EPIG-Seq: Extracting Patterns and Identifying Co-expressed Genes from RNA-Seq Data

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Abstract

RNA-sequencing (RNA-seq) provides genome-wide representation of gene expression. RNA-Seq data is count-based rendering many normal distribution models inappropriate. We adapted our extracting patterns and identifying co-expressed genes methodology for RNA-seq (EPIG-Seq). To identify patterns, a count-based correlation measures similarity between expression profiles, a quasibinomial model estimates dispersion and the Wilcoxon rank sum Z-statistic indicates the magnitude of differential expression. EPIG-Seq categorizes genes to the patterns that they correlation with. Using simulated data, we show that EPIG-Seq is superior to EPIG and the CLICK clustering algorithm when the data is transformed by popular RNA-Seq normalization methods and performs equally as well as other pattern analysis methods when count data is used. We applied EPIG-Seq to toxicogenomics and cancer datasets to identify co-expressed genes related to the underlying biology of the conditions. EPIG-Seq is available at: www.niehs.nih.gov/research/resources/software/biostatistics/epig-seq.

Key words: gene expression, RNA-seq, clustering, pattern analysis, toxicogenomics, EPIG

Introduction

The advantages of RNA-sequencing (RNA-Seq) over microarray to measure gene expression have been widely reported (refs). Recently, methods have been developed to analyze RNA-Seq data based on normalization of the read counts or using the raw count data (refs). The former has advantages in that it adjusts the data according to sequencing library size (read depth), accounts for the length of transcripts and allows the use of analysis tool designed specifically for microarray data. However, normalized RNA-Seq data has limitations which can adversely impact the analysis (inflated variation among replicates, zeros in the data, low and highly expressed genes squelching, etc.) and there is no clear consensus of which normalization method is best (ref). On the other hand, using the raw read counts avoids the shortcomings of normalization but requires modeling of the data to estimate overdisperion, accounting for the read depth and filtering to avoid cases of inflated zeros. In addition, statistical models of count data based on Poisson or negative-binomial distributions can be severely impacted by outliers in the data although recently developed approaches overcomes this pitfall by analyzing the data with a log-linear model or Wicoxon statistic and resampling in a non-parametric manner (Biostatistics. 2012 Jul;13(3):523-38. doi: 10.1093/biostatistics/kxr031, SAMSeq: Stat Methods Med Res. 2013 Oct;22(5):519-36. doi: 10.1177/0962280211428386). Unfortunately, there is a paucity of methodologies that can identify correlated gene expression patterns from RNA-Seq count data across biological conditions (i.e., time course, dose response, multiclass study designs).

Extracting patterns and identifying co-expressed genes (EPIG) was designed for microarray data to detecting genes with similar expression profiles across biological conditions (ref). EPIG uses Pearson correlation to group genes by similarity of expression across treatment groups, a signal-to-noise ratio (SNR) to compare the level of a genes expression to background noise and magnitude of change to assess the level of differential gene expression between test samples and controls/baseline. Normalizing RNA-Seq data to transform it into microarray gene expression space permits it to be analyzed by EPIG but comes with the aforementioned caveats.

We adapted the EPIG methodology for the identification of co-expressed genes from RNA-Seq data (EPIG-Seq). Patterns of gene expression across experimental groups are determined using a similarity measure for count data (ref) to ascertain similarity between expression profiles, a quasibinomial model (ref) to estimate overdispersion in the data and the Wilcoxon rank sum Z-statistic (ref) as a measure of the magnitude of difference between test samples and control/baseline. EPIG-Seq then categorizes each gene expression profile to the pattern for which it has the highest correlation. The EPIG-Seq approach is impervious to variations in read depths, inflated zeros, outliers in the data and overdispersion. Using simulated data, we show that EPIG-Seq is superior to EPIG and the CLICK clustering algorithm when the data is transformed by popular RNA-Seq normalization methods and performs equally as well as other pattern analysis methods when count data is used. We also apply EPIG-Seq to a publicly available toxicogenomics data set and The Cancer Genome Atlas (TCGA) breast cancer RNA-seq data. We identify several co-expressed genes related to modes of action of the chemical agents in the toxicogenomics project; we also determine genes that are associated with breast cancers subtypes classifications (ref) and some actively involved in crucial pathways (ref).

