WEB TOOL

shRNA-seq data analysis with *edgeR* [version 1; peer review: 3 approved]

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Abstract

Pooled short hairpin RNA sequencing (shRNA-seq) screens are becoming increasingly popular in functional genomics research, and there is a need to establish optimal analysis tools to handle such data. Our open-source shRNA processing pipeline in *edgeR* provides a complete analysis solution for shRNA-seq screen data, that begins with the raw sequence reads and ends with a ranked lists of candidate shRNAs for downstream biological validation. We first summarize the raw data contained in a fastq file into a matrix of counts (samples in the columns, hairpins in the rows) with options for allowing mismatches and small shifts in hairpin position. Diagnostic plots, normalization and differential representation analysis can then be performed using established methods to prioritize results in a statistically rigorous way, with the choice of either the classic exact testing methodology or a generalized linear modelling that can handle complex experimental designs. A detailed users' guide that demonstrates how to analyze screen data in *edgeR* along with a point-and-click implementation of this workflow in Galaxy are also provided. The *edgeR* package is freely available from <http://www.bioconductor.org>.

This article is included in the **Bioconductor** gateway.

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Any reports and responses or comments on the article can be found at the end of the article.

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Introduction

Pooled short hairpin RNA sequencing (shRNA-seq) screens couple RNA interference (RNAi) with second generation sequencing to enable researchers to elucidate gene function in an unbiased, high-throughput manner¹. Several recent high impact studies have exploited this technology to discover novel genes involved in processes including cell fate decisions of normal and cancer cells, and to generate genetic interaction maps in mammalian cells²⁻⁴.

Pooled shRNA screening relies on the stable genomic integration (often by viral transduction) of expression cassettes that allow stable or inducible expression of shRNAs targeting specific genes in a large population of cells. Depending on the biological question of interest, typically two or more cell populations are compared either in the presence or absence of a selective pressure, or as a time-course before and after a selective pressure is applied. Gain of shRNA representation within a pool suggests that target gene knockdown confers some sort of advantage to a cell. Similarly, genes whose knockdown is disadvantageous may be identified through loss of shRNA representation. Screening requires a library of shRNA constructs in a lentiviral or retroviral vector backbone that is used to generate a pool of virus for transducing cells of interest. The relative abundance of these shRNAs in transduced cells is then quantified by PCR amplification of proviral integrants from genomic DNA using primers designed to amplify all shRNA cassettes equally, followed by second-generation amplicon sequencing (Figure 1A). Sample-specific primer indexing allows many different conditions to be analyzed in parallel.

As the popularity of this approach grows, there is a need to develop suitable analysis pipelines to handle the large volumes of raw data that each screen generates. The major steps in an analysis involve processing the raw sequence reads, assessing the data quality and determining representational differences in the screen in a statistically rigorous way.

Two pipelines are currently available for this task. The **shALIGN program**⁵ is a custom Perl script that trims the sequence reads to the pre-defined base positions and then matches these to a library of hairpin sequences. Mismatch bases are permitted, and any ambiguous matches are ignored from the final hairpin count. Statistical analysis of the data is then performed using the **shRNAseq R package**⁵, which calculates log-ratios of the counts from each screen replicate, normalizes these values and ranks hairpins by their median, mean or *t*-statistic. Another solution is the **BiNGS!SL-seq program**⁶ that uses Bowtie to perform sequence mapping followed by statistical analysis in **edgeR**⁷.

In this article, we describe a complete analysis solution for shRNA-seq screens accessible from within the **edgeR** package available from **Bioconductor**⁸.

Implementation

A summary of the main steps in a typical shRNA-seq analysis alongside the functions in **edgeR** that perform each task is given in Figure 1B.

Sequence pre-processing

Our sequence counting procedure has been tailored for screens where PCR amplified shRNA constructs of known structure are sequenced using second generation sequencing technology (Figure 1A). The location of each index and hairpin sequence is used to determine matches between a specified list of index and hairpin sequences expected in the screen with the sequences in the fastq file. Mismatches in the hairpin sequence are allowed to accommodate sequencing errors, as are small shifts in the position of the hairpin sequence within the read. Analysis of unpublished in-house data reveals that allowing for mismatches can yield up to 4.4% additional reads, and shifting an extra 2.6%. This simple searching strategy is implemented in C, with the user interface provided by the **processHairpinReads** function in **edgeR**. Input to this function consists of a fastq file/s, a second file containing sample IDs and their index sequences and a third file listing hairpin IDs and their sequences (the latter files are tab-delimited). A screen with 100 million reads (one lane from an Illumina HiSeq 2000) can be processed in 2-15 minutes depending on the processing parameters. Fastq processing requires minimal RAM, allowing analysis to be completed on any standard computer with **R**⁹ installed.

The matrix of counts returned by the **processHairpinReads** function, which contains hairpins in the rows and samples in the columns, is stored as a **DGEList** object so that it is fully interoperable with the downstream analysis options available in **edgeR**. Such an object can also be created directly by the user in the event that the hairpin counts have been summarized by alternate means.

Next, the data quality of a screen can be assessed conveniently using multidimensional scaling (MDS) plots via **plotMDS** (Figure 1C) and access to a range of normalization options is available through the **calcNormFactors** function.

