Title: Principled, Comprehensive Analytic Platform for Extracellular RNA Analysis

Summary:

Extracellular RNAs (exRNA) are gaining considerable interest for their role in intra-cellular communication and their potential use as biomarkers, but the field faces unique technical challenges. In particular, exRNAs are more vulnerable to contamination and artifacts from different isolation techniques, have lower concentrations than in cellular samples, and some have exogenous elements. To address these challenges we present extracellular RNA processing tool (exceRpt), the standardized small RNA-seq analysis pipeline of the NIH Extracellular RNA Communication Consortium (ERCC) optimized for exRNA analysis. exceRpt is structured as a series of filtering and quantifications prioritized based on the confidence of a given set of annotated RNA. The pipeline generates sample-level quality control metrics and abundance estimates for RNA biotypes, and is also capable of characterizing alignments to exogenous genomes, which can be used to generate phylogenetic trees. exceRpt has uniformly processed all (currently ~2500) exRNA-Seq datasets currently in the public exRNA atlas, and is available at genboree.org and github.gersteinlab.org/exceRpt.

Highlights and eTOC Blurb:

- exceRpt processes and analyzes exRNA profiling data
- Generates quality control metrics, RNA biotype abundance estimates, and processing reports
- User-friendly, browser-based graphical interface available
- Processes all RNA-seq datasets in the exRNA Atlas

Introduction:

Recent discoveries of extracellular RNA (exRNA) in the blood and other body fluids have added a new dimension to the paradigm of intercellular signaling (Patton et al., 2015; Skog et al., 2008). The relative stability of exRNA within extracelluar vesicles (EVs) or bound to proteins or lipids (Yanez-Mo et al., 2015), coupled to the availability of sensitive and specific tools such as RNA-seq, underpins the emergence of exRNA profiling as an approach for biomarker discovery. exRNA-based liquid biopsy is particularly attractive as a non-invasive mode for monitoring disease due to the significantly increased accessibility of biofluids over tissues, thereby allowing more frequent and longitudinal sampling (Byron et al., 2016). With better characterization of the differences between profiles secreted by diseased and healthy tissues, the diagnostic and prognostic utility of exRNA-based profiling is increasingly becoming a reality (Akat et al., 2014; Yuan et al., 2016).

However, exRNA profiling faces unique challenges. Biochemical methods for extraction, purification, and sequencing of exRNAs are much more vulnerable to contamination and artifacts than cellular RNA preparations, in large part due to relative low abundance (Danielson et al., 2017). Quality control prior to sequencing for samples derived from EV or exosome preparations is difficult due to the lack of reliable 'housekeeping' markers, such as the ratio of 18S and 28S ribosomal RNAs (Tataruch-Weinert et al., 2016). The variable presence of rRNA in mixtures of low- and high-density EVs (Lasser et al., 2017); deterministic cleavage of structured smallRNA (tRNAs and piRNAs) and longer RNA molecules; and imperfect annotation of miRNAs, piRNAs, and tRNAs all pose challenges for quantification and functional interpretation. Furthermore, it has been suggested that exogenous exRNAs may be also present at detectable levels in some biofluids (Freedman et al., 2016; Yeri et al., 2017), and careful analysis is required to differentiate these sequences from endogenous RNA molecules. For these reasons,

existing computational tools capable of analyzing smallRNA-seq data are not as well suited to the new field of exRNA analysis.

To address these analytical challenges, we present here the extracellular RNA processing tool (exceRpt). exceRpt is the primary smallRNA analysis pipeline of the NIH Extracellular RNA Communication Consortium (ERCC). By providing an optimized and standardized bioinformatics platform, exceRpt reduces technical bias and allows for cross-study analyses to potentiate meaningful insights into exRNA biology.

