Hierarchical clustering of DNA k-mer counts in RNA-seq Fastq files reveals batch-effects

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1 Abstract

Background: Batch effects, artificial sources of variation due to experimental design, are a widespread phenomenon in high-throughput data. Therefore, mechanisms for detection of batch effects are needed requiring comparison of multiple samples. We apply hierarchical clustering (HC) on DNA k-mer counts of multiple RNAseq derived Fastq files. Ideally, HC generated trees reflect experimental treatment groups and thus may indicate experimental effects, but clustering of preparation groups indicates the presence of batch effects. In order to provide a simple applicable tool we implemented sequential analysis of Fastq reads with low memory usage in an R package (seqTools) available on Bioconductor. DNA k-mer counts were analysed on 61 Fastq files containing RNAseq data from two cell types (dermal fibroblasts and Jurkat cells) sequenced on 8 different Illumina Flowcells. Results: Pairwise comparison of all Flowcells with hierarchical clustering revealed strong Flowcell based tree separation in 6 (21 %) and detectable Flowcell based clustering in 17 (60.7 %) of 28 Flowcell comparisons. In our samples, batch effects were also present in reads mapped to the human genome. Filtering reads for high quality (Phred >30) did not remove the batch effects.

Conclusions Hierarchical clustering of DNA k-mer counts provides a quality criterion and an unspecific diagnostic tool for RNAseq experiments.
2 Introduction

In hierarchical clustering (HC), different entities are located in bi-parting trees according to their pairwise similarity (quantified by a distance measure). Although the trees provide no absolute measure, accumulation of biological or technical related samples in different sub-trees indicates sample similarities which possibly influence downstream analysis. The interpretation of dendrograms requires (at least to some degree) subjective validation.

DNA k-mer’s are short sequence patterns in DNA sequence of length $k$. Counting of DNA k-mer’s in Fastq files results in count vectors of length $4^k$ to which distance measures (for example the Canberra distance) can be applied.

Transcriptome sequencing data is prone to disturbing effects resulting from experimental design for example ozone levels [1], random hexamer priming [3], GC-content [9], and transcript length [2]. Batch effects, variation due to experimental design, is a prevalent phenomenon and clustering according to surrogate values (processing date or batch) is a common approach for recognition of underlying sources of variation [7].

We apply hierarchical clustering to DNA k-mer content in Fastq files (HcKmer) and analyse transcriptome sequencing data of 61 samples. Sample clustering from raw Fastq files, (Phred) quality filtered sequences and from aligned sequences are analysed. A putative effect on analysis results of differential gene expression is explored. Additionally, the sensitivity of HcKmer is examined in a simulation study.

2.1 Algorithmic framework

For a DNA sequence of length $k$, $4^k$ different sequence motifs (k-mers) exist each defining a category for which occurrences can be counted. The value of $k$ is usually chosen in the range of 5 to 9 (resulting in 1,024 to 262,144 different k-mers) as shorter motifs impair separation capabilities and longer motives increase computational demands.

The algorithms for performing HcKmer are available in R package seqTools on Bioconductor [4]. DNA k-mer counts are collected with other quality measures (for example Phred scores and GC content) from Fastq files. Data from multiple samples (for example from whole Illumina Flowcells) can be collected at once into merge-able objects. Combined objects then serve as input for hierarchical clustering. The Canberra distance, defined as

\[
f_{cb}(x_i, y_i) = \frac{|x_i - y_i|}{|x_i| + |y_i|}
\]

\[
d(x, y) = \sum_{i=1}^{k} f_{cb}(x_i, y_i), \quad (x_i)_{i=1,...,k}, (y_i)_{i=1,...,k} \in \mathbb{R}^k,
\]

is used as distance measure between samples. Total read numbers ($\sum_{i=1}^{m} x_i$) are scaled to a common value in order to compensate a systematic offset caused by different sequencing depth’s (thereby increasing $|x_i - y_i|$ values). Subsequent cluster-analysis was calculated using the hclust function (CRAN stats package) [8].

