# Local ORF Detector Manual
## (LOD)

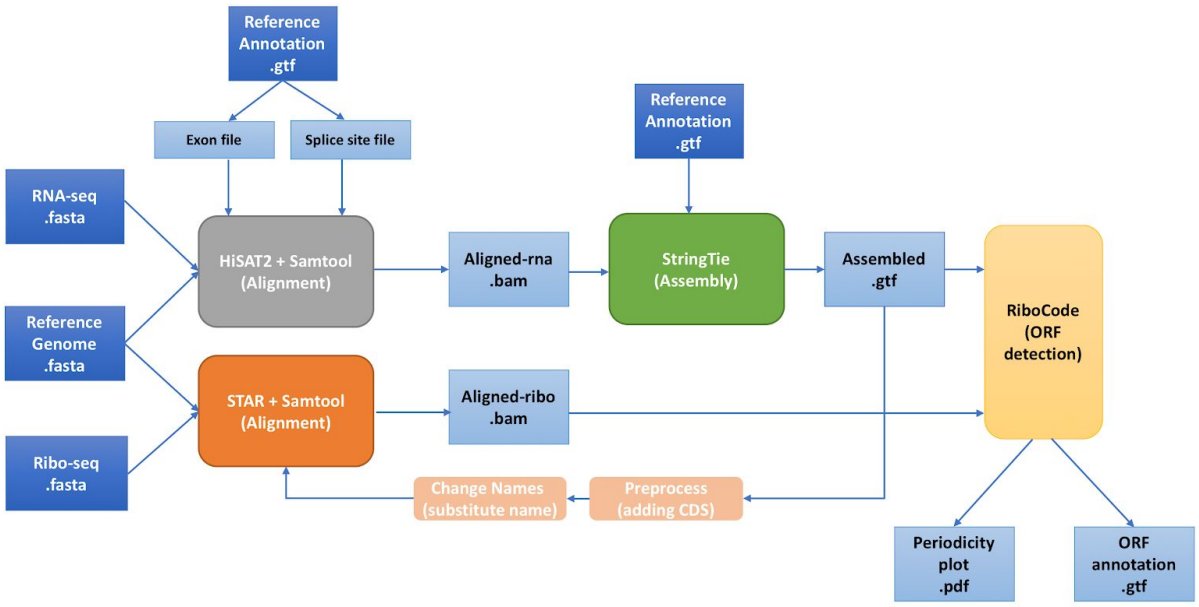
# Table of Contents

# Introduction

## About this Pipeline

This new pipeline of annotating open reading frames (ORFs) using both RNA-seq and Ribo-seq data integrates multiple bioinformatics tools. *RiboCode* is a newly-invented public tool that can make use of Ribo-seq data to identify ORFs. The reference .gtf file that RiboCode requires normally comes from the public database and therefore loses the specificity in terms of annotating samples from a specific cell type or condition. Here we introduce a solution of making a tailored experimental reference transcript based on the RNA-seq data that are gathered from the same experiment. We use *HiSAT2* for RNA-seq alignment and *StringTie* for transcript assembly to generate the experimental reference .gtf file. Then the Ribo-seq reads are aligned with *STAR* and the resulting alignment files are fed into RiboCode together with our newly assembled transcript to call ORFs.

## Workflow




Alternatively, if the users do not have enough memory to run HiSAT2, user will eventually be able to use STAR as a backup aligner to carry out the first round of

RNA-seq alignment too. However, at the moment, this has not been implemented yet.

# Packages

Note: Our pipeline incorporates all the following packages in binary format except for RiboCode. User could only try to install RiboCode manually to set up all the environment needed. For parameters please refer to Running Pipeline-Parameters section.

## Samtools

Sequence Alignment/Map tools(Samtools) is a package that takes .sam and .bam files as inputs to implement utilizes for post-processing alignment. The most frequently used functions of samtools are viewing the aligned files, sorting the aligned files (which is required before assembly and many other following operations), and converting between .sam files and .bam files.

