

XPRESSyourself: Automating and Democratizing High-Throughput Sequencing

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With the advent of high-throughput sequencing platforms, expression profiling is becoming common-place in medical research. However, for the general user, a computational overhead often exists. The XPRESSyourself suite aims to remove some of these barriers for users with limited or advanced computational experience alike and create a series of tools aimed at standardizing and increasing throughput of data processing and analysis. The XPRESSyourself suite is currently broken down into two software packages. The first, XPRESSpipe, automates the pre-processing, alignment, quantification, normalization, and quality control of single-end, paired-end RNAseq, and ribosome profiling sequence data. The second, XPRESStools, is a Python toolkit for expression data analysis, compatible with private or RNAseq datasets. This software suite is designed where features can easily be modified, and additional packages can be included for processing of other data types in the future, such as CHIPseq or genome alignment. In addition, this package offers several new tools useful in processing RNA-seq data, specifically for ribosome profiling. We validated the performance of this suite by processing and analyzing publicly available datasets and comparing the output with published results.

XPRESSyourself is freely available on GitHub: <https://github.com/XPRESSyourself>

1 Introduction

High-throughput profiling of gene expression data has revolutionized biomedical, industrial, and basic science research. Within the last two decades, RNA-seq has found itself the forerunner technology for highest quality expression profiling, as it can measure relative transcript abundance, differential splice variants, sequence polymorphisms, and more (1). This technology has also been adopted to create technologies such as single-cell RNA-seq, capable of assaying the transcriptional profile cell by cell; and ribosome profiling, which measures ribosome occupancy and translation efficiency (2).

While vast strides have been made to these technologies, various bottlenecks still exist. For example, while more and more researchers are becoming accustomed to these technologies, learning the intricacies of the different tools used in processing RNA-seq data can be inhibitory if users are not aware of the proper tools to use

or use outdated software (3, 4). For the experienced user, developing robust pipelines that process and check datasets can be laborious and introduce variability to data processing.

While several pipelines have emerged over the last several years that have been built to tackle various aspects of these bottlenecks, many suffer from usability issues, or are not easily modifiable. Additionally, few if any offer a thorough set of integrated tools for handling common quality control issues or reference creation. For example, a common bias in ribosome profiling libraries is a 5' transcript pile-up (5–7). It is recommended that this region of each transcript not be quantified when processing ribosome profiling libraries; however, currently no tools exist to aid the general user in doing so (8, 9).

In response to these issues surrounding the automation and democratization of sequencing technology, we created the XPRESSyourself bioinformatics suite for processing and analyzing high-throughput expression data. In creating this tool, we focused on five aspects in order to create an easy, reliable tool where large barriers-to-entry would be eliminated. These were create a tool that was useful, usable, reliable, efficient, and flexible (10).

With XPRESSyourself, the user is provided with a complete suite of software to handle pre-processing, aligning, and quantifying reads, performing quality control via various meta-analyses of pre- and post-processed reads, and tools to perform the bulk of sequence analysis and generation of figures for publication.

2 Materials and Methods

2.1 XPRESSpipe

XPRESSpipe pipelines for single-end RNA-seq, paired-end RNA-seq, and ribosome profiling offer a handful of tunable parameters to the user, while keeping most hidden to maintain TCGA alignment standards. In the future it is feasible that additional tunable parameters will be added. For the purposes of this manuscript, we will focus on ribosome profiling examples, while the majority of statements are applicable to single- and paired-end RNA-seq. More details can be found in the documentation (<https://xpresspipe.readthedocs.io/en/latest/>). Table 1 outlines these parameters.

Table 1: Summary of XPRESSpipe pipeline arguments.

