

# RNA-Seq as a Relative Abundance Measure: oportunities afforded by a compositional analysis framework.

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## 1 Introduction

The rapid rise in the use of RNA sequencing technology (RNA-seq) for scientific discovery has led to its consideration as a clinical diagnostic tool. However, as a new technology, the analytical accuracy and reproducibility of RNA-seq must be established before it can realize its full clinical utility [22, 26]. Recent studies evaluating RNA-seq have found generally high intra-platform and inter-platform congruence across multiple laboratories [Li2013, 24, 22]. Despite these promising results, there remains a need to establish reliable diagnostics and quality control metrics to improve the reproducibility of RNA-seq data. Understanding, and capatilizing on, the relative frequency nature of RNA-Seq data provides tools for creating quality control metrics and identifying batch effects leading to improved reproducibility.

This research is focused on developing diagnostics, using the theory of compositions, for targeted RNA-Seq. Targeted sequencing allows researchers to efficiently measure transcripts of interest for a particular disease by focusing sequencing efforts on a select subset of transcript targets. Targeted sequencing offers several benefits over traditional whole-transcriptome RNA-Seq for clinical use including the elmination of amplification bias, reduced sequencing cost, and a simplified bioinformatics workflow. Moreover, extraction-free targeted sequencing technologies, such as HTG EdgeSeq, permit the use of very small sample volumes. However, extraction free technologies create the need for post-sequencing quality control metrics since poor quality samples, which would likely be removed after unsuccessful RNA extraction in extraction-based technologies, can still be sequenced. The post-sequencing methods described here should be easily extensible to

traditional extraction-based RNA-Seq because targeted and traditional RNA-Seq data share many of the same properties.

Relative frequency measures are characterized as a vector of proportions of some whole. These proportions are necessarily positive and sum to a constant which is determined by the measurement system and not the measurand. Targeted and whole transcriptome RNA-Seq measurements from NGS-based instruments provide only relative frequencies of the measured transcripts. The measurement technology, along with sample preparation, preclude the measurement of absolute abundance. The total number of reads in a sequencing run for high-throughput RNA-Seq instruments is determined by the maximum number of reads available per run and not the absolute number of reads in a sample. For example, the Illumina Mi-Seq is limited to 25 million reads in a sequencing run while the Roche 454 GS Junior <sup>(TM)</sup>, with longer read lengths, claims approximately 100,000 reads per run for shotgun sequencing. These reads are distributed across all of the samples included in a sequencing run and, therefore, impose a total sum constraint on the data. This constraint cascades down to each probe or tag within a sample which is, in turn, constrained by the total number of reads allocated to the sample thereby creating a natural hierarchical structure to RNA-Seq data.

Previous authors have identified the relative abundance nature of RNA-Seq data [20, 6, 21, 12, 14]. For example, Robinson and Smyth (2007) [20] consider counts of RNA tags as relative abundances in their development of a model for estimating differential gene expression implemented in the Bioconductor package edgeR. Similarly, Robinson and Oshlack (2010) explicitly acknowledge the mapped-read constraint when developing their widely used Trimmed-Mean of M-values (TMM) normalization method for RNA-Seq data. Finally, the commonly used  $\log_2$  Counts per Million (CPM) re-scaling transformation proposed by Law et al. (2014) [12] divides each sequence count by the total number of reads allocated to the sample thereby transforming the data for each sample into a vector of proportions.

The positivity and summation constraint complicate the analysis of relative frequency data. As early as 1896 Karl Pearson [19] identified the spurious correlation problem associated with compositions. John Aitchison observed that relative frequency data is compositional and developed a methodology based on the geometric constraints of compositions [1]. Recent authors have argued that ignoring the sum constraint can lead to unexpected results and erroneous inference [15]. Despite the evidence that RNA-Seq data are compositional in nature, few researchers have extended the broad set of compositional data analysis theory and

operations for use in RNA-Seq analysis problems. We provide a brief background on compositional methods. We then extend existing compositional data methodology to develop a simple quality control metric and improve batch effect detection for RNA-Seq data. Finally, we show how compositional properties can be exploited to facilitate exploration of high-dimensional RNA-Seq data.