Results:

The exceRpt pipeline is composed of a cascade of computational steps (Fig. 1A) where the input reads at a given step are aligned against a set of annotations and the unmapped reads are the input to the next step, with the prioritization of the steps based on our level of confidence in the annotations. For example, in order to combat potential contamination in a library, mapping to known contaminants occurs before the host genome, since if the steps were reversed contaminant sequences could be incorrectly quantified as endogenous RNAs which is less preferable than having false positive contaminant reads. The pipeline is also highly modular (constructed as a makefile file containing both shell and R scripts), allowing the user to define the order of which smallRNA annotations are used during read-mapping; it includes support for random-barcoded libraries and spike-in sequences for calibration or titration. The general workflow comprises steps for preprocessing, endogenous alignment, and exogenous alignment (Figure 1A).

First, exceRpt begins the preprocessing step by automatically identifying and removing 3' adapter sequences as well as random barcodes sequences, if used, which are increasingly being used in smallRNA sequencing in an attempt to identify and compensate for ligation and/or amplification artifacts that have the potential to affect downstream quantification (Fu et al., 2014). The pipeline than aligns against user-specified spike-ins sequences if used in the library construction, followed by a filter to remove low-quality reads. As the final preprocessing step, exceRpt aligns reads to likely sequences in the UniVec database in order to filter out common laboratory contaminants and to endogenous ribosomal RNAs, both of which are highly variable in abundance in EV preparations.

Second, reads are aligned to the endogenous genome and transcriptome of either human or mouse, and transcript abundances are calculated (RNAs are quantified using both raw read counts and normalized reads per million (RPM)). Based on the variety of RNA preparations available (totalRNA, smallRNA, miRNA), the user can prioritize the order that the annotations (miRBase, tRNAscan, piRNA, GENCODE, circRNA) are used for quantification based on our confidence in the presence of a given annotation in a given sample. For example, reads from a miRNA-seq prep can be assigned to miRBase miRNA annotations before piRNA annotations. Likewise, reads from long or total RNA preparations can be assigned to longer GENCODE transcripts before (or instead of) the other smallRNA libraries. This feature is particularly relevant for lower-confidence annotations; piRNAs, for example, are generally given lower priority than tRNAs to ensure correct read assignments.

Third, we designed exceRpt from the beginning to enable confident assessment of non-human sequences in biofluids after careful, explicit removal of as many known or likely contaminants as possible. Before we analyze the remaining reads for potential exogenous sequences, we perform a second pass alignment against the host genome using a more relaxed mapping criteria and known repetitive sequences. This serves to remove sequences that could potentially be from the host genome and to be fairly conservative in the identification of exogenous sequences. Reads are then aligned to

curated libraries of annotated exogenous miRNAs in miRBase and exogenous rRNA sequences in the Ribosomal Database Project (RDP), followed by alignment to the full genomes of all sequenced bacteria, viruses, plants, fungi, protists, metazoa, and selected vertebrates that are potentially part of the host diet. Existing approaches for exogenous sequence alignment remove degenerate sequences (i.e. those that co-occur across multiple species), which results in a loss of potentially valuable data as reads frequently align to multiple species/strains. By characterizing exogenous genome alignments generated by exceRpt in terms of the NCBI taxonomy tree and assigning reads to the most specific node in the phylogenetic tree (many reads can only be assigned to nodes higher up in the phylogenetic tree due to not uniquely mapping to a specific genome of a sub-species), users may obtain valuable information regarding the contribution of the flora to various exRNA samples and generate phylogenies for cross-sample comparison.

In Figure 2C and 2D we present the phylogenetic trees of the reads that we assign to bacterial ribosomal and genome sequences for a specific saliva sample. Saliva biofluids are distinguished from other biofluids by their exposure to a robust and complex bacterial community in the oral cavity (Hasan et al., 2014, Kaczor-Urbanowicz et al., 2017), which causes a greater contribution of reads of bacterial origin (and not human genome) to the sample. In both the phylogenetic trees constructed using bacterial ribosomal and genome mapped reads, we find an abundance of reads assigned to the node corresponding to the genus *Streptococcus.* We have a high degree of confidence in these results given that the reads used for constructing these two trees are disjoint.