**Methods and Materials**

**Data**

**TCGA breast cancer RNA-Seq data**

The count-level breast cancer RNA-Seq data ([2012](#_ENREF_1)) from The Cancer Genome Altas (TCGA) was downloaded from NCI portal: <https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm>. This is part of the comprehensive cancer research cross many different types of cancer, and it involves different research platforms, i.e. mRNA expression, DNA methylation, DNA copy number, etc. We focused on the mRNAseq data produced on Illumina GAII. Raw sequences were analyzed by the TCGA team with SeqWare <https://seqware.github.io/> (Version=0.7.0) using RNASeqAlignmentBWAWorfklow (Version=0.7.10) and RNASeqQuantificationWorfklow (Version=0.7.2). Tumor samples were classified by the mRNA subtypes ([Perou, Sorlie et al. 2000](#_ENREF_6); [Sorlie, Perou et al. 2001](#_ENREF_8); [Parker, Mullins et al. 2009](#_ENREF_5)). In our study, we only used data of the following four subtypes with enough replicates: luminal A, luminal B, Her2-enriched, and basal-like. To test the robustness of our algorithm and further explore the biological implication, we randomly sampled 10 lanes from each tumor subtypes plus 10 lanes from normal samples and constructed four such balanced data-matrices for this research.

**Simulated RNA-Seq data**

To simulate RNA-Seq count data for each *k*th pattern where k{1,…,K}, we adopted and extended a method published previously ([Soneson and Delorenzi 2013](#_ENREF_7)). Briefly, the count was simulated based on a negative binomial distribution with dispersion parameter estimated from a known data set. Let denote the count of a gene *g* {1,…,*G*}, belonging to a group *m* {1,…,*M*} of a sample *s*{S1, S2,…,SN} such that:



~NB (mean = , var= ( 1 + ))



Where, is the parameter measuring the dispersion in the data and is the true mean of the data. The dispersion parameter was estimated from the aforementioned TCGA cancer data and was set to be the same for all samples within a group. Thus, = . Here,



where, is the sequencing depth for the *m*th group in sample *s*. = 107for ~Unif[0.7,1.4]. The estimation of the sample mean and are obtained from our aforementioned sampled RNA-Seq raw count carried out with DEseq ([Anders and Huber 2010](#_ENREF_2)). Then, to generate simulated patterns of genes across groups of samples, we defined, where S1 is the control group of samples, Sm is a group of samples with a particular phenotype, and



.



The parameter denotes the lower bound of differential expression for the *g*th gene between Sm and S1; it was set at different levels for the respective groups. We let denote the set of genes that are up-regulated and denote the set of genes that are down-regulated.



**Toxicogenomics RNA-Seq data**

RNA-Seq data from the MicroArray Quality Control phase III (Sequence Quality Control (SEQC)) crowd source toxicogenomics (TGxSEQC) effort (ref) is available in the Sequence Read Archive (SRA) under accession number SRP024314. We used the training set data containing RNA-seq data from the livers of male Sprague-Dawley rats exposed to one of 15 chemicals or vehicle and route matched controls. Sets of three chemicals share one of five modes of action (MOA). Three MOAs are associated with well-defined receptor-mediated processes—peroxisome proliferator-activated receptor alpha (PPARA), orphan nuclear hormone receptors (CAR/PXR) and aryl hydrocarbon receptor (AhR). The other two are non-receptor-mediated—DNA damage (DNA\_Damage) or cytotoxicity (Cytotoxic). The data is comprised of paired-end 100 bp reads from Illumina HiScanSQ or HiSeq2000 systems with depths between 23 – 25 million reads. Specific details of the study design and sample collection are available in the TGxSEQC publication (ref). For each sample, a two-step alignment was performed using the fastq files. In the first step, raw reads were aligned with Novoalign v2.08.01 (www.novocraft.com) against rat genome rn4 downloaded from the UCSC ftp server (ftp://hgdownload.cse.ucsc.edu/goldenPath/rn4). The intermediate bam files generated with Novoalign were then parsed through customized scripts to summarize mapping results. Unmapped reads were passed to Novoalign again and were mapped to the rat RefSeq transcriptome gene model (release version 52, March 5, 2012) downloaded from the NCBI ftp server (ftp://ftp.ncbi.nih.gov/refseq). The alignment results were parsed through customized scripts to summarize mapping results and then merged with the results generated in step one.