## 2 Methods

### 2.1 Compositional Data

We begin with a brief introduction to compositional data, its properties, and some established analytical methods. Compositional data is defined as any data in which all elements are non-negative and sum to a fixed constant [1]. For RNA-seq data, the total sum constraint is imposed by the limited number of available reads in each sequencing run. Since this total differs between sequencing platforms we will refer to the total number of available reads as  $\mathbb{T}$ . These reads are distributed among the  $D$  samples in a sequencing run such that:

$$\sum_{i=1}^D t_i = \mathbb{T} \quad (1)$$

where  $t_i$  represents the total reads for sample  $i$ . Because of the total sum constraint, the vector  $\mathbf{t}$  is completely determined by  $D-1$  elements since the  $D^{th}$  element of  $\mathbf{t}$  can be determined from the other  $d = D-1$  elements and the total  $\mathbb{T}$ :

$$t_D = \mathbb{T} - \sum_{i=1}^d t_i \quad (2)$$

In 2, any of the elements can be chosen for  $t_D$  with the remaining elements labeled  $1, \dots, d$  in any order [1]. Similarly, the total reads for each sample ( $t_i$ ) are distributed among the  $P$  transcript targets in the assay such that  $\sum_{j=1}^P p_{ij} = t_i$ , where  $p_{ij}$  is the total reads allocated to target  $j$  in sample  $i$ . We highlight the hierarchical structure of RNA-Seq data as it leads to useful properties when developing quality control metrics.

From equations 1 and 2 it is clear that the total reads allocated to each of the  $D$  samples represent a  $D-1 = d$  dimensional simplex ( $\mathcal{S}^d$ ). This leads to a difficulty in interpreting the traditional  $D \times D$  covariance structure. In particular, it is clear that for a D-part composition  $\mathbf{x}$ ,  $\text{cov}(x_1, x_1 + \dots + x_D) = 0$

since  $x_1 + \dots + x_D$  is a constant. Moreover, the sum constraint induces negativity in the covariance matrix,

$$\text{cov}(x_1, x_2) + \dots + \text{cov}(x_1, x_D) = -\text{var}(x_1). \quad (3)$$

Equation 3 shows that at least one element of each row of the covariance matrix must be negative. Aitchison refers to this as the “negative bias difficulty” (although ‘bias’ is not used in the traditional sense; [1], p. 53). The structurally induced negative values create problems for the interpretation of the covariance matrix.

Because of the difficulties outlined above, standard statistical methodology is not always appropriate [1] and can produce misleading results [14]. To overcome these obstacles, Aitchison [2] proposed working in ratios of components. We focus on the Centered Log-Ratio (CLR) which treats the parts of the composition symmetrically and provides an informative covariance structure. The CLR transformation is defined for a  $D$ -part composition  $\mathbf{x}$  as:

$$y_i = \text{CLR}(x_i) = \log \left( \frac{x_i}{g(\mathbf{x})} \right), \quad (4)$$

where  $g(\mathbf{x})$  is the geometric mean of  $\mathbf{x}$ . The  $D \times D$  covariance matrix is then defined as:

$$\Gamma = [\text{cov}(y_i, y_j) : i, j = 1, \dots, D] \quad (5)$$

To avoid numerical difficulties arising from sequence targets with 0 reads, Martin-Fernandez et al. (2000) [17] suggest an additive-multiplicative hybrid transformation. If zeros are present in the data We recommend using the Martin-Fernandez transformation with a threshold value of  $\delta = \frac{0.55}{\text{Total Reads}}$  to account for differences in library size. The CLR transformation is then applied to the Martin-Fernandez transformed data which contains no zeros.

The CLR transformation is similar to the familiar Counts per Million (CPM) transformation [12] defined as,  $\log_2 \left( \frac{r_{gi} + 0.5}{t_i + 1} \times 10^6 \right)$ , where  $r_{gi}$  is the number of sequence reads for each probe ( $g$ ) and sample ( $i$ ), (scaled to avoid zero counts), adjusted for the number of mapped reads (library count) for each sample  $t_i$  (scaled by a constant 1 to ensure the proportional read to library size ratio is greater than zero). The primary difference between the CLR and log(CPM) transformations is in the use of the geometric mean in the denominator of the

CLR transformation. The use of the geometric mean results in subtracting the mean of the log transformed values from each element thereby centering the vector of log-ratio transformed read counts. The difference appears minor but has important implications for the application of several common statistical methods.

The compositional geometry must be accounted for when measuring the distance between two compositions or finding the center of a group of compositions [3]. Aitchison [4] outlined several properties for any compositional difference metric which must be met: scale invariance, permutation invariance, perturbation invariance (similar to translation invariance for Euclidean distance), and subcompositional dominance (similar to subspace dominance of Euclidean distance). The scale invariance requirement is ignorable if the difference metric is applied to data on the same scale (which is generally not satisfied in raw RNA-seq data due to differences in read depth). The permutation invariance is generally satisfied by existing methods such as Euclidean distance [18]. However, the perturbation invariance and subcompositional dominance are not generally satisfied [18].