For more details, please refer to:
https://www.ncbi.nlm.nih.gov/pubmed/19505943
Download and build the environment of Samtools from:
https://github.com/samtools/samtools

## HiSAT2

HiSAT2 is an aligner that maps sequenced reads to the reference genome considering the alternative splicing.

For more details, please refer to:
https://www.ncbi.nlm.nih.gov/pubmed/25751142
Download and build the environment of HiSAT2 from:
https://ccb.jhu.edu/software/hisat2/manual.shtml#building-from-source

### STAR

Spliced Transcripts Alignment to a Reference (STAR) is an algorithm designed to align high-throughput RNA-seq data to a reference file using a novel strategy for spliced alignments.

For more detail, please refer to:
https://www.ncbi.nlm.nih.gov/pubmed/23104886
Download and build the environment of STAR from:
https://github.com/alexdobin/STAR

### StringTie

StringTie is an assembler of RNA-Seq alignments into potential transcripts. It takes the alignments of raw reads as input and can be run in either reference-guide or *de novo* mode. In our pipeline, transcripts are assembled only in a reference-guide mode. By doing so, we sacrifice the ability to identify new transcripts to gain benefits in specificity.

For more detail, please refer to:
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4643835/
Download and build the environment of StringTie from:
https://github.com/gpertea/stringtie

### RiboCode

RiboCode, a newly developed statistical method, used for gene annotation and characterization. RiboCode aimed to identify translated ORFs using the ribosome-profiling data.

For more detail, please refer to:
https://www.ncbi.nlm.nih.gov/pubmed/29538776
Download and build the environment of riboCode from:
https://github.com/xryanglab/RiboCode

## Package installation

The only package the users need to install manually is RiboCode. To do so, pip must have write privileges for folders in the path, python-devel must be installed, and pip must be installed

pip install RiboCode

For full documentation please see: https://github.com/xryanglab/RiboCode

# Running Pipeline

## Required Input File:

All the reference files can be downloaded from e!Ensemble:
http://useast.ensembl.org/info/data/ftp/index.html
All the experimental data can be generated either by the users or downloaded from the public database like NCBI: https://www.ncbi.nlm.nih.gov/

1. Reference genome fasta file: Serves as reference genome file for STAR and RiboCode.
2. Reference annotation gtf file: Serves as reference annotation file for StringTie. Our newly assembled transcript reference gtf file is built in a reference-guided mode.
3. RNA-seq fasta file: experimental RNA profiling to build our own transcripts.
4. Ribo-seq fasta file: experimental Ribosome profiling to generate ORF annotation.

## Usage:

The LOD.py script can NOT be moved from the project folder. The relative path to the binaries is used.
The user can use LOD in two ways. The full path to the executable can be used, or the user can create a sym-link in their path for convenience. By creating a sym-link, LOD.py can be called from anywhere without having to specify the full path.

```
ln -s /path/to/LOD.py /usr/local/bin/
```

## Command Line:

Example:

```
python LOD.py --refFa <Mus_musculus.GRCm38.75.dna.primary_assembly.fa> --refGTF <Mus_musculus.GRCm38.75.gtf> --RNASeq <RnaSeq.fa> --RiboSeq <RiboSeq.fa> --f 0.99 --n 25
```

### Parameters:

Required:
--refFa: Reference genome fasta file.
--refGTF: Reference annotation gtf file
--RNASeq: RNA-seq fasta file
--RiboSeq: Ribo-seq fasta file

Optional:
--n: numThreads. If using a large number of threads (~20-30) be aware of the max number of files your system allows. You may need to adjust this to avoid harddrive bomb. https://github.com/alexdobin/STAR/issues/269

--f: when assembling transcripts using StringTie, the user can set the minimum isoform abundance of the predicted transcripts as a fraction of the most abundant transcript assembled at a given locus <0.0-0.1>. The default fraction is set at a relatively high level to eliminate transcript isoforms with low confidence. Default: 0.9

## Getting Started with Pipeline

For your convenience, we put all the intermediate files in the ./outputs/ folder, only the final .gtf file, some Log files we haven't gotten around to removing yet, and the PDF file would be in the current directory.