The pipeline generates bulk statistics for differential abundance of the various RNA biotypes in addition to sample-level quality control (QC) metrics and processing reports. Because of the heterogeneity of exRNA isolation techniques, we developed ERCC QC metrics for identification of clear experimental outliers. These metrics are based on read counts and the proportion of mapped reads that overlap with annotated RNA transcripts, since enrichment of RNA can be used to distinguish exRNA samples from DNA-enriched cellular contaminants (see STAR Methods). Descriptions of the post-processing output files and diagnostic plots generated by exceRpt are listed in Table 1. As a performance evaluation, we found that the endogenous miRNA abundance estimates produced by exceRpt are in close agreement with existing tools. Comparing exceRpt-filtered read counts for miRBase miRNAs, we obtain an average Pearson correlation of 99.99% to the counts produced by miRDeep2 (Figure S1). As another performance evaluation, running the same sample through the pipeline with individual steps excluded shows the effect of the filters and alignments on downstream quantifications (Figure 1B). Most obvious from this analysis is that the pre-filtering of low quality and low-complexity reads and reads that align to UniVec or rRNA sequences account for a sizeable fraction of the total number sequenced and, without explicit removal, do align to the human genome leading to potential confounding and added quantification variability. UniVec has the largest effect on the fraction of reads aligning to exogenous genomes, and leaving it out substantially increases the number of reads that appear to be, but are not, exogenous in origin.

These bulk statistics can be used to differentiate biofluids (or tissues, if exceRpt is run on cellular samples) on the basis of their RNA distribution. For example, results from samples selected from the exRNA Atlas (Figure 2A) show that, relative to other biofluids, saliva samples tend to have more reads that are unmapped or that map to exogenous genomes, which is consistent with saliva's high potential for bacterial contamination and exposure to the external environment. Moreover, abundance quantifications for specific RNA biotypes can show which miRNAs (or other RNA biotype) are most highly represented in a particular sample (Figure S2). This information

is critical for understanding the composition of particular exRNA profiles and for interrogating their biological significance.

Discussion:

The exceRpt pipeline was built to address the need for a standardized bioinformatics processing platform in extracellular RNA research, and is structured as a series of filtering and quantification steps where unmapped reads are used as inputs to the next step. The prioritization of steps is biased towards conservative estimates for RNA quantifications, with higher confidence libraries (by degree of expectation or annotation quality) having higher priority. The exceRpt has uniformly processed all of the datasets in the ERCC exRNA Atlas (http://exrna-atlas.org/) in a principled, comprehensive manner. The pipeline applies ERCC-defined QC standards, allows for user-specification for library prioritization, offers barcoding and spike-in support, and generates detailed quantification reports, all of which can be done with the source code available in a Github repository (github.gersteinlab.org/exceRpt) or in a user-friendly, browser-based graphical interface available at Genboree.org.

Tables:

Table 1: Description of Output Files

File Name	Description of File
QC Data	
exceRpt_DiagnosticPlots.pdf	All diagnostic plots automatically generated by the tool
exceRpt_readMappingSummary.txt	Read-alignment summary including total counts for each library
exceRpt_ReadLengths.txt	Read-lengths (after 3' adapters/barcodes are removed)
Raw Transcriptome Quantifications	
exceRpt_miRNA_ReadCounts.txt	miRNA read-counts quantifications
exceRpt_tRNA_ReadCounts.txt	tRNA read-counts quantifications
exceRpt_piRNA_ReadCounts.txt	piRNA read-counts quantifications
exceRpt_gencode_ReadCounts.txt	gencode read-counts quantifications
exceRpt_circularRNA_ReadCounts.txt	circularRNA read-count quantifications
Normalized Transcriptome Quantifications	
exceRpt_miRNA_ReadsPerMillion.txt	miRNA RPM quantifications
exceRpt_tRNA_ReadsPerMillion.txt	tRNA RPM quantifications
exceRpt_piRNA_ReadsPerMillion.txt	piRNA RPM quantifications
exceRpt_gencode_ReadsPerMillion.txt	gencode RPM quantifications
exceRpt_circularRNA_ReadsPerMillion.txt	circularRNA RPM quantifications
R Objects	
exceRpt_smallRNAQuants_ReadCounts.RData	All raw data (binary R object)
exceRpt_smallRNAQuants_ReadsPerMillion.RData	All normalized data (binary R object)