Arguments	Description
Required	
-i, --input	Path to input directory
-o, --output	Path to output directory
-r, --reference	Path to parent organism reference directory
-g, --gtf	Path and file name to GTF used for alignment quantification
-e, --experiment	Experiment name
Optional	
-a, --adaptors	Specify adaptor as string (only one allowed) – if “None” is provided, software will attempt to auto-detect adaptors – if “POLYX” is provided as a single string in the list, polyX adaptors will be trimmed
-q, --quality	PHRED read quality threshold (default: 28)
--min_length	Minimum read length threshold to keep for reads (default: 18)
--output_bed	Include option to output BED files for each aligned file
--output_bigwig	Include flag to output bigwig files for each aligned file
--method	Normalization method to perform (options: “RPM”, “TPM”, “RPKM”, “FPKM”, “LOG”)
--batch	Include path and filename of dataframe with batch normalization parameters
--sjdbOverhang	Sequencing platform read-length for constructing splice-aware reference previously (see documentation for more information)
-m, --max_processors	Number of max processors to use for tasks (default: No limit)

2.1.1 Installation

XPRESSpipe can be compiled from source (<https://github.com/XPRESSyourself/XPRESSpipe>) or a version-controlled Docker image (<https://www.docker.com/>) can be loaded using the following commands:

Listing 1: curateReference example

```
$ docker image pull jordanberg/xpresspipe:latest
```

Table 2: Summary of dependency software, accession location, and purpose in the XPRESSpipe package.

Package	Purpose	Reference
fastp	Read pre-processing	(11)
STAR	Reference curation and read alignment	(12)
samtools	Alignment file manipulation	(13)
bedtools	Alignment file manipulation	(14)
deeptools	Alignment file manipulation	(15)
htseq	Read quantification	(16)
fastqc	Quality Control	(17)
multiqc	Quality Control	(18)
DESeq2	Perform differential expression analysis	(19)
dupRadar	Measure library complexity	(20)
pandas	Data manipulation	(21)
numpy	Data manipulation	(22, 23)
scipy	Data manipulation	(24)
matplotlib	Plotting	(25)
xpresstools	Normalization and matrix manipulation	This paper

XPRESSpipe is built upon several pre-established software packages, listed in Table 2. These dependencies are included in any Docker images for XPRESSpipe; however, if installing manually, these packages will need to be installed by the user.

2.1.2 Inputs

While inputs will vary sub-module to sub-module, and further information can be found in the documentation (<https://xpresspipe.readthedocs.io/en/latest/>) or by entering `xpresspipe <sub-module name> --help`, a few points of guidance are important to consider.

- Single-end reads should end in `.fa`, `.fasta`, `.txt`
- Paired-end reads should end in `.read1/2.fa` or `.r1/2.fa`, where `.fa` could also be `.fasta` or `.txt`
- The transcriptome reference file should be a valid GTF file and should be named `transcripts.gtf`
- If specifying a group of fasta files to use for alignment or reference curation, the directory containing this files cannot contain any other files ending in `.txt` or `.fa`

Other sub-modules that handle a point in the middle of sequence processing need to be of appropriate file type, explained more in the help menu or documentation.

2.1.3 Reference Curation

One of the first preparatory steps of RNAseq alignment is curating a reference for the alignment software to map reads. For the purposes of the current version of XPRESSpipe, a STAR (12) reference should be created. A Ensembl-formatted GTF should also be placed in the reference directory and be named `transcripts.gtf`. Additional modifications are recommended to this file, which can be beforded using the `modifyGTF` sub-module,

where the GTF can be formatted to only contain protein-coding genes and/or contain only the longest annotated transcript. For the purposes of ribosome profiling, where 5' and 3' biases are frequent, the 5' and 3' ends of each gene record can be trimmed using the same function (8,9). While each of these steps are available as stand-alone sub-modules, all reference files used by XPRESSpipe, including STAR files and modified transcriptome files can be created by running the `curateReference` command. An example is shown below for creating a ribosome profiling-ready reference XPRESSpipe directory. The following assumes one is avoiding mapping to the first 45 nucleotides and last 15 nucleotides of the longest transcript for each gene, will only quantify to protein coding regions, and is tailored for mapping 50-bp single-end RNA-seq reads. As this can be a time-consuming process, we will leave the `--max_processors` argument as default in order to utilize all cores available to the computing unit.

