Bioinformatics, 37(1), 2021, 126–128 doi: 10.1093/bioinformatics/btaa1063 Advance Access Publication Date: 26 December 2020 Applications Note

OXFORD

## Gene expression **ProkSeq for complete analysis of RNA-Seq data from prokaryotes**

# A. K. M. Firoj Mahmud<sup>1</sup>, Nicolas Delhomme (2, Soumyadeep Nandi<sup>3,\*</sup> and Maria Fällman<sup>1,\*</sup>

<sup>1</sup>Laboratory for Molecular Infection Medicine Sweden (MIMS), Umeå Centre for Microbial Research (UCMR), Department of Molecular Biology, Umeå University, SE-901 87 Umeå, Sweden, <sup>2</sup>Department of Forest Genetics and Plant Physiology, Umeå Plant Science Centre (UPSC), Swedish University of Agricultural Sciences, Umeå, Sweden and <sup>3</sup>Amity Institute of Integrative Sciences and Health, Amity University Haryana, Gurgaon, 122413 Haryana, India

\*To whom correspondence should be addressed. Associate Editor: Jan Gorodkin Received on June 4, 2020; revised on November 19, 2020; editorial decision on December 6, 2020; accepted on December 11, 2020

#### Abstract

**Summary:** Since its introduction, RNA-Seq technology has been used extensively in studies of pathogenic bacteria to identify and quantify differences in gene expression across multiple samples from bacteria exposed to different conditions. With some exceptions, tools for studying gene expression, determination of differential gene expression, downstream pathway analysis and normalization of data collected in extreme biological conditions is still lacking. Here, we describe ProkSeq, a user-friendly, fully automated RNA-Seq data analysis pipeline designed for prokaryotes. ProkSeq provides a wide variety of options for analysing differential expression, normalizing expression data and visualizing data and results.

**Availability and implementation:** ProkSeq is implemented in Python and is published under the MIT source license. The pipeline is available as a Docker container https://hub.docker.com/repository/docker/snandids/prokseq-v2.0, or can be used through Anaconda: https://anaconda.org/snandiDS/prokseq. The code is available on Github: https://github.com/snandiDS/prokseq and a detailed user documentation, including a manual and tutorial can be found at https://prokseqV20.readthedocs.io.

**Contact**: snandi@ggn.amity.edu. or maria.fallmamn@umu.se **Supplementary information**: Supplementary data are available at *Bioinformatics* online.

#### **1** Motivation

The advancement of massive parallel sequencing and dramatic reduction in sequencing costs have made deep sequencing of RNA (RNA-Seq) a primary tool for identifying and quantifying RNA transcripts. Today RNA-Seq is widely used to analyse bacterial gene expression in studies that aim to identify drug targets, predict novel gene regulatory mechanisms, etc. Such studies often require profound knowledge of both computational data handling and biology. There are some stand-alone pipelines and tools that require only moderate knowledge of bioinformatics (Delhomme *et al.*, 2012; Prieto and Barrios, 2020), but these are not designed for analyses of bacterial gene expression.

Prokaryotic RNA-Seq analysis is challenging because most available RNA-Seq packages assume the input data reflect eukaryotic gene structures, which in many aspects differ from those of prokaryotes (Johnson *et al.*, 2016). Bacterial transcripts do not have introns and are not alternatively spliced; therefore, using an aligner developed to consider splice junctions often increases falsely assigned reads in the genome (Magoc *et al.*, 2013). Moreover, unlike in eukaryotes, under specific stresses, the expression of almost all prokaryotic genes can change (Creecy and Conway, 2015). Furthermore, quality trimming, adapter removal and normalization of skewed data are often required for prokaryotic data due to variations in experimental setups, the presence and overexpression of plasmid genes and differences in RNA-Seq protocols (Magoc *et al.*, 2013; McClure *et al.*, 2013).

Although there are a few software packages available for prokaryotes that can facilitate the analysis of RNA-Seq data, such as SPARTA (Johnson *et al.*, 2016), EDGE-pro (Magoc *et al.*, 2013) and RockHopper (McClure *et al.*, 2013), all require substantial knowledge of data handling. Therefore, to reduce human intervention in conducting RNA-Seq data analysis for prokaryotes, we developed ProkSeq, a fully automated command-line based workflow by integrating various available tools and built-in functions written in Python. ProkSeq integrates short read aligner bowtie2 (Langmead and Salzberg, 2012) with its default parameter as well as Salmon (Berghoff et al., 2017) as an option for (pseudo-)alignment. It provides normalized expression value to compare within and between samples, options to remove unwanted variation (RUV) (Risso

126

 $\ensuremath{\mathbb{C}}$  The Author(s) 2020. Published by Oxford University Press.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.