Figure Legends:

Figure 1

(A): exceRpt schema

Sample inputs in FASTA or SRA file formats for the input to excerpt. Adapter and random barcode sequences are removed, followed by a read-quality filter, optional spike-in library removal, and contaminant library removal. Unmapped reads then enter the endogenous quantification engine, with RNA library prioritization defined by the user. After a second-pass endogenous genome and repetitive elements filter, reads are mapped to the exogenous libraries.

(B): Leave-one-out analysis
Running the pipeline multiple times with individual steps removed show the effect of those steps on subsequent alignments. The sample used for this analysis is SRR822433 exRNA plasma sample from a non-pregnant woman. Low quality and low-complexity reads and reads that align to UniVec or rRNA sequences account for a sizeable fraction of the total number sequenced. Removing the UniVec alignment step significantly increases the number of reads that map to the exogenous genomes.

Figure 2
(A): Read distributions
exceRpt outputs endogenous alignment quantifications which can be used to see RNA type distributions in exRNA samples. Here, saliva has a higher proportion of exogenous sequences than other samples, and urine has a higher proportion of tRNA sequences. Quantifications can also be performed for cellular datasets, such as ENCODE samples.

(B): Exogenous alignment phylogeny with genome reads
Exogenous sequence quantifications can be used to construct phylogenetic trees using exogenous genome reads and rRNA reads. This tree was constructed using 1.74 M genome reads from a saliva small exRNA-seq sample.

(C): Exogenous alignment phylogeny with ribosomal reads
This tree was constructed using 1127 K ribosomal reads from the same saliva small exRNA-seq sample as in Fig. 2B.

Figure S1: miRNA quantification comparison with miRDeep2
miRDeep2 is a commonly used software package for identification of miRNAs in deep sequencing data, Read quantifications for miRNAs based on results from processing an exRNA plasma sample with miRDeep2 and exceRpt are highly correlated, with an average Pearson correlation of 99.99%. This suggests that results from exceRpt is consistent with existing tools.

Figure S2: miRNA abundance for exRNA samples
exceRpt produces miRNA quantifications for exRNA samples, which can be used to see which miRNAs are enriched within which samples. Here, enriched miRNAs in samples from four different biofluids are shown.

Figure S3: Quality Control metrics
ERCC QC metrics are based on number of transcriptome reads and ratio of RNA annotated reads to the genome reads. The horizontal and vertical lines define QC cutoffs, and most exRNA Atlas samples meet the standards in the upper right quadrant.

Figure S4:
(A): Genboree interface for exceRpt
Signing up fro a graphical, browser-based interface at Genboree.org is the quickest way to start processing exRNA samples using exceRpt. Shown is the working interface for Genboree, including navigation functionalities and locations for inputs and outputs.

(B): Docker implementation for exceRpt
exceRpt is also available as a Docker image at github.com/gersteinlab/exceRpt/. Shown are example commands for running a sample to the exceRpt pipeline using Docker. Detailed instructions are on the Github webpage.

Figure S5: Read distributions with unmapped reads
By including unmapped reads into the visualization of the read distributions for exRNA and ENCODE samples (Fig. 2A), we see that saliva also has a higher proportion of unmapped reads compared to the other biofluids.

Figure S6: Exogenous sequence characterization algorithm
For exogenous sequence alignment, reads assigned to most specific node possible.