**EPIG-Seq**

Pattern extraction

**Correlation**

A compiled RNA-seq gene expression dataset consists of a 2-dimensional matrix, in which each row represents a gene expression profile and each column represents a sample. We denote x*ij* as the count of reads from sample *j* mapped to a gene *i* and *xkj* as the count of reads from sample *j* mapped to a gene *k*. To measure the count level correlation between two gene profiles, we adopted the similarity measure for count data as defined by Cao et al. ([Cao, Williams et al. 1997](#_ENREF_3)){Cao, 1997 #20} is:



where



and *a* is the total number of samples with read counts mapped to either profile. *CYd* is maximized by assigning *xij* = 1 and *xkj* = *Dk* for *a*/2 times and likewise *xkj* = 1 and *xij* = *Di* *a*/2 times. Here

, for *xij* ≥ 1 and *xkj* ≥ 1. Thus, maximum *CYd* = *D1* + *D2* + *D3*, where



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*b* is the total number of samples with read counts present in profile *i* only and *c* is the total number of samples with read counts present in profile *k* only. As such,= 0 when two profiles are totally difference and = 1 when the two are identical.



**Magnitude of change**

We define the strength of a gene expression profile’s signal according to the value of the test-statistic obtained from a Wilcoxon rank sum non-parametric test ([Hollander 2013](#_ENREF_4)) measuring the difference between the ranks of the expression of the genes in sample *X* vs those in sample *Y*. Here, sample *X* is the biological replicates from the treated, perturbed or diseased group and sample *Y* is the biological replicates from the controls. The *g*th gene expression profile’s signal is therefore:



When the sample size for each group is small (i.e., ≤ 30), the estimation of the Z-statistic from the Wilcoxon rank sum test can be spurious. In such a case, we default to measure the strength of the *g*th gene’s differential expression according to the value of the Hodges-Lehmann estimator for the difference between two groups([Hollander 2013](#_ENREF_4)). Briefly, is the median of the Walsh averages, the average of all possible pairs of differences between the ranks of the *g*th gene RNA-Seq counts in sample X vs the ranks of the RNA-Seq counts in sample Y. Thus, for a set of N paired differences observations, there will be N(N+1)/2 Walsh averages. The median of all the Walsh averages is equal to. Hence,



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**Dispersion**

Count data is known to be dispersed (ref). The variance-to-mean ratio (*VMR*) is a measure of dispersion () and is the inverse of signal to noise (SNR). If is larger than 1, a dataset is said to be overdispersed. For each *g*th gene expression profile, we estimate using a quasi-Poisson regression to model the data. For Poisson distributed data, the variance is equal to the mean, i.e., V(Yg) = E(Yg) = *g*. The quasi-Poisson likelihood model is commonly used for overdispersed count data as it incorporates the dispersion parameter into the Poisson model (McCullagh and Nelder 1989 generalized linear models). In doing so, the variance of the response (Yg) is a linear function of the mean, V(Yg) = and dispersion estimated as



where *n* is the sample size, *c* is the number of estimated parameters andis an inverse function of the linear predictors. Here, the inverse function is a “log” link in the form of a generalized linear model:



, where for the *g*th gene expression profile in the *j*th sample, *Y* is the read count, *X* is the independent variable and ** is the random error term. In our study, one set of dispersion parameters were estimated from the TCGA data and was used in simulating the research data. In general modeling, dispersion was often estimated from the aforementioned Quasi-Poisson likelihood model for individual profile.