*et al.*, 2014) and average nucleotide count normalization for differential expression (Creecy and Conway, 2015). In addition, ProkSeq supports downstream Gene Ontology (GO) (Gene Ontology Consortium, 2008) and KEGG pathway enrichment analyses (Kanehisa and Goto, 2000). ProkSeq processes RNA-Seq data from quality control steps to pathway enrichment analysis of differentially expressed genes (Fig. 1). It provides a wide variety of options for differential expression, normalized expression and visualization, and produces figures. Reduced human intervention and multithreading feature makes the use of ProkSeq less time consuming than the sequential application of separate tools, which often requires reformatting data.

#### 2 Implementation

ProkSeq runs in a Linux-based command-line environment and depends on user-defined parameters and sample files. The tools used in the pipeline are set with their default parameters. However, more advanced users can adjust the parameters of the tools to control the functionality. The sample file indicates the names of the fastq files to be included in the analysis, and also defines the experimental classes, such as treatment and control samples. ProkSeq first checks the quality of reads and filters out low-quality reads using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and afterQC (Chen et al., 2017). It maps the reads to the reference genome using bowtie2 and its default parameters for both single and paired-end reads. ProkSeq then generates a report on the alignment quality for each library, as both figures and text, providing information about coverage uniformity, distribution along protein-coding sequences, and 5' and 3' UTR regions, as well as the read duplication rate and strand specificity generated by RSeQC (Wang et al., 2012). Total reads per gene are calculated with featureCounts (Liao et al., 2014), which provides a high efficiency of read assignments across the genome. ProkSeq also calculates normalized gene expression values for each gene, in the form of transcripts per million

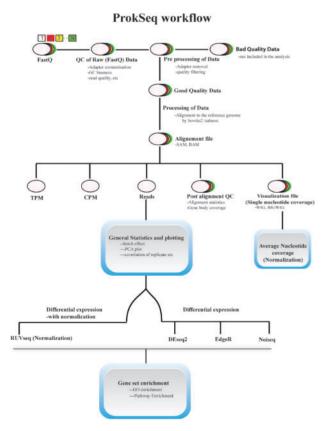


Fig. 1. Workflow of ProkSeq showing major steps and tools

(TPM) and counts per million (CPM) (Wagner *et al.*, 2012). The formulas by which these are calculated are explained in the Supplementary Methods (S1).

ProkSeq integrates several tools for differential expression analysis, such as DESeq2 (Love *et al.*, 2014), edgeR (Robinson *et al.*, 2010) and NOISeq (Tarazona *et al.*, 2015). For downstream analysis of differentially expressed genes, ProkSeq uses GO enrichment and pathway enrichment by integrating clusterProfiler (Yu *et al.*, 2012). Reports on pre- and post-alignment quality statistics and graphical visualization are created in pdf and HTML formats. One important unique feature of ProkSeq is the integration of RUV normalization and average nucleotide count methods for skewed data (Creecy and Conway, 2015; Zhu *et al.*, 2019). Furthermore, the package generates a single-nucleotide resolution wiggle file for visualization in any genome browser. ProkSeq generates graphics and figures at every step of data analysis to give the user more confidence in and understanding of their data. The methods are described in detail in the Supplementary Methods (S1).

#### **3 Discussion**

ProkSeq has been designed to meet researchers with moderate bioinformatics knowledge for analysing RNA-Seq data in a reliable and time-efficient way. RNA-Seq data can provide much more information than simply the differential expression of known coding sequences. Exploring RNA-Seq reads to single-nucleotide resolution across the genome can provide information about biological events other than gene expression. ProkSeq offers easy access to genome-wide visualization of RNA-Seq data. Visualization of read mapping will reveal expression from unannotated genomic regions and intergenic regions, including 5' and 3' UTRs, which is of great interest in relation to novel transcriptional and translational regulation. Other tools for revealing this type of information that are available today (Supplementary Table S1) usually require substantial competence in bioinformatics and lack some of the options available in ProkSeq. Furthermore, integration of Salmon in the process gives the user one of the most up-to-date methods of estimating transcript abundance. Salmon uses a realistic model of RNA-Seq data that takes into account not only experimental attributes but also biases commonly observed in RNA-Seq data (Bergoff et al., 2017). Users can quickly extract transcript abundance and subsequent differential expression data by opting to use salmon.