Aitchison [1, 4] suggests using the sum of squares of all log-ratio differences. Billheimer, Guttorp, and Fagan [8] use the geometry of compositions to define a norm which, along with the perturbation operator defined by Aitchison [1], allow the interpretation of differences in compositions. Martin-Fernandez et al. [18] showed that applying either Euclidean distance or Mahalanobis distance metric to CLR transformed data satisfies all the requirements of a compositional distance metric. Euclidean distance on CLR transformed compositions is referred to as Aitchison distance:

$$d_A(x_i, x_j) = \left[ \sum_{k=1}^D \left( \log \left( \frac{x_{ik}}{g(x_i)} \right) - \log \left( \frac{x_{jk}}{g(x_j)} \right) \right)^2 \right]^{\frac{1}{2}}$$

or

$$d_A(x_i, x_j) = \left[ \sum_{k=1}^D (clr(x_{ik}) - clr(x_{jk}))^2 \right]^{\frac{1}{2}}.$$

Up to this point we have referred to the total reads available per sequencing run,  $\mathbb{T}$ . However, it is more typical to work with the aligned reads in practice. The total aligned reads,  $T$ , is always a fraction of the total reads available for a sequencing run,  $\mathbb{T}$ . The fraction of the total reads aligned can be affected by

multiple factors, including the choice of alignment algorithm, which we do not address here. We assume that  $T$  imposes the same constraints on the data as outlined above for  $\mathbb{T}$  and will refer exclusively to  $T$  hereafter.

### 3 Sample Quality Control

Problems with sample quality, library preparation, or sequencing may result in a low number of reads allocated to a given sample within a sequencing run. The Percent Pass Filter (% PF) metric provided on Illumina sequencers provides a subjective measure that can identify problems with sequencing that result in a low number of reads allocated to a sample. However, % PF will not necessarily catch problems associated with poor sample quality or problems with sample pre-processing since these processes may affect cluster generation, and not just cluster quality. This is particularly important for extraction-free RNA-Seq technologies, such as the HTG EdgeSeq<sup>(tm)</sup>, which allow for the use of smaller input amounts but lack the intermediate steps for checking sample quality. There is currently no objective way to evaluate sample quality based on the total number of reads attributed to a sample. We propose a method for objectively identifying problematic samples based on the total number of reads allocated to the sample.

For most experimental designs we expect the number of reads allocated to each sample in a sequencing run to arise from the same general data generating mechanism, namely the chemistry of the NGS-based measurement system, regardless of experimental condition. The objective is then to determine which samples arise from a different mechanism. Outlier detection is well suited for discovering observations that deviate so much from other observations that they are likely to have arisen from a different mechanism [11]. We base our method off Tukey’s box-plots [25], which is a well used and robust method for detecting outliers [7].

We expect the total number of reads allocated to each sample,  $t_i$ , to be equivalent notwithstanding random variation. For a given sequencing run with  $D$  samples we define the vector of total reads allocated to each sample as  $\mathbf{t}$ . Since the  $D$  dimensional vector  $\mathbf{t}$  is a composition we have  $\mathbf{t} \in \mathcal{S}^{D-1}$ , the  $D-1$ -dimensional simplex. As noted above, traditional statistical methods may not be appropriate for data in the simplex. Therefore, we map  $\mathbf{t} \in \mathcal{S}^{D-1} \rightarrow \mathbf{x} = CLR(\mathbf{t}) \in \mathcal{R}^D$  using the Centered Log Ratio transformation 4. We then apply Tukey’s method for detecting outliers to  $\mathbf{x}$ , which simply identifies those observations which lie outside 1.5 times the inter-quartile range.

**Definition 1.**  $x_i$  is a quality control sample failure if  $x_i < \text{lower-quartile} - 1.5 \times \text{IQR}$  or  $x_i > \text{upper-quartile} + 1.5 \times \text{IQR}$ , where IQR is the interquartile range of  $\mathbf{x}$ .

We demonstrate the utility of our sample quality control measure using two sets of targeted RNA-Seq data: 1) 120 mRNA technical replicate universal-RNA samples prepared with the HTG EdgeSeq Immuno-Oncology assay and sequenced in 5 different equally sized runs, and 2) 105 miRNA technical replicate samples of human plasma, FFPE tissue, and Brain RNA prepared with the HTG EdgeSeq Whole Transcriptome miRNA assay. These two data sets differ in both the type of RNA (mRNA versus miRNA) and the number of sequence targets in each assay (558 versus 2,280 targets, for the mRNA and miRNA assays respectively). All samples were prepared for sequencing using the HTG EdgeSeq Processor and sequenced with an Illumina Mi-Seq sequencer.