Differential representation analysis

The **shRNAseq** software⁵ assumes simple experimental set-ups (e.g. comparing two conditions) that are unsuitable in more complicated situations, such as time-course designs. In **edgeR**, screens can be analyzed using either the classic method¹⁰, ideal for simple two-group comparisons, or generalized linear models (GLMs)¹¹ for more complex screens with multiple conditions (using the **glmFit** function). This framework can accommodate hairpin-specific variation of both a technical and biological nature as estimated via the **estimatedDisp** function and visualized using **plotBCV**, which plots biological variability as a function of average hairpin abundance. Statistical testing for changes in shRNA abundance between conditions of interest (typically over time) is carried out using exact (see **exactTest** function) or likelihood ratio (**glmLRT**) tests that allow results to be ranked by significance using the **topTags** function and plotted using the **plotSmear** function (Figure 1D).

Gene set analysis tools available via **roast**¹² and **camera**¹³ allow researchers to further test and prioritize screen results. This capability can be used to obtain a gene-by-gene ranking, rather than a hairpin-specific one, which can be helpful when shRNA libraries contain multiple hairpins targeting each gene.

Figure 1. Summary of the raw data, workflow and diagnostic plots from edgeR. (A) Structure of the amplicons sequenced in a typical shRNA-seq screen. Each one contains sample and hairpin specific sequences at predetermined locations. After sequencing, the raw data is available in a fastq file. (B) The main steps and functions used in an analysis of shRNA-seq screen data in edgeR are shown. (C) Example of a multidimensional scaling (MDS) plot showing the relationships between replicate dimethyl sulfoxide (DMSO) and Nutlin treated samples (data from Sullivan *et al.* (2012)³). MDS plots provide a quick display of overall variability in the screen and can highlight inconsistent samples. (D) Plot of $\log_2$ -fold-change versus hairpin abundance ($\log_2$ CPM) for the same data. Hairpins with a false discovery rate < 0.05 from an exact test analysis in edgeR (highlighted in red) may be prioritized for further validation.

Case studies and further extensions

We provide example [data sets](#) and a [complete analysis script](#) that demonstrate how to use the edgeR package to prioritize data from four different pooled shRNA-seq screens¹⁴. These examples were chosen to showcase edgeR's ability to deal with experiments of varying size (from tens to thousands of hairpins) and complexity, from

two-group situations, to settings with four groups, or a time-course design, where a GLM with a slope and intercept term is most appropriate. We have also developed a Galaxy tool^{15–17} that implements this workflow as a point-and-click application to improve accessibility for researchers who are unfamiliar with the R programming environment (Figure 2).

Figure 2. Screenshots of the Galaxy tool for shRNA-seq screen analysis powered by edgeR. (A) From the main screen, the user selects the appropriate input files and analysis options. (B) The results of an analysis are summarized in an HTML page that includes various diagnostic plots. (C) Output also includes a table of ranked results at the hairpin and gene-level (where appropriate) as well as barcode plots (D) that highlight the ranks of hairpins targeting a specific gene relative to all other hairpins in the data set.

Discussion

Although the major functionality of edgeR has been developed with RNA-seq data in mind, the analysis of numerous in-house data sets¹⁴ and the results of others³ have demonstrated its utility for count data derived from shRNA-seq screens. edgeR provides users with a unique tool for the analysis of data from this emerging application of second generation sequencing technology, that is capable of handling both the biological variability and experimental complexity inherent in these screens. Provision of a Galaxy module puts these powerful statistical methods within reach of experimentalists. Future work will be focused on the use of a suitable control data set to compare this analysis pipeline with other approaches such as shRNAseq⁵. We anticipate that the approach for differential representation analysis described in this paper will also be useful in the analysis of short-guided RNA-seq (sgRNA-seq) knockout screens as facilitated by the clustered regularly interspaced short palindromic repeats-Cas9 (CRISPR-Cas9) system^{18,19}.

Software availability

edgeR is an R⁹ package distributed as part of the Bioconductor project⁸ (<http://www.bioconductor.org>). The Galaxy tool that implements this workflow is available from <http://toolshed.g2.bx.psu.edu/view/shians/shrnaseq>.

Author contributions

ZD and MER developed the hairpin counting software and SS developed the Galaxy tool. JMS, LJG, DLM and MEB generated the screen data analyzed in the user guide that accompanies this article and RAD developed the hairpin technology. All authors wrote and approved the manuscript.

Competing interests

No competing interests were disclosed.

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The authors introduce the multiplex shRNA screening approach as commonly used currently to find new drug targets or other similar applications. They also provide a literature overview on the relevant bioinformatics tools used in this context. With rapid improvements in the shRNA technology, data analysis methods for this are high in demand. The manuscript provides a good framework for analysis of data on pooled shRNA screens. There is a strong need to have such user friendly, open-source programs dedicated to analysis of shRNA screens that can easily be used by experimental biologists. The manuscript provides a coherent platform that nicely incorporates analytic tools for the current requirements of the RNAi or other similar functional genetic screens. As part of the review process, we have analyzed our own data using the software and found it fully functional and very easy to use. Based on our experience, we recommend the manuscript for indexation.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 08 May 2014

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This is a well written paper describing a potentially useful application for the analysis shRNA-seq screening data. It includes a brief description of this relatively novel technique and some competing methods as well as a description of the method used in this implementation. The authors are to be commended for providing a wrapper for Galaxy which will make it very easy for biologists to access the method in a reproducible analysis environment and this seems likely to improve the real availability and eventual impact of their work.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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This is a useful manuscript, detailing the author's extensions of existing functionality within the Bioconductor edgeR package to include shRNA screen data.

The manuscript itself provides an overview of shRNA screens, competing analysis pipelines, and the methods available in edgeR. In addition, the authors provide a link to a vignette that gives example analyses of four different shRNA experiments that vary in depth of sequencing and complexity, along with the data so potential users can recapitulate the analyses provided, before making an attempt to analyze their own data.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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