2.2 Sample preparation, sequencing and alignment

In this study, transcriptome data from 61 samples is analysed. Therefrom, 57 samples of human short term cultured human dermal fibroblasts were obtained from 27 human healthy individuals and sequenced for a study on human ageing [5]. Collection and processing of dermal samples from donors was approved by the Ethical Committee of the Medical Faculty of the University of Düsseldorf (# 3361) in 2011. The Fastq files from these samples are available under ArrayExpress accession E-MTAB-4652 (ENA study ERP015294). Also, 4 samples from cultured human Jurkat cell lines were sequenced for a study on HIV infection.

Sample preparation and sequencing has been described elsewhere [5]. In short, cellular mRNA was amplified on 8 Illumina Flowcells (v1.5) and sequenced on an Illumina HiSeq 2000 sequencer. From each lane, the resulting 101-nucleotide sequence reads were converted to Fastq by CASAVA 1.8.2. A Fastq file contained in average $162.2 \times 10^6$ reads. In total the 61 Fastq files contained $9.8 \times 10^9$ reads.
Subsequent alignments were calculated on unprocessed Fastq files with TopHat (v 2.0.14) using human GRCh38 assembly. In order to compare batch effects between raw Fastq files and mapped reads, BAM file content was transformed back into Fastq (using bam2fastq from CRAN package rbamtools [6]). All DNA k-mer counts were collected using \( k=9 \) (\( 4^9 = 262,144 \) DNA motifs), except for the simulation study (\( k=6 \) is used for HcKmer). Differential gene expression analysis was performed using Quasi-likelihood F-Tests from the edgeR (3.12.0) framework [10]. Genes with a reported FDR < 0.1 were considered to be significantly differential expressed. The same analytic procedure performed on the whole dataset resulted in no significantly differential expressed gene [5].

3 Results

3.1 Data collection

The 61 samples had been sequenced on 8 Flowcells. Data from each Flowcell was collected into one data-set. Processing of \( 9.8 \times 10^9 \) reads took 8.96 hours (\( 3.04 \times 10^6 \) reads/second) in a single thread with approximately 1 Gigabyte working memory consumption. The saved raw data was 60.8 megabytes in size.

![HC on DNA k-mer profile](image.png)

Figure 1: **Strong batch effect in comparison of a Flowcell pair.** Leaf labels denote cell type (fib, jur) and number of individual, lane number and Flowcell label (d24a and c0yr). Samples from Jurkat cells are highlighted in grey. The tree clearly separates Flowcell d24a and c0yr although Flowcell c0yr contains two different cell types.

3.2 Identification of batch effects.

Figure 1 shows a dendrogram where 16 samples from two Flowcells (d24a and c0yr) and two cell types (dermal fibroblasts and Jurkat cells) are analysed using HcKmer. The partition tree consists of two equally sized sub-trees. Each of them only contains samples sequenced on one Flowcell although on Flowcell c0yr, two different cell types are present. Thus, DNA motif dissimilarity is greater between Flowcells d24a and c0yr than between fibroblasts and Jurkat cells. Figure 2 indicates that median Phred scores in sequences from both Flowcells are sufficiently high.
3.3 Filtering for sufficient Phred scores

In order to explore, whether the observed dissimilarity can be removed, reads containing at least one Phred score < 30 were discarded. The filter was applied to Fastq files sequenced on Flowcells d24a and c0yr Flowcells. From Flowcell d24a, 14.3 % of reads in Fastq files and from Flowcell c0yr, 15.1 % of reads in Fastq files were excluded thereby. Finally, the filtered reads were re-analysed.

In the HC-tree, the position of one Flowcell (fib13 lane03 c0yr) changed to the opposite subtree but still, three fibroblast samples cluster together with the Jurkat samples (Figure 3).

Figure 2: Median Phred score values. For each read position, median Phred scores are shown for samples sequenced on Flowcells d24a and c0yr. All median Phred scores are > 28.
Figure 3: **Clustering of Fastq files containing filtered reads.** All reads containing Phred scores < 30 had been discarded before HcKmer analysis. On top level clade, the Jurkat cell samples still exclusively cluster together with samples from the same Flowcell.

Thus, filtering based on Phred scores induced only a minor change in cluster formation.