Categorization of gene expression profiles to patterns

Once the patterns have been extracted, the measure is used to correlate the *i*th profile to the *k*th pattern, which is often represented by the profile initialized as the seed. The profile is assigned to the pattern to which it has the highest similarity to. Once all the profiles are assigned, a representative profile for each of the patterns is determined by the highest average correlation to the other profiles in the pattern. Briefly, for the *i*th gene expression profile and for the *k*th pattern it is assigned to, a Pattern Correlation Score



is computed as the sum of the correlations among the *i*th profile (*xi*) to all other profiles (*xj*) assigned to pattern *k* (*Pk*) divided by the size of the pattern (i.e., the number of profiles in the pattern minus 1). Until no more profiles are reassigned, the measure is used to correlate the *i*th profile to the *k*th pattern and assign it to the pattern with which it has the highest correlation unless the profile exhibits low or high dispersion or a small magnitude of change.



Assessing the significance of extracted pattern

To assess the significance for a pattern extracted in the research, we adopted an empirical bootstrapping-like approach. Basically, for a given pattern contains k gene profiles, we randomly select k number of gene profiles from the initial whole dataset. Then for the selected k profiles, we compute PCS for each gene profile against the remainder k-1 profiles. Then we store the average PCS as the normalized measurement for this round of sampling. We shall empirically perform such practice for 10000 times, in the end we retained 10000 average PCSs from the randomly sampled “patterns”. A nominal p-value for the real average PCS score from the “real” pattern was defined as the probability getting it by chance.

Assessing the pattern extraction performance across replicated passes

To assess the reproducibility of pattern extraction performance, Mutual Information (MI) between two random variables, which is dimensionless and with log base 2 and interpreted in units of bits (ref??) was used. MI in terms of comparing two clustering outcomes (i.e., A and B), is the reduction in uncertainty about the cluster groupings in one outcome given knowledge of another (ref). A high MI value indicates that the two clusterings are similar where zero MI between the two cluster outcomes means that they are independent. Normalized MI = MI/(Entropy\_A+EntropyB)/2. NMI ranges between 0 and 1.

The EPIG-Seq algorithm pseudo code

**Extract patterns**

1. Define the parameter set θ for pattern extracting;
2. **for** i = 1..n **do**

**for** j = i +1..n **do**

Calculate all pairwise CYs correlation

Perform Wilcoxon Rank sum test

Model data to estimate dispersion

Categorize profiles and assign to seeded-clusters (patterns)

**end**

**end**

1. Tally candidate patterns against the parameter threshold
   1. Remove patterns with similar correlation (>=0.8)
   2. Delete the patterns failed to pass the parameter
   3. Delete the profiles failed to pass the threshold

**Categorize profiles to patterns**

1. Define the parameter set θ for categorization;
2. **for** i = 1..k **do**

Use extracted patterns with the seeded representing profile

Correlate profiles to patterns using CYs similarity

Update patterns with profiles with the highest correlation

Until no more moves (i.e., if 1-NMI < 0.001)

**end**

1. Report the PCS for each pattern

**Results**

**A co-expressed profile pattern extraction platform is established**

In contrast to common analysis strategies for genome level monitoring of responses from experimental specimens at different treatment conditions or perturbation stresses, we explored a route to reveal the hidden mechanism through assessing the systematic behavior crossing across multiple conditions. Our hypothesis is that a significant profile/pattern supported by a group of co-expressed genes could serve as a key signature of the responses to outside exposures. As shown in table 1, four methods were showed side by side. our newly proposed EPIG-Seq method, although shares some similarities with other highly-remarked methods, stands out by itself with its special advantages, not only provides researcher a new approach for monitoring the systematic responses across multiple conditions, it also avoids unjustified data pre-processing and normalization procedures.

