similar or highly different tissues. PS normalization led to highest decrease in MSE values in 3 of 4 datasets we tested, and retained the variability between tissues. PS can be considered as a novel method to normalize qPCR data.

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