Listing 2: `curateReference` example

```
$ xpresspipe curateReference -o /path/to/output/location/ \
                             -f /path/to/fastq/genome/files/ \
                             -g /path/transcripts.gtf \
                             --protein_coding \
                             --longest_transcript \
                             --truncate \
                             --truncate_5prime 45 \
                             --truncate_3prime 15 \
                             --sjdbOverhang 49 \
                             --max_processors 12
```

While current available arguments are limited, the design is simple enough where arguments could be added easily to give more control over STAR reference creation. Current setting are designed to follow with The Cancer Genome Atlas (TCGA) standards (<https://www.cancer.gov/tcga>).

2.1.4 Read Processing

While all intermediate steps of the pipelines can be run singly, we will describe the outline of the software in the context of the pipelines. Pipelines and individual sub-modules are capable of being run in a parallel manner for each input file, thus speeding up the overall process.

1. **Trim:** First, read need to be cleaned of artifacts for the library preparation and sequencing process. These include adaptors, unique molecular identifier (UMI) sequences, and base calls with low confidence. By doing so, non-native sequences are removed and reads can align properly to the reference sequence. XPRESSpipe uses `fastp`, a faster, more accurate trimming package that has improved alignable read output (11). Adaptor sequence, base quality, and read length are all adjustable parameters available to the user. Descriptions of the options can be found in Table 1.
2. **Align:** After trimming, reads are then aligned to a reference genome. XPRESSpipe uses STAR, which, while taking a memory intensive approach, is fast and one of the most accurate aligners currently available (12, 26). XPRESSpipe adheres to TCGA standards and performs a two-pass alignment of reads wherein first splice junctions are mapped and built into the reference; and second, reads are mapped accounting for these junctions. A sorted by coordinate and indexed BAM file is output and only unique alignments pass on to the next steps. Optionally, bed and bigwig files can also be output. PCR duplicates are also detected and marked or removed for downstream processing.
3. **Count:** XPRESSpipe quantifies read alignments for each de-duplicated input file using `htseq-count` (16).

If a modified GTF was provided as input, it is used here to avoid potential pitfalls with read quantification (i.e. a read being counted as a multi-mapper when belonging to multiple transcripts for the same gene). By default, htseq behavior conforms to TCGA standards by being strand agnostic, mapping assuming reads were sorted by name, and by using the `intersection-nonempty` method for handling reads overlapping multiple genes.

4. **Normalize:** Methods for count normalization are available within XPRESSpipe. For normalizations involving transcript length, the appropriate GTF (transcripts-only recommended) must be provided. For samples sequenced on different chips, prepared by different individuals, or on different days, the `--batch` argument should be provided along with the appropriate metadata matrix. Sample normalization methods available include reads-per-million (RPM), Reads-per-kilobase-million (RPKM) or Fragments-per-kilobase-million (FPKM), and transcripts per million (TPM) normalization, as outlined in equations 1-4 (27) (see Table 1 for more information on arguments).

$$RPM = \frac{(\# \text{ number reads per gene}) \cdot 1e6}{(\# \text{ mapped reads per sample})} \quad (1)$$

$$RPKM = \frac{(\# \text{ number reads per gene}) \cdot 1e6 \cdot 1e3}{((\# \text{ mapped reads per sample}) \cdot (\text{gene length (bp)}))} \quad (2)$$

$$FPKM = \frac{(\# \text{ number fragments per gene}) \cdot 1e6 \cdot 1e3}{(\# \text{ mapped fragments per sample}) \cdot (\text{gene length (bp)})} \quad (3)$$

$$TPM = \frac{(\# \text{ number fragments per gene}) \cdot 1e3 \cdot 1e6}{(\text{gene length (bp)}) \cdot (\# \text{ mapped fragments per sample})} \quad (4)$$

5. **Quality Control:** It is important to perform quality control of sequencing sample to ensure they are reliable and their biological insight can be trusted. XPRESSpipe performs a variety of quality control measures. For each analysis type, high-resolution summary plots are output for all samples in a given experiment.