## 4 Testing for Compositional Invariance

Normalization and standardization methods for RNA-Seq generally assume that the total number of reads assigned to a sample does not affect the observed relative frequencies of probes within an assay. For example, implicit in the CPM transformation is the idea that if you re-scale the counts (by dividing by the total for each sample) then the resulting counts are comparable and any differences are due to underlying differences in expression. Other methods which apply a scaling factor to each sample, such as Trimmed-mean of M values (TMM) or Quantile normalization, also rely on this assumption. In the parlance of compositional data these methods assume *Compositional Invariance*, i.e. the underlying composition is statistically independent of the total size of the composition (the total counts for a sample,  $t$ ).

Compositional invariance is an important property for RNA-Seq data which enables the comparison of samples with differing read depths. However, it is well documented that the quality of RNA-Seq depends on the read depth of the sequencing run with higher read-depths associated with higher quality data [23]. Read depth may affect the measurement of relative abundances for the target RNA sequences as some targets may receive proportionally more reads as the read depth increases. This would be a direct violation of compositional invariance and could lead to spurious results.

Testing for compositional invariance in high-dimensional RNA-Seq assays is important to ensure that the differences in expression among samples is independent of the differences in total counts among samples.

Aitchison [1] outlined a simple model for testing compositional covariance using the ALR transformation.

1. transform using ALR so that we can use multivariate normal methods 1b. results to not depend on choice of divisor 2. test hypothesis that  $H_0: \beta_1 = \dots = \beta_d = 0$  2b.  $P(\text{reject } H_0 \mid Y, t) \neq 0.05$  due to multiple testing so need to apply a correction 2c. Likely to have small sample size so low power to reject null i. not enough power to demonstrate equivalence ii. Compare effect sizes? i.e. is the DE more related to total than treatment? How to do this with discrete treatment groups? iii. Create a model which simultaneously estimates effects and controls for size effects?

$$[y_1 \dots y_d] = \begin{bmatrix} 1 & t \end{bmatrix} \begin{bmatrix} \alpha_1 & \dots & \alpha_d \\ \beta_1 & \dots & \beta_d \end{bmatrix} + [e_1 \dots e_d] \quad (6)$$

## 5 Batch Effects and Normalization

Batch effects arising from differing laboratory conditions or operator differences have been identified as a problem in high-throughput measurement systems [13, 9]. Identifying and controlling for batch effects is a critical step in the transition of RNA-Seq from the lab to the clinic. Batch effects are typically identified with a hierarchical clustering (HC) method or principal components analysis (PCA). For both methods, the multivariate distance between the samples is visualized, either in a biplot for PCA or a dendrogram for HC, to check for the existence of clusters of samples related to batch. The compositional nature of RNA-Seq data has important implications for the detection of batch effects because of the difficulty of interpreting the covariance matrix [1] and the incompatibility with standard measures of distance between compositions as noted above [1, 18].

The next generation sequencing process results in arbitrary differences in scale among samples as some samples will receive more total reads than others. Principle components analysis is sensitive to differences in scale among the variables, failure to remove these difference can mask potential batch effects and leave unwanted technical variation in the data. Most normalization methods use a scaling factor calculated for each sample to rescale the read count for each gene within the sample [10]. The CLR transformation can also be viewed as a scaling factor (the inverse of the geometric mean  $1/g(x)$ ), which is multiplied to each gene read count in the sample. Unlike other normalization methods, the CLR transformation has the added benefit of being applied at the individual sample level, not experiment wise. This makes it particularly suited for the



clinic where there are generally no reference samples to normalize to.

Aitchison demonstrated that the CLR transformation has several other useful properties in addition to rescaling the data [1], particularly with respect to PCA biplots [5]. Most notably for the detection of batch effects, the distance between any two points representing samples in the form-biplot approximates the Euclidean distance between the two samples. The CLR transformation retains the property that this distance is at least as great as the distance between any corresponding subset of these two compositions (subspace dominance). Other scaling methods do not necessarily satisfy this property when used with compositions, and therefore batch effects may be masked.

We demonstrate the use of the compositional biplot to detect batch effects using technical replicates of three sample types: brain, plasma, and fresh frozen paraffin embedded (FFPE). Each sample is replicated 8 times in each of 5 sequencing runs for a total of 120 samples. Samples were prepared using the EdgeSeq Whole Transcriptome miRNA assay which measures 2,280 targets including including 11 control probes and 2,269 unique miRNA probes. All sequencing was performed on an Illumina Mi-seq<sup>(tm)</sup> sequencer.

We create a second data set, by re-scaling the original data, to better illustrate the effects of changes in read depth on batch effect detection. To re-scale the samples from the original we multiply every read count in a given sample by a factor, ranging from 0.5 to 1.5, randomly generated from the uniform distribution. We then obtain a new data set in which the proportions between the read counts remains unchanged but the variance in the total number of reads among the samples is increased.