ProkSeq provides an option for batch effect identification and normalization. An essential difference between eukaryotes and prokaryotes that can cause problems when analysing prokaryotic gene expression using tools optimized for analyses of eukaryotic cells is the relative number of differentially expressed genes. Most often, tools such as DESeq2, edgeR and Limma (Dillies *et al.*, 2013) are designed with the assumption that the number of genes is constant in eukaryotes. But in prokaryotes, the expression of the majority of genes can be altered under specific stress conditions (Berghoff *et al.*, 2017; Creecy and Conway, 2015). To address this bias, ProkSeq normalizes the data at the level of nucleotide base count making the data comparable across samples. ProkSeq provides two normalization options that can handle differential expression analyses of this type of data, which are described in detail in the Supplementary Methods (S1).

The built-in automatic sequential handling of the data from differential gene expression analysis to downstream functional analyses allows researchers to focus on complex biological mechanisms instead of tackling bioinformatics obstacles. The flexibility that comes with built-in options for certain steps and the visualization of mapped reads across genomes opens a path to new discoveries in gene regulation as well as in RNA biology.

#### Acknowledgement

The authors thank Dr Rikki Frederiksen and Chayan Kumar Saha for testing and Dr Roberto Navais for feed-back.

#### Funding

The work was supported by funding from Knut and Alice Wallenberg foundation [2016.0063], Swedish research Council [2018-02855] and the Medical faculty at Umea University.

Conflict of Interest: none declared.

### References

- Berghoff,B.A. et al. (2017) RNA-sequence data normalization through in silico prediction of reference genes: the bacterial response to DNA damage as case study. BioData Min., 10, 30. doi: 10.1186/s13040-017-0150-8.
- Chen, S. et al. (2017) AfterQC: automatic filtering, trimming, error removing and quality control for fastq data. BMC Bioinf., 18, 80.
- Creecy, J.P. and Conway, T. (2015) Quantitative bacterial transcriptomics with RNA-seq. *Curr. Opin. Microbiol.*, 23, 133–140.
- Delhomme, N. et al. (2012) easyRNASeq: a bioconductor package for processing RNA-Seq data. Bioinformatics, 28, 2532–2533.
- Dillies, M.A. et al.; on behalf of The French StatOmique Consortium. (2013) A comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis. Brief. Bioinf., 14, 671–683.
- Gene Ontology Consortium. (2008) The Gene Ontology project in 2008. Nucleic Acids Res., 36, D440–D444.
- Johnson, B.K. et al. (2016) SPARTA: simple program for automated reference-based bacterial RNA-seq transcriptome analysis. BMC Bioinf., 17, 66.
- Kanehisa, M. and Goto, S. (2000) KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res., 28, 27–30.

- Langmead, B. and Salzberg, S.L. (2012) Fast gapped-read alignment with Bowtie 2. Nat. Methods, 9, 357–359.
- Liao, Y. et al. (2014) featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics, 30, 923–930.
- Love, M.I. et al. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol., 15, 550.
- Magoc, T. et al. (2013) EDGE-pro: estimated degree of gene expression in prokaryotic genomes. Evol. Bioinf. Online, 9, 127–136.
- McClure, R. et al. (2013) Computational analysis of bacterial RNA-Seq data. *Nucleic Acids Res.*, 41, e140.
- Prieto, C. and Barrios, D. (2020) RaNA-Seq: interactive RNA-Seq analysis from FASTQ files to functional analysis. *Bioinformatics*, **36**, 1955–1956.
- Risso, D. et al. (2014) Normalization of RNA-seq data using factor analysis of control genes or samples. Nat. Biotechnol., 32, 896–902.
- Robinson, M.D. et al. (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, 26, 139–140.
- Tarazona, S. *et al.* (2015) Data quality aware analysis of differential expression in RNA-seq with NOISeq R/Bioc package. *Nucleic Acids Res.*, **43**, e140.
- Wagner, G.P. et al. (2012) Measurement of mRNA abundance using RNA-seq data: RPKM measure is inconsistent among samples. *Theory Biosci.*, 131, 281–285.
- Wang,L. et al. (2012) RSeQC: quality control of RNA-seq experiments. Bioinformatics, 28, 2184–2185.
- Yu,G. et al. (2012) clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS, 16, 284–287.
- Zhu, A. *et al.* (2019) Heavy-tailed prior distributions for sequence count data: removing the noise and preserving large differences. *Bioinformatics*, 35, 2084–2092.