- **Read Length Distribution:** Per sample, the lengths of all reads are analyzed by FastQC (17). This is another useful way to look for biases in a sequencing library, or make sure that ribosome fragments were enriched in a ribosome profiling experiment.
- **Library Complexity:** Analyzing library complexity is an effective method for analyzing the robustness of a sequencing experiment of capturing various mRNA species. The analysis is performed using the PCR duplicate-tagged BAM files for each sample by dupRadar (20).
- **Metagene Profile:** In order to identify any general 5' or 3' biases in captured transcripts, a metagene profile can be created for each sample. This is done by determining the middle genomic coordinate for all aligned reads and mapping them to exon space for any mapped transcripts. Required inputs are an indexed bam file and an unmodified GTF reference file. For each mapped coordinate, the metagene position is calculated as:

$$p = \frac{e \cdot 100}{l} \quad (5)$$

Where p is the meta-position, e is the coordinate position from the start of the transcript in exon space, and l is the cumulative length of all exons for the given transcript.

In the case where a mapped coordinate falls within multiple genes, a penalty is assigned as:

$$c = \frac{1}{n} \quad (6)$$

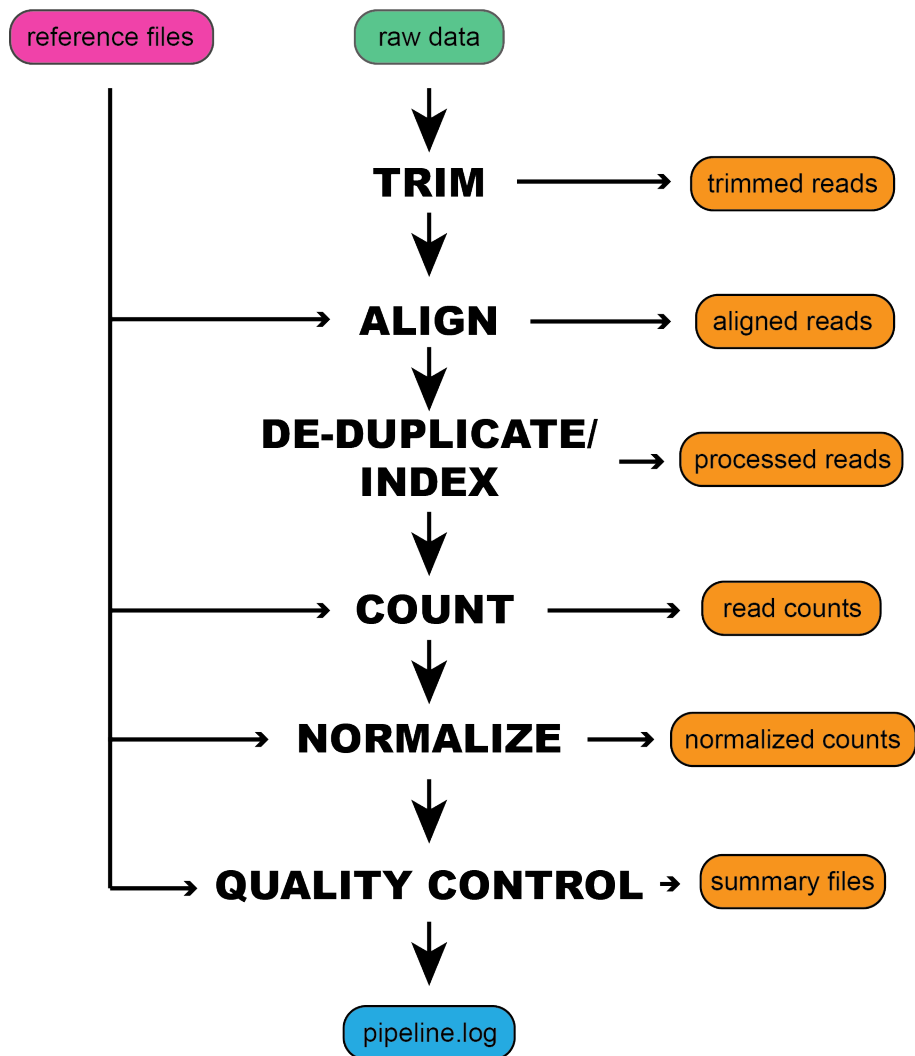


Figure 1: An example schematic of the inputs required by XPRESSpipe and organization of the outputs.

Where c is the count score for a given metaposition and n is the number of transcripts a given coordinate mapped to in exon space.

- **Codon Phasing/Periodicity:** In ribosome profiling, a useful measure of a successful experiment is by investigating the codon phasing of ribosome footprints (). To do so, the P-site is calculated for each mapped ribosome footprint by taking the genomic coordinate 16 nucleotides upstream of the 3' end of each transcript and mapping it in exon space from the start of each transcript. The same inputs are required as for the `metagene` sub-module, and the penalty is calculated in the same manner. P-sites are calculated as:

$$p = \frac{e}{l} \quad (7)$$

Where p is the meta-position, e is the coordinate position from the start of the transcript in exon space, and l is the cumulative length of all exons for the given transcript.

2.1.5 Outputs

While outputs will vary sub-module to sub-module, generally, the user will specify a parent output directory and necessary sub-directories will be created based on the step in the pipeline. Further information can be found in the documentation (<https://xpresspipe.readthedocs.io/en/latest/>) or by entering `xpresspipe <sub-module name> --help`. Figure 1 provides an example of output file scheme for XPRESSpipe.