STAR Methods:

KEY RESOURCES TABLE:

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited Data		
exRNA Atlas	ERCC	https://exrna-atlas.org/
Human reference genome build GRCh38 (UCSC hg38)	Genome Reference Consortium	https://www.ncbi.nlm.nih.gov/grc/human
Human reference genome build GRCh37 (UCSC hg19)	Genome Reference Consortium	https://www.ncbi.nlm.nih.gov/grc/human
Mouse reference genome build GRCm38 (UCSC mm10)	Genome Reference Consortium	http://www.ncbi.nlm.nih.gov/grc/mouse
miRBase version 21	(Griffiths-Jones, 2004)	http://www.mirbase.org/
GtRNAdb	(Chan and Lowe, 2009)	http://gtrnadb.ucsc.edu/
piRNABank	(Sai Lakshmi and Agrawal, 2008)	http://pirnabank.ibab.ac.in/
Gencode version 24 (hg38)	(Harrow et al., 2012)	http://www.gencodegenes.org/
Gencode version 18 (hg19)	(Harrow et al., 2012)	http://www.gencodegenes.org/
Gencode version M9 (mm10)	(Mudge and Harrow, 2015)	http://www.gencodegenes.org/
circBase	(Glazar et al., 2014)	http://www.circbase.org/
UniVec	NCBI	ftp://ftp.ncbi.nlm.nih.gov/pub/UniVec/
Ribosomal Database Project	(Cole et al., 2014)	http://rdp.cme.msu.edu/
Software and Algorithms		
exceRpt version 4.6.2	This paper	http://genboree.org/theCommons/projects/exrna-tools-may2014/wiki/Small%20RNA-seq%20Pipeline
Java	Oracle Corporation	https://www.java.com/
R version 3.2	The R Project	https://www.r-project.org/
FASTX version 0.0.14	Hannon Lab	http://hannonlab.cshl.edu/fastx_toolkit/
STAR version 2.4.2a	(Dobin et al., 2013)	https://github.com/alexdobin/STAR/releases
Bowtie 2 version 2.2.6	(Langmead and Salzberg, 2012)	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
Samtools version 1.3.1	(Li et al., 2009)	http://www.htslib.org/
FastQC v0.11.2	Babraham Bioinformatics	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
SRA-Toolkit version 2.3	NCBI	https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=software

Preprocessing

Input files of sequenced reads can either be in the form of FASTQ, BAM or SRA formats. exceRpt begins the preprocessing step by automatically identifying and removing 3' adapter sequences. Randomly barcoded 5' and/or 3' adapter sequences are increasingly being used in smallRNA sequencing in an attempt to identify and compensate for ligation and/or amplification artifacts. exceRpt is capable of removing and quantifying these biases at both the insert level, which reveals ligation/amplification bias, and the transcript level, which provides an opportunity to compensate for the bias by counting unique N-mer barcodes rather than counting the number of inserts. The pipeline than aligns against an input set of known spike-ins sequences if used in the

library construction, followed by a filter to remove low-quality reads and reads with large homopolymer repeats using the FASTX toolkit (QFILTER_MIN_READ_FRAC = 80, QFILTER_MIN_QUAL = 20). As the final preprocessing step, exceRpt aligns reads annotated sequences in the UniVec database (designed for filtration of common laboratory contaminants) and to endogenous ribosomal RNAs, both of which are highly variable in abundance in EV preparations. Bowtie2 is used for the alignments in the preprocessing steps of the exceRpt pipeline.

Endogenous Quantification

Reads that were not filtered out in the preprocessing steps of the pipeline are aligned to the endogenous genome and transcriptome of either human or mouse, using STAR. STAR uses the following parameters for the endogenous alignments by default, MIN_READ_LENGTH = 18, MAX_MISMISMATCH = 1, MISMATCH_OVER_L_MAX = 0.3, MATCH_N_MIN_OVER_L_READ = 0.9. Transcript abundances are calculated (RNAs are quantified using both raw read counts and normalized reads per million (RPM)). The user can prioritize the order that the annotations (miRBase, tRNAscan, piRNA, GENCODE, circRNA) are used for quantification based on our confidence in the presence of a given annotation in a given sample. By default the order for quantification is miRNAs, tRNAs, piRNAs, GENCODE transcripts (snRNAs, snoRNAs, miscRNAs, protein coding genes and lncRNAs) followed by circular RNAs based on our confidence of the likelihood of these annotation to be present in a small exRNA-Seq sample. The exceRpt pipeline can be used with the human host endogenous genome (either hg19 or grch38) or the mouse host endogenous genome (mm10) with corresponding annotations.