## 6 Results

### 6.1 Sample Quality Control

We compare the utility of our method to evaluation of the untransformed total counts. Figure 1 shows a boxplot and heatmap of the total number of reads allocated to each sample for each of 5 sequencing runs. Figure 2 shows the same data after CLR transformation. After transformation the poor samples become much more visually evident in the heat maps. Additionally, the ability to detect outlying values increases and the number of poor samples detected increases from 1 to 6.

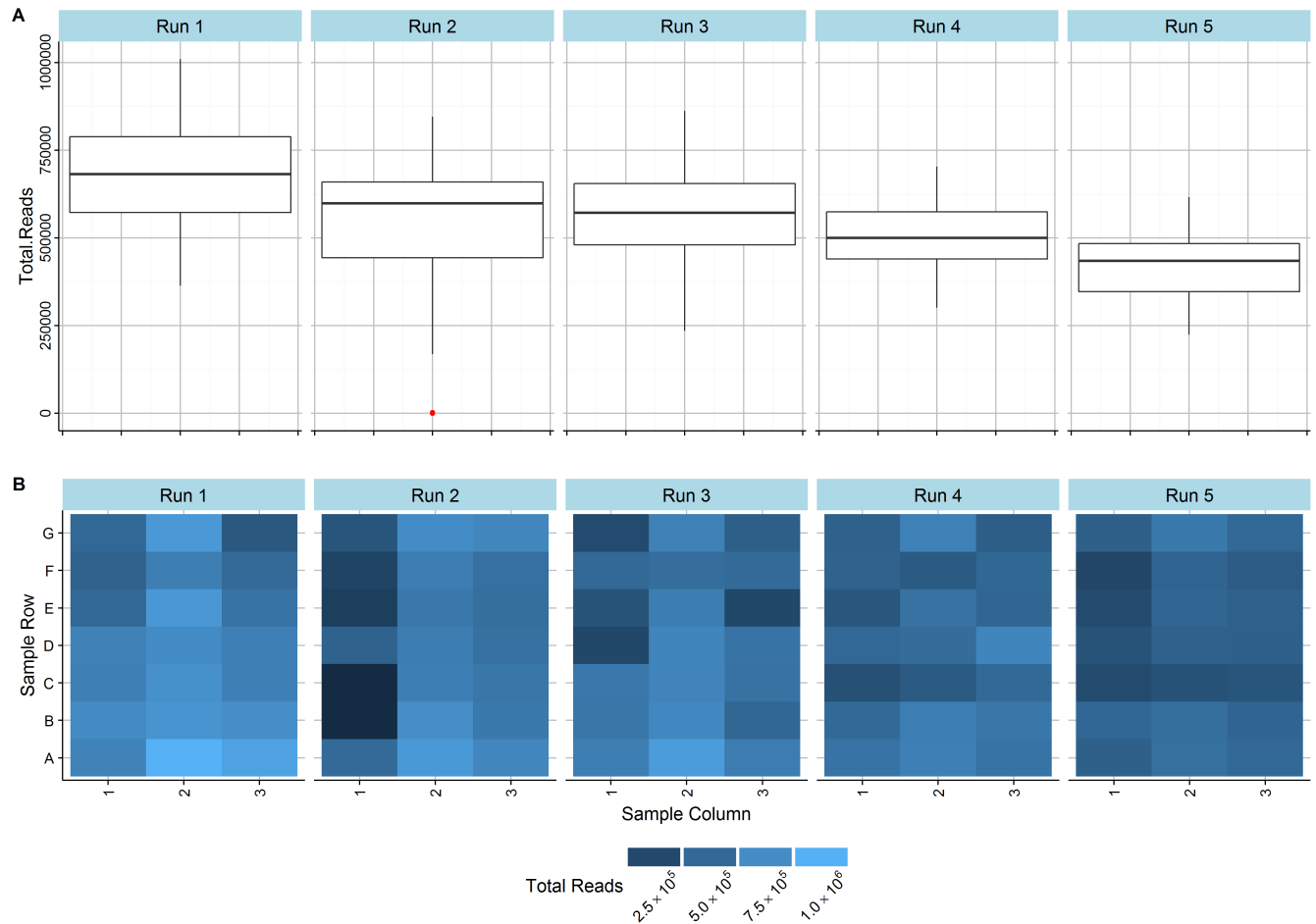


Figure 1: A) Distributions of total reads allocated to each sample in 5 runs on an Illumina Mi-Seq sequencer. Only 1 sample is identified as a problematic sample. B) Heatmaps showing the relative totals for each sample within each run. The darker heatmaps for runs 4 and 5 reflect the generally lower number of total reads in those sequencing runs as compared to runs 1 and 2. This is caused by normal variation in the number of reads available in a sequencing run.

how would we demonstrate the increase in power mathematically?