2.1.6 Analyses

XPRESSpipe has two analytical sub-modules. The first simplifies the R package, DESeq2 (19), for the user and acts as a command line interfaced option to access this statistical package.

The second, new analytical tool introduced in XPRESSpipe is `rrnaProbe`. Ribosomal RNA (rRNA) contamination is common in RNA-seq library preparation and the bulk of RNA in a cell at any given time is dedicated to rRNA. As unique rRNA sequences are relatively few and therefore highly repeated in sequencing libraries without intervention, depletion of these sequences is often desired in order to have better depth of coverage of non-rRNA sequences. In order to facilitate this depletion, many commercial kits are available that target specific rRNA sequences for depletion, or that enrich mRNA polyA tails. However, and especially in the case of ribosome profiling experiments, where RNA is digested to create ribosome footprints that commercial depletion kits won't detect and polyA selection kits are inoperable as footprints will not have the requisite polyA sequence. To this end, custom rRNA probes are recommended (2, 8). `rrnaProbe` works on a directory containing fastqc (17) zip compressed files to detect over-represented sequences for each sample. These sequences are then collated to create consensus fragments. A rank ordered list of over-represented fragments within the appropriate length range to target for depletion is then output. A BLAST (28) search on consensus sequences intended for probe useage can then be performed to verify the fragment maps to an rRNA sequence.

2.2 XPRESStools

XPRESStools is a python library of analysis features that builds upon existing packages, such as Matplotlib (25) and Seaborn (29) to generate flexible, specific analyses frequently used by biological researchers that can each be executed in a single line of code. Additionally, many of the introduced analyses are currently available in the R environment but libraries in Python, a programming language becoming more and more common in biological research, have not yet been developed. A summary of these tools is summarized below and methods are discussed in subsequent sections. For the sake of brevity, methods and capabilities new or more automated for a Python toolkit will be discussed in detail. We refer the reader to the documentation (<https://xpresstools.readthedocs.io/en/latest/?badge=latest>) for more details instructions for other features currently in the toolkit, as well as for future features to be added.