**Overall work flow of EPIG-Seq algorithm**

In test our overall research hypothesis, we implemented such algorithm and integrated series of steps into a well-tuned work-flow as figure1 shows, we have successfully established a framework to extract expression patterns, which often include co-expressed genes from an RNA-seq experiment with multiple conditions. Along the pipeline workflow, a novel similarity and dissimilarity method was adopted to compute the CYs, which range between 0 and 1, across all available gene profiles. By consulting the *a priori* defined parameter set, seeded patterns emerged in the right next step. At this stage, each seeded pattern is supported by one representing profile, which ranked on the top according the metrics thresholds. The following step is to categorize all profiles into the seeded patterns. During this step, the number of co-expressed genes for each seeded pattern starts to grow and the process is getting more complicated and the speed starts to slow down as more and more gene profiles are added. Basically, for each gene profile, it will get an opportunity to be tested against all available patterns. We introduce a new metric, Pattern Correlation Score (PCS), which was computed against each seeded pattern. The candidate gene profile will be assigned to a pattern where the highest PCS was determined comparing to those computed against other patterns; or dropped if none of the PCS passed the threshold.

The last step in the workflow before a thumbnail plot and individual plots are produced is to apply statistical significance as final assessment for the results. To get such a significance measure, we used the p-value obtained from an empirical bootstrapping like approach.

**Dissimilarity and similarity measurement for correlation of profiles (need to run Matlab to get CYs comparison on simulated data)**

The main motivation for the EPIG-seq method is to extend the existing EPIG to handle the RNA-Seq data when the count level measurement is used and common normalization procedure is hard to be justified. In the EPIG-Seq, we adopted CY as the dissimilarity and similarity measurement, which provides us the viable assessment and basis to seed the significant pattern(s) and to cluster profiles to their belonging patterns.

**Assessing the robustness of the EPIG-seq method with simulated RNA-Seq data**

To illustrate the robustness of the EPIG-seq method, we simulated RNA-seq count data cross four conditions, namely four groups. Overall six patterns were simulated (figure xx) with the main parameters setting shown in table xx. The simulation was done based on a negative binomial model with a single same dispersion, which was empirically estimated from the TCGA breast cancer RNA-seq data. The mean varies for each group at either upper or lower level so that an overall pattern is created crossing all four groups. For each group, 35 lanes of data were simulated; then, we set *G* = 200 for each *k*th pattern, resulting in a total of 1000 simulated genes for the first five patterns, and 19000 genes for the last pattern. As shown in figure xx, the first pattern reflected a monotone increase in “expression level”; the second pattern reflected a monotone decrease in “expression level”. Pattern 3 and 4 showed the elevated “expression” at different group by setting the largest mean for one of the middle group. Pattern 5 had a dramatic increase at group2 then it leveled off throughout the remaining groups. Pattern 6 was specifically designed to challenge the EPIG-seq algorithm, where no real signal existed. In addition, the last pattern had the most number of “gene profiles” (19000), therefore it basically served as the background noises. If the algorithm is robust enough, it shall be able to extract meaningful patterns from the background noise. At the same time, it should not extract the last pattern based on the pre-defined parameter thresholds.

As seen in figure xxx, through the EPIG-seq processing, all five patterns were successfully extracted; no pattern was reported that matched our “noise pattern”, pattern6. In the figure showed, samples belong to different groups were color coded with: group1 in red, group2 in green; group3 in blue and group4 in purple. Pattern 1 had ten gene profiles; pattern 2 had eight gene profiles; pattern 3 had 13 gene profiles; pattern 4 had 19 gene profiles and pattern 5 had four probes. Within each group, although the expected mean under a condition in a given pattern was defined at the simulation, we did observe clear oscillation of replicated sample points owing to the impact imposed by dispersion in the model. Regardless, neither the artificially introduced noises or the dispersion in data prevented the EPIG-Seq from recovering all the simulated patterns with pvalue fall between [**some type of range..]**

As part of the confirmation of the validity using the CY as the dissimilarity and similarity measurement, we performed PCA analysis on both the whole simulated data with 20000 genes and genes existed only in the reported patterns. To perform the PCA analysis, we used the “correlation matrix”, which consisted in reality of all pair-wise CYs. Despite that the main parameters varied among different simulated patterns, the overall 140 samples belong to four different groups tended to cluster together in a 3D plot of the first three PCs (figure xx). The other similar PCA plot, which was obtained from the analysis, only based on the extracted pattern simulated profiles (figure xx), showed clear separation of four different groups. Noticeably, the first three PCs explained almost 90% of the total variability in the data, which sufficient captured the variability in the data. This delivered a much stronger confirmation and ensured the validity of separation of groups showed in the PCA plot. A hierarchical plot, which was obtained from genes selected in the extracted pattern, was also showed (figure xx). Clear separation and clustering reflected the differences in the expected sample means within a sample group. The expression level was reflected in the conventional green-red color pallet, and similar expression across profiles within the simulated pattern was also clearly recovered and revealed.