### HiSAT2:

HiSAT2 will generate .sam file from the RNA-seq data, reference genome, and the reference annotation file. It is the alignment result of RNA-seq reads to the reference genome. Samtools is then used to make a .bam file from the alignment result.

### STAR (Alternative):

The first time through STAR will generate .bam file which would be in the ./intermediates/ folder for aligning the RNA-seq reads to the reference genome. Samtools is then used to make a bam file. The second time through STAR after StringTie takes the assemble gtf file as input and generates a .bam file in the ./intermediates folder that aligned the Ribo-seq data to the experimental genome.

STAR will first index the reference genome using the reference gtf file. To do this, we will create a reference_genome directory to hold these index file. Then in the next step, STAR aligns the RNAseq reads to the indexed reference genome, creating a sam file (shown below). The sam file is then converted to a bam file and sorted using samtools.

Sample output:

In a sam file, each line stands for a linear segment that is aligned. The standard format of a sam file include 11 fields for each entry. These fields are mandatory and should always appear in the same order. If the information is missing, the default values can be imputed with either '0' or '*' depending on the specific field. The following table shows an overview of the 11 fields in the SAM format:

Please refer to samtool manual for more information:
https://samtools.github.io/hts-specs/SAMv1.pdf

## StringTie:

StringTie would generate .gtf file from the alignment results provided in .bam format, the assembly is carried out in a guided mode, therefore another reference gtf file is also required. The resulting .gtf file would be in the ./outputs/ folder. The standard format of a .gtf file contains the following 9 fields in the exact order: seqname, source, feature, start_position, end_position, score, strand, frame and attributes as a string including information such as gene_id, transcript_id, exon_number, reference_id, ref_gene_id, ref_gene_name, coverage, FPKM, and TPM.

Sample output:

Please refer to StringTie manual for more information:
https://ccb.jhu.edu/software/stringtie/index.shtml?t=manual

## preprocessGTF:

A python script that adds the CDS annotation and the start/stop codon annotations in the reference to the newly assembled gtf file after StringTie. The entries are added whenever there's a matching transcript found in the reference gtf file. The CDS and start/stop codon information are required in order to build the periodicity when trying RiboCode.

## ChangeName:

A python script that changes the gene_id and the transcript_id to the reference_gene_id and reference_id respectively whenever there's a matching transcript/exon found in the reference gtf file. It keeps the newly discovered transcripts with the names that the StringTie randomly assigned, possibly starting with ‘STRG’.

## STAR:

STAR will first index the reference genome using the newly assembled experimental gtf file. To do this, we will create a directory to hold these index file. Then STAR aligns the Ribo-seq reads to the indexed experimental genome, creating a sam file. The sam file is then converted to a bam file and sorted using samtools.

## RiboCode:

RiboCode would first generate a <RiboCode_annot> folder in the ./outputs/ folder that contains <transcript_cds.txt>, <transcripts_sequence.fa>, and <transcripts.pickle> when running ‘prepare_transcripts’. These are only intermediate files for following steps, ignore them if you only want the final results. The second step, ‘metaplots’, would output a PDF file in current folder which plots the aggregate profiles of the distance from the 5'-end of reads to the annotated start codons (or stop codons). The last step is running ‘ribocode’ and this would generate a <RiboCode_ORFs_result.gtf> file in current folder which contains the predicted ORFs.

Sample output:

## Pitfalls and Limitations:

Currently the pipeline requires a large amount of computational requirements. When Hisat is provided a gtf file for indexing, it can take 200GB of RAM to index. When aligning reads to the indexed genome, if the number of threads is too high, thousands of temporary files can be written. Most computers have a limit on the number of open files at a time to prevent fork/recursion bombs. This will cause the program to crash.

The nature of the novel ORFs detected has not been evaluated. It is entirely possible that many of these novel ORFs have a similar regions in the reference annotations and stringtie failed to create them during the assembly step.