## 6.2 Batch Effects and Normalization

We perform a PCA on log-transformed and CLR transformed data. We then construct form-biplots of the first two principle components for each transformed data set (Fig. 6.2). The differences between the 3 samples types (brain, plasma, and FFPE) dominate the first two principle componenets for both data sets. However, the CLR transformed data provides tighter clusters, relative to the distance between the clusters, than the log-transformed raw data. There is also a single FFPE sample which is closer to the brain samples

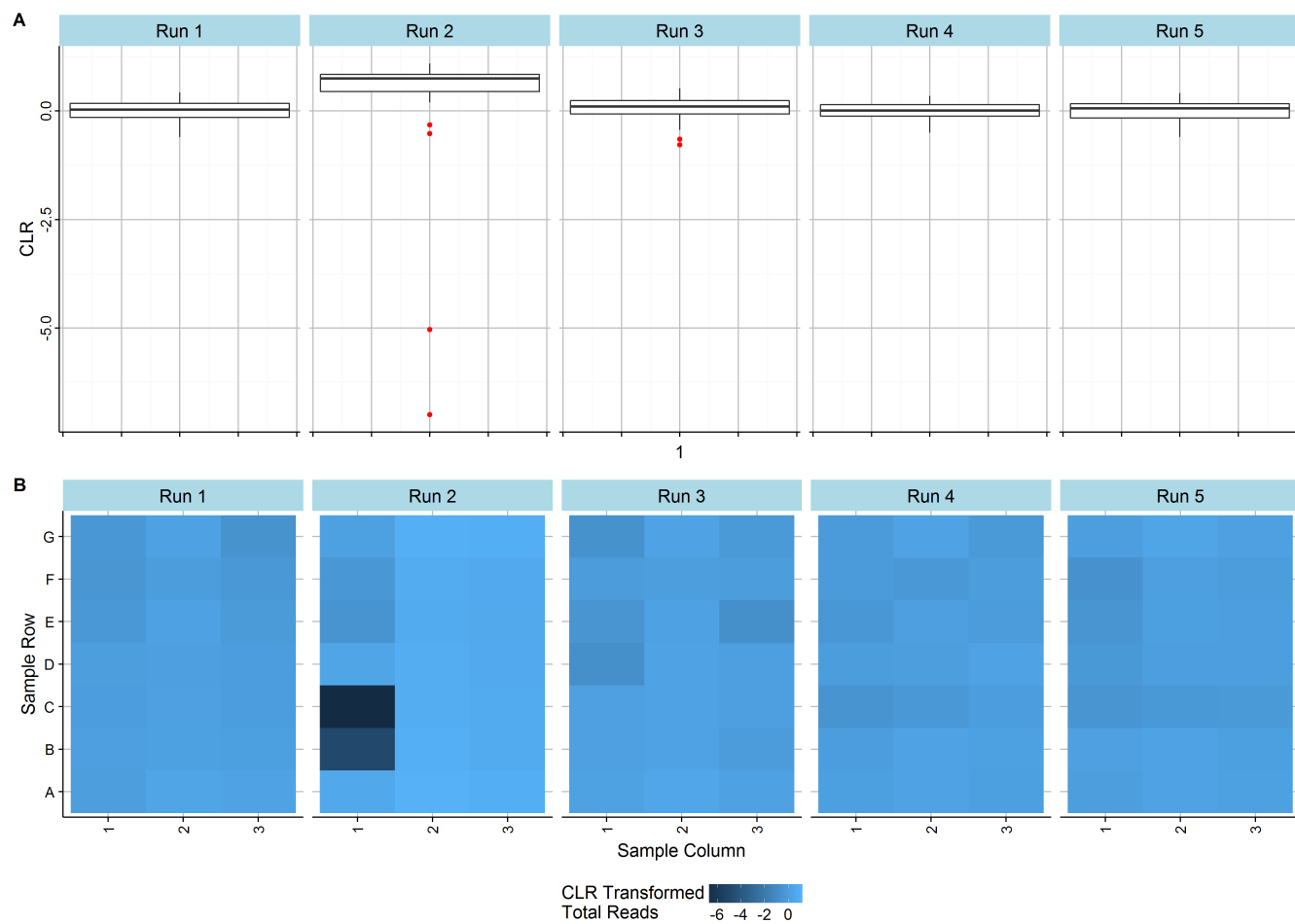


Figure 2: A) Distributions of CLR tranformed total reads allocated to each sample in 5 runs on an Illumina Mi-Seq sequencer. After CLR transformation, 6 samples are identified as a problematic. B) Heatmaps showing the relative CLR transformed totals for each sample within each run.