**Comparing against available methods EPIG, ORIGEN, DEseq and SAMSeq**

To show the validity of the newly proposed EPIG-Seq method, we compared two highly regarded count level RNA-Seq analysis method: DEseq and SAMseq; we also compared the analysis with the EPIG on the RPM normalized data and ORIGEN on the squared rooted count data. To better address the advantages and disadvantages, all the comparison was performed on the aforementioned simulated dataset with known expected expression values. In the DEseq comparison, we followed author’s suggestion (ref) using “pooled” method to estimate the dispersion while using the locfit package to fit a dispersion-mean relation as described in the paper (ref). To get the “differentially expressed genes” (DEGs), we used a negative binomial test between each “experimental” group and the “basal” line. In other words, groups 2 – 4 were each compared to “group 1”; and DEGs were extracted with adjusted p-value < 0.05 and fold change requirement was at 1.5, 2.5, and 4 for the corresponding comparison respectively. In the end, we got 1130, 1006, and 758 DEGS from those three comparisons. Not surprisingly, all 758 DEGs from the comparison between group 4 and group 1 were the subset of 1006, which was got from the comparison between group 3 and group 1. Owing the fold change requirement set for comparison between group 2 and group1, the test results were a little inflated with approximate 10% as the falsely discovered. In addition to those falsely discovered DEGs, there were 96 DEGs pulled from the comparison between group3 and group1 were not included in the set between group2 and group1. On the other hand, about another 100 DEGs from comparison between group2 and group1 were not recovered in comparison between group3 and group1. Although, DEseq performed reasonably well in term of recovering the known DEGs, obviously, the co-expressed genes that primarily make the pattern 3 & 4 were easily missed with the pair-wise comparisons.

SAMseq (ref) is anther efficient non-parametric testing methods extended from its predecessor SAM (ref) to directly apply to count level RNA-seq data. We compared the analysis with SAMseq using the mutli-class comparison with default parameter setting at FDR = 0.20. As expected, SAMseq worked very efficiently and reported 1256 DEGs including all those 1000 simulated genes that made up the first five informative patterns. Not surprisingly, it included 256 false discovered DEGs, which is a little more than the claimed false discovery rate (20%). When we lowered the FDR to 0.05, SAMseq reported 1035 DEGs (data now shown), and it again included all those 1000 simulated genes that made up the first five informative patterns. When we re-visited the method comparison metrics (table xx), SAMseq used similar approaches in its main statistical assumption, except the CYs as the correlation measurement in. It also is also proved very efficient on our simulated RNA-seq data.

EPIG performed reasonably well (figure xx) with RPM normalized count data. It was able to recovered the five main simulated patterns, however the patterns tended to contain noisy gene profiles, which were known not belonging to the patterns. i.e. the last pattern had 96 noise genes categorized, which was an FDR of 23.4%. ORIGEN was also able to recovere the five known patterns (figure xx), but it also reported many more sub-patterns, which were not simulated. (enough **Comparing to EPIG or ORIGEN??)**

**EPIG-Seq on TCGA breast cancer study (need the results from the actual runs)**

[Need NMI measurement and biological retuls

**Discussion**

In contrast to common analysis strategies for genome level monitoring of responses from experimental specimens at different treatment conditions or perturbation stresses, we explored a route to reveal the hidden mechanism through assessing the systematic behavior crossing across multiple conditions. Our hypothesis is that a significant profile/pattern supported by a group of co-expressed genes could serve as a key signature of the responses to outside exposures. As shown in table 1, our newly proposed EPIG-Seq method, although shares some similarities with other highly-remarked methods, stands out by itself with its special advantages, not only provides researcher a new approach for monitoring the systematic responses across multiple conditions, it also avoids unjustified data pre-processing and normalization procedures.

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