2.2.1 Getting Data

XPRESStools is designed for analyzing gene expression data, but it tractable to most data types assuming two conditions are met:

1. **Expression Matrix:** It is assumed the data matrix is $i * j$ where i (columns) are samples and j (rows) are genes or other relative measurement points.
2. **Metagene Table:** It is assumed the metagene table is a two column data matrix where column 0 is the sample ID (as specified in i of the expression matrix) and column 1 is the sample group (for example, wild-type and treatment).

Several functions are built into XPRESStools for importing and formatting this data. Options include imported microarray and RNAseq expression data and metadata from the GEO database, as well as custom data and metadata that follows required formatting. Several methods for normalization, such as RPM, RPKM, FPKM, TPM and log-scale normalization are accessible within this toolkit.

2.2.2 Analyzing Data

While a litany of analysis tools are included in XPRESStools as of time of writing, we will focus on tools unique to this Python library and refer to reader to the documentation for further details and examples of analysis features, current and future.

- **Principle Components Analysis:** Principle components analysis (PCA) for the data matrix are performed with Python's scikit-learn package (30) and desired principle components are plotted in a scatter plot via the matplotlib (25) and seaborn (29). The XPRESStools PCA module, as in many other analysis modules within XPRESStools, samples are color-coded by cross-referencing the data matrix with the metagene table to determine sample label. A dictionary is additionally passed into the function that maps a particular color to each sample label. Confidence intervals are plotted over the scatterplot using numpy (22, 23) as follows:

1. Compute the covariance of the two principle component arrays, x and y using the `numpy.cov()` function.
2. Compute the eigenvalues and normalized eigenvectors of the covariance matrix using the `numpy.linalg.eig()` function.
3. Compute the θ of the normalized eigenvectors using the `numpy.arctan2()` function and converting the output from radians to degrees using `numpy.deg()`.
4. Compute the λ of the eigenvalues by taking the square root of the eigenvalues.
5. Plot the confidence intervals over the scatter plot: The center point of the confidence interval is determined from the means of the x and y arrays. The angle is set equal to θ . The width of the confidence interval is calculated by

$$w = \lambda_x \cdot ci \cdot 2$$

where ci is equal to the corresponding confidence level (i.e. 68% = 1, 95% = 2, 99% = 3). The height is similarly computed by

$$h = \lambda_y \cdot ci \cdot 2$$

- **Volcano Plotting:** Volcano plots are an efficient method for plotting magnitude, direction, and significance of changes in expression or other measurements between two conditions. Providing the categorical names for samples of two conditions in the metadata matrix, XPRESStools will automate the calculation and plotting of this plotting method. For each gene, expression levels are averaged between the two conditions and the $\log_2(\text{fold change})$ is calculated. Additionally, for each gene, the P-value between the two conditions is calculated using scipy's individual T-test function (24). The $\log_2(\text{fold change})$ and $-\log_{10}(\text{P-value})$ is then plotted for each gene between the two conditions. Additional features available are the ability to plot threshold lines, highlight subsets of genes within the plot, and label specific genes by name.

When these computations are performed with a metadata table containing several sample types, these computations will be performed for each label type individually and plotted on the scatter plot.

2.3 Availability

The source code for these packages is open source protected under the GPL-3.0 license and can be publically accessed at <https://github.com/XPRESSyourself>. Updates to the software are version controlled and maintained

on GitHub. Packages are pip installable in addition to source installable. XPRESSpipe is available as a Docker image. Jupyter notebooks and video walkthroughs are included on <https://github.com/XPRESSyourself> for guiding a user through use of the packages. Documentation is hosted on readthedocs (31).

2.4 Cost Analysis

3 Results and Discussion

3.1 Validation

3.1.1 Ribosome Profiling Dataset

3.1.2 TCGA Dataset

3.1.3 Ease of Use Case Study

3.2 New Insights into Metabolism

4 Conclusions

We have described herein a new software suite, XPRESSyourself, a collection of tools and pipelines to aid in expression data processing and analysis. The software was designed to be flexible and modular so that in the future, new modules and tools can be easily added to and tested in the software suite.

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