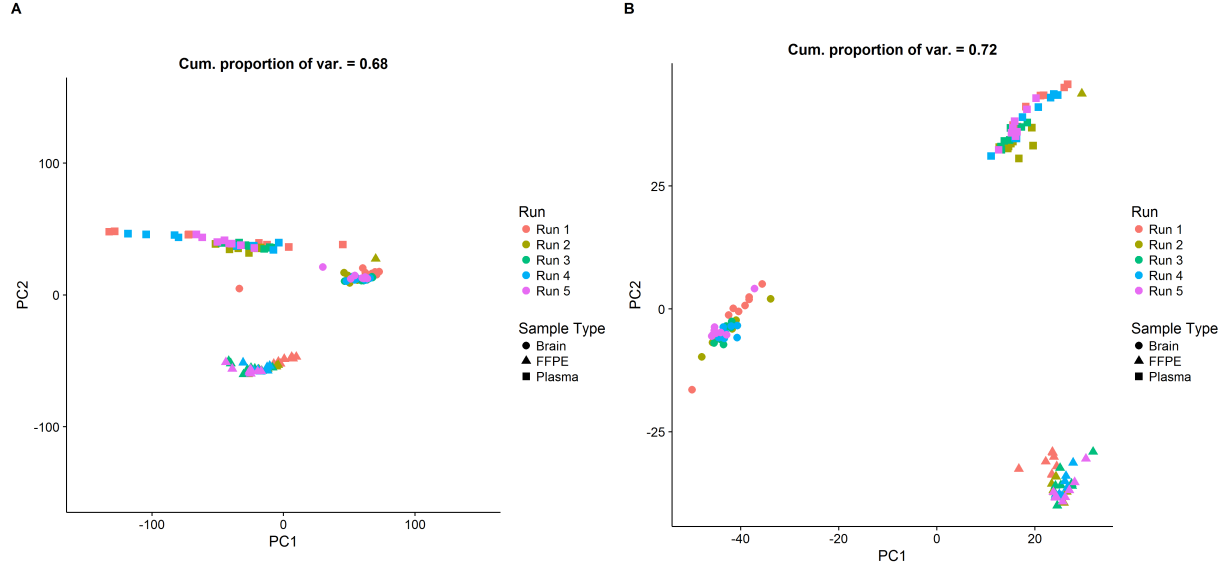


Figure 3: Principle component analysis of A) log-transformed and B) CLR-transformed read count data. The differences between sample types is much greater than the batch effects in both transformation. The CLR transformation results in tighter sample type clusters resulting from less variation along the first principle component.

than the other samples. It is worth noting that this sample would have been removed using our proposed quality control metric.

Since the sample type differences overwhelm the potential batch effects we performed a second PCA on only the brain samples for both transformed data sets (Fig. 6.2). Both biplots exhibit clustering by batch but the CLR transformed data shows better separation between the batches, although batches are still overlapping.

Some of the batch effects detected in the log-transformed data may be attributable to the differences in total reads between batches. By randomly re-scaling each sample by a constant we are able to break the relationship between batch and the total reads in a sample. Figure ?? gives the biplots for log-transformed and CLR-transformed randomly re-scaled data. The sample type clusters in the log-transformed data become more diffuse while the CLR-transformed biplot remains unchanged. Most notably, the batch effects previously visible in the log-transformed brain samples become completely obscured in the randomly re-scaled data but remain unchanged in the CLR-transformed data (Fig. 6.2).