Exogenous Quantification

Before we analyze the remaining reads for potential exogenous sequences, we perform a second pass alignment against the host genome (allowing for novel gapped alignments) using STAR, with a more relaxed mapping criteria and a database of known repetitive sequences. Reads are then aligned to curated libraries of annotated exogenous miRNAs in miRBase and exogenous rRNA sequences in the Ribosomal Database Project (RDP), followed by alignment to the full genomes of all sequenced bacteria, viruses, plants, fungi, protists, metazoa, and the following 12 vertebrate genomes: chicken, cod, cow, dog, duck, frog, horse, rabbit, pig, sheep, tilapia, and turkey. Multiple STAR indexes are constructed for the bacterial genomes, the plant genomes, the metazoan genomes, fungi, protest and virus genomes and the vertebrate genomes so that exogenous genome alignment, which is the most time-consuming step of the exceRpt pipeline, can be parallelized. We then use STAR to align the remaining reads to these genomes allowing for no mismatches (in order to be as conservative as possible in identifying possible exogenous sequences).

Since many exogenous genomes have a high degree of sequence similarity based on evolution, we find many reads that align to a exogenous genome alignment to multiple genomes. We assign reads that align to exogenous genomes to the position in the phylogenetic taxonomy tree based on the node is most parsimonious with the different genomes that the read aligns (see Supp. Fig. S6). Reads that align to a unique genome are aligned to a leaf node in the phylogenetic tree while reads that align to multiple genomes are assigned to nodes high up in the tree. We also independently perform this assignment for the reads that align to exogenous rRNA sequences in the context of the phylogenetic taxonomy tree constructed using rRNA sequences. The structures of the phylogenetic taxonomy trees constructed independently using the disjoint set of exogenous genome and rRNA reads can be used for comparison.

Quality Control

To evaluate the samples themselves and identify outliers, the ERCC developed QC data standards which exceRpt evaluates uniformly on all input samples: (1) datasets are required to have at least 100,000 reads that overlap with any annotated RNA transcript in the host genome, and (2) over 50% of the reads that map to host genome also align to any RNA annotation. The first criterion ensures that enough reads are generated for quantification (the minimal read depth required for the minimal normalized expression of an annotated RNA to be greater than 1 RPM) and the second ensures that the reads mostly align to RNA, as opposed to DNA contamination from cellular sources. We find that 95% of the ~2500 exRNA-Seq datasets that have been uniformly processed in the exRNA Atlas with exceRpt meet both criteria (Figure S3), with most datasets well above both thresholds.

QUANTIFICATION AND STATISTICAL ANALYSIS:

All quantification analyses were performed in R. Annotated RNAs are quantified using both raw read counts and are also normalized by sample across all RNA biotypes to reads per million mapped reads (RPM)). Diagnostic plots and output statistics are also automatically generated by exceRpt.

DATA AND SOFTWARE AVAILABILITY:

The graphical, browser-based, user-friendly interface for uploading and processing of exRNA-seq datasets with exceRpt is available at the Genboree Workbench: http://genboree.org/theCommons/projects/exrna-tools-may2014/wiki/Small%20RNA-seq%20Pipeline. The exceRpt source code may be downloaded and installed manually for the greatest degree of flexibility (github.gersteinlab.org/exceRpt). For this option, the dependencies and databases used by the pipeline must be downloaded and installed, and the makefile must be properly configured for use (instructions for installation and use are available from the Github page). exceRpt includes a script (mergePipelineRuns.R) to combine outputs from multiple samples for downstream comparative analysis. Alternatively, the exceRpt Docker image with all required dependencies may be used for installation on the user's own machine or cluster (github.com/gersteinlab/exceRpt/).

ADDITIONAL RESOURCES:

The ERCC exRNA Atlas can be found here: https://exrna-atlas.org/
The ERCC quality control standards can be found here:
https://exrna.org/resources/data/data-quality-control-standards/

Supplemental Information:

Document S1. Figures S1–S5

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