### 3.4 Detection of experimental effects

Likewise batch effects, clustering of experimental groups may be indicative for experimental effects. The analysed fibroblast samples had been collected from donors in different age groups (Young: 19 to 25 years, Middle: 36 to 45 years, Old: 60 to 66 years) [5]. Figure 4 shows a dendrogram, where all samples from donors aged < 60 years are located within the same subtree while two samples sequenced on the same flowcell (c0yr) are located outside this subtree. As appearance of these clusters may indicate experimental effects, putative experimental sources should be explored. As DNA k-mer content is an unspecific indication, potential causes are not restricted to differential gene expression but may include differential (alternative) splicing events.
3.5 HcKmer on sequences aligned to the human genome

Fastq reads causative for HcKmer tree separation may not match to the reference genome and thus would be filtered out by alignment. In order to evaluate this eventuality, clustering of raw Fastq files and mapped reads are compared. Pairs of Flowcells containing 8 fibroblast samples (d24a, d10r, c0g9, c0yt, c2uk, d1pd) are analysed by HcKmer on raw reads and on mapped reads. An example is shown in Figure 5 where raw and mapped reads cluster in similar patterns. In both trees, the size of the largest subtree containing samples from only one Flowcell is 7. The mean sizes of these subtrees in all 15 possible pairs is 7.21 in raw reads and 6.57 in mapped reads. Mapping thus reduces Flowcell cluster sizes by 8.9 % but still, clusters of size \( \geq 6 \) are prevalent. Detailed results are shown in supplemental data.
3.6 Separation sensitivity on simulated data

The degree of sequence dissimilarity required to produce HcKmer trees with top level separation of two sample groups (e.g. Flowcells) is determined in simulation where Fastq files with random DNA are analysed. A pure random group is compared to a group in which a variable percentage of random DNA reads is contaminated with (one or multiple) fixed DNA 6-mers. Clustering of groups is quantified using a score (Contralaterality Score, CS). A decrease of CS from 41.1% (mean value for pure random sequences) to 0% is regarded as indicative for clustering of groups and in the setting of the simulation, a CS < 10% can considered to be statistical significant (p < 0.05).

Figure 6 shows separation capabilities of HcKmer for contamination of 0 - 6% of Fastq reads with one fixed DNA 6-mer. The results indicate that with a contamination of 4% of Fastq reads, significant sample separation reaches a power of 80%. Details of simulation, definition of CS and results are shown in supplemental data.
3.7 Prevalence of batch effects in RNAseq data

The prevalence of detectable batch effects is analysed on the whole set of 61 samples sequenced on 8 Flowcells. Pairwise comparison of 8 Flowcells results in 28 pairs. All Flowcell pairs are analysed for presence of batch effects using HcKmer and a semi-quantitative score ranging from strong batch effect (b1a = top-level separation of Flowcells; shown in Figure 1) and detectable batch effect (b1a, b1b, b2a or b2b) to absence of batch effects (es). From the analysed Flowcell pairs, 6 (21.4 %) show strong batch effects and 17 (60.7 %) show detectable batch effects. Thus, batch effects are present in a considerable fraction of Flowcell pairs. The definitions and details on analysis are shown in supplemental material.

3.8 Influence of batch effects on false discovery rate

Clustering according to sample preparation batches potentially influences differentially Expressed Gene (DEG) analysis in RNAseq data. To address this question, results from DEG analysis are compared between Flowcells with different HcKmer dissimilarity using a two-way ANOVA. Classification as batch effect (b1 or b2 vs. es) is significantly associated with increased number of differentially expressed (DE) genes (see supplemental material for details). The ANOVA predicts 3,848 DE genes for b1a dissimilarity and 695 DE genes for idf/es (no batch effect detectable). Thus, increased number of false positives in DEG analysis are found when batch effects are identified by HcKmer.

3.9 K-mer spectrum responsible for tree separation

Standard quality control tools (for example FastQC) only report a small number of over-represented k-mers which may not represent a sufficient sample for explanation of batch effects diagnosed by...
HcKmer. We therefore estimate how many k-mers are needed in order to evoke the observed batch effects using an example.