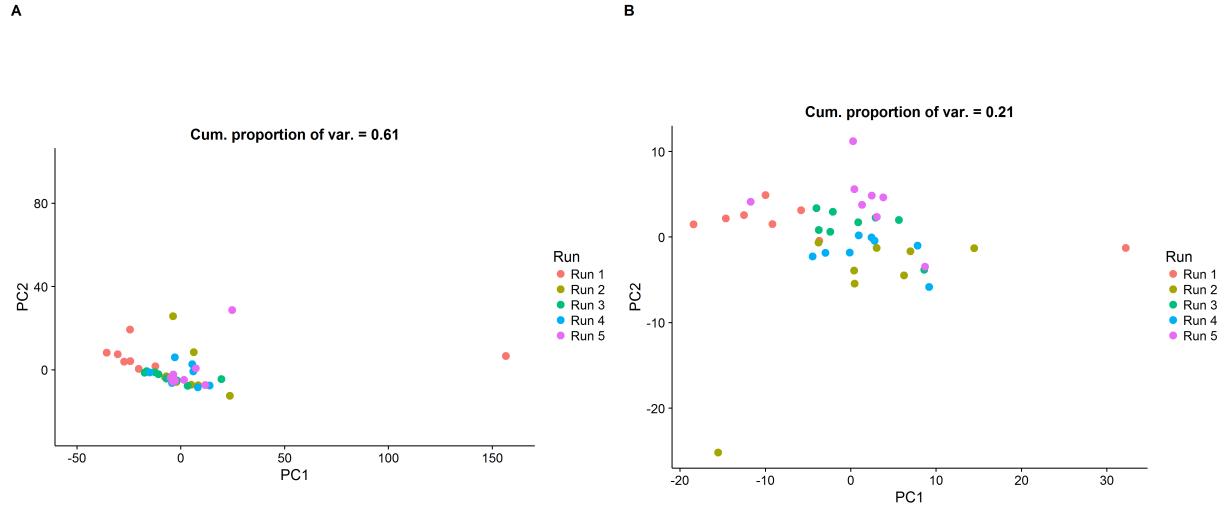


Figure 4: Principle component analysis of only brain samples from A) log-transformed and B) CLR-transformed read count data. The batch effects are more easily identified in the CLR transformed data.

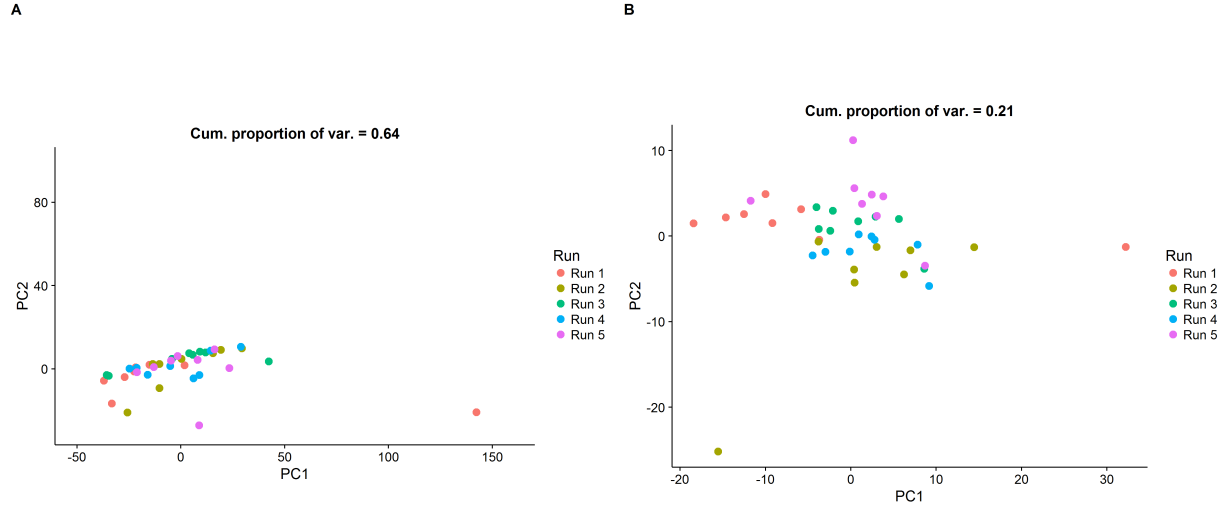


Figure 5: Principle components analysis of the randomly re-scaled brain samples for A) log-transformed and B) CLR-transformed read count data. The batch effects visual in the log-transformed raw data disappear after random re-scaling whereas the batch effects remain identifiable in the CLR transformed data.

## 7 Discussion

Our sample quality control metric can identify problematic samples which arise from multiple failure modes, e.g. a low quality sample or a sequencing problem. However, it is conceivable that a sample might have an unusually low (or high) number of reads and still provide quality information. In certain experimental designs one might be able to further evaluate these samples with a PCA biplot on the CLR transformed data. In our PCA analysis we identified a FFPE sample which would have failed our quality control and was clearly very different from the other technical replicates. However, if this sample had remained quite similar to the other FFPE replicates this would have provided information that the sample may still be valuable. In this way, the quality control metric and PCA biplot can be used in tandem to provide additional information about the quality of a sample.

The principal components analysis biplot is a well know dimension reduction visualization. For the current data the dimension is reduced from 2,280 probes to 2 principle components. The utility of the data reduction, including the quality of the approximation of the multivariate distance between the samples, is proportional to the amount of variance explained by these two principle components. In our data the first two principle components explain between 72 and 21 percent of the variation in the data. The analysis with the lowest percent of variation explained by the first 2 components is of the CLR-transformed brain samples. Surprisingly, batch effects are still visible in this plot, in which case they can be removed [16].

As RNA-Seq makes the transition from the research laboratory to the clinic there is a need for robust quality control metrics. The realization that RNA-Seq data are compositional opens the door to the existing body of theory and methods developed by John Atchison and others. We show that the properties of compositional data can be leveraged to develop new metrics and enhance existing methods.

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