From the simulation data on separation sensitivity, it is assumed that \( \approx 3\% \) contamination (where the median CS falls below 12.5 % in Figure 6) is required in order to produce a strong batch effect (b1a). Two pairs of Flowcells are selected, one with complete tree separation due to batch effect (Flowcells d24a and c0yr) and one pair of Flowcells without tree separation due to batch effect (Flowcells d24a and d1pd). For each k-mer, the logarithmised and normalised sum of k-mer counts on whole Flowcells are calculated and compared for both pairs and are shown in Figure 7. In the example shown in left panel (d24a/c0yr comparison), differences from the 3,678 k-mers with the largest difference in k-mer count need to be accumulated in order to attain a 3 % contamination rate.

Due to this large number it is unlikely, that strong batch effects (b1a) diagnosed by HcKmer can be identified by inspecting few k-mers and that they can be eliminated by removing reads containing a small set of selected k-mers.

4 Discussion

Differences identified by HcKmer should initiate exploration of underlying effects because comparison of raw and mapped reads as well as differential expression analysis on our samples indicate, potentially influential effects on downstream analysis.

Clustering of samples according to biological or experimental entities is an unspecific criterion as different K-mer spectra may be caused by differential gene expression, differing splicing patterns as well as the mentioned disturbing factors (for example random hexamer priming, GC-content or transcript length). Thus etiologic factors may not be apparent.

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**Figure 7: K-mer spectrum responsible for tree separation.**

Comparison of K-mer counts in two Flowcell pairs using density scatter plots: All axes represent normalised and \( \log_{10} \) transformed k-mer counts on a whole Flowcell (each 8 Fastq files). The diagonal (red line) indicates equal normalised counted numbers for k-mers in both Flowcells. **Left panel:** Comparison of k-mer counts on Flowcells d24a/c0yr (strong batch effect (=b1a) identified by HcKmer). **Right panel:** Comparison of k-mer counts on Flowcells d24a/d1pd (no batch effect (=es) identified by HcKmer). **Result:** The k-mer count differences are larger for d24a/c0yr (mean= 0.30, sd= 0.12) than for d24a/d1pd (mean=0.01, sd=0.07). The HcKmer diagnosed difference of sample similarity between the Flowcell pairs is due to larger deviation of k-mer counts from the diagonal for a broad variety of k-mers (thus not generated by a small group k-mers with large deviation).
Analysis of our samples shows, that clustering of preparation batches may be prevalent (21 % - 61 %) and that resulting disturbances may not be removed by Phred based filters or by alignment to a genome. Also, batch effects potentially affect results of DEG analysis.

Due to analysis results of differential gene expression on our fibroblast samples [5], we assume that there are no consistent differences between different groups and thus, GC-content and transcript length are unlikely to evoke the observed batch effects. Also, observed sample dissimilarities seem to result from a larger number of small differences in k-mer counts which can not be diagnosed by identification of few over- or underrepresented k-mers. Based on the shown results, our group decided to discard data from samples sequenced on two other Flowcells (data not shown here) exhibiting strong batch effects when compared with all Flowcells in the shown analysis.

HcKmer provides no quantitative measure for similarity so that subjective judgement is required. Also, a minimum of four samples per group are required for assessment. But, as HcKmer can easily be applied (using seqTools) and HcKmer can identify influential effects not detected by other QC procedures, it appears advisable to include HcKmer into analysis standards.

5 Conclusions

HcKmer provides an unprejudiced view onto the raw data produced by a sequencing experiment with the capability of detecting unwanted variation which is prevalent and potentially influential. Experimental designs allowing HcKmer analysis (for example by sequencing samples together on a defined set of Flowcells and to avoid multiplexing) thus are favourable. Based on contrasts identified by HcKmer, further exploration of results from sequencing experiments as well as exclusion of contaminating samples may be reasonable.

5.0.1 List of abbreviations

HC Hierarchical clustering
HcKmer Hierarchical clustering of DNA k-mer counts
DEG Differential expressed gene(s)
QC Quality control

5.0.2 Ethics approval

The study was approved by the Ethical Committee of the Medical Faculty of the University of Düsseldorf (# 3360) in 2011.

5.0.3 Availability of data and materials

The used software (seqTools) is available from the Bioconductor web site. The raw Fastq files for fibroblast samples are available from ArrayExpress under accession E-MTAB-4652 (ENA study ERP015294).

5.0.4 Competing interests

The authors declare that they have no competing interests.

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