RBP Functional Target Predictor using Multiple Peak Callers (RFM)

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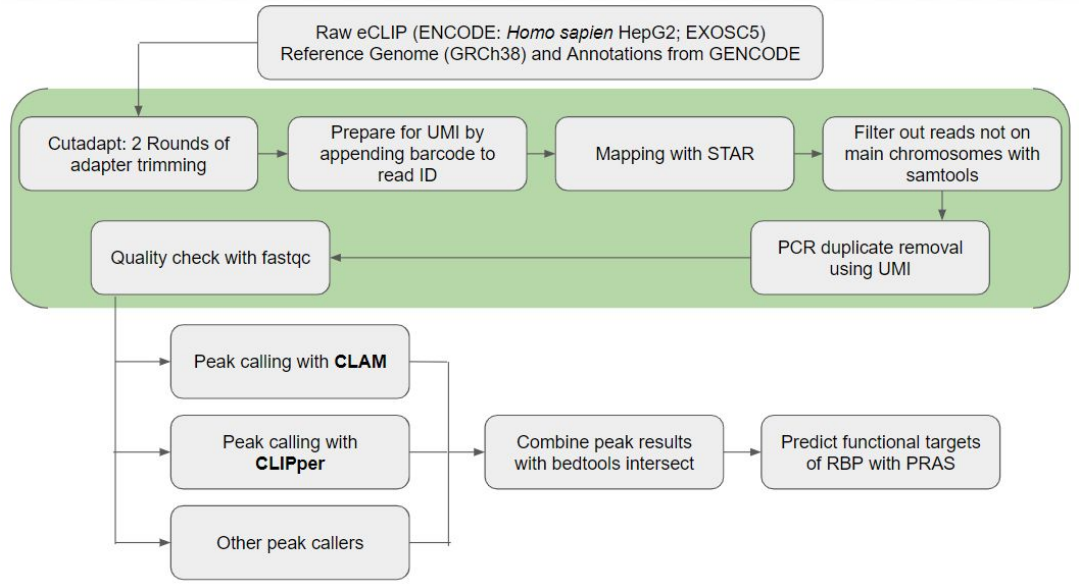
I. Introduction

About this Pipeline

RNA binding proteins (RBPs) are essential for the cell, playing a large role in the regulation of transcription, RNA processing, and gene expression (e.g. alternative splicing, mRNA export, translation, turnover, and localization). In order to learn more about these proteins, studies have focused on identifying RBP binding sites and interactions by utilizing methods such as cross-linking immunoprecipitation (CLIP) and other variations. The basic protocol for processing the data includes three main steps: Preprocessing, peak calling, and postprocessing. Preprocessing involves trimming off the adapters from the raw sequence reads and mapping them on a reference genome. Peak calling algorithms attempt to extract authentic binding sites and identify motifs. Postprocessing involves integrating the data and making predictions on potential binding sites. Unfortunately, the peak calling process is not perfectly accurate, containing false negatives and positives. The primary purpose of this pipeline is to utilize and combine peak calls from different software (maximum of three) as a method to improve accuracy and reduce the number of false negatives and positives. This pipeline will generate peak data using *CLAM*, so the user will need to provide one or two more peak data (bed format). In our example, we utilize raw eCLIP data of two replicates (*Homo sapien* HepG2, *EXOSC5*) and *CLIPper* data from *ENCODE*. The *CLIPper* data is then combined with the generated *CLAM* data and used in *PRAS* to make predictions on the functional targets of RBP.

Workflow

We arbitrarily chose to utilize eCLIP data of RBP EXOSC5 from *Homo sapien* HepG2 cell line.



II. Packages/Installation

We suggest installing the tools into a python environment (anaconda3).

Cutadapt

Cutadapt removes unwanted sequence elements such as adapters, primers, and poly-A tails from raw sequence reads used as inputs. Cleaning of the input data in this way is crucial, as it removes elements which may result in false positives in read data.
https://cutadapt.readthedocs.io/en/stable/index.html

Umi_tools

UMI is responsible for removing PCR duplicates in the data by using mapping position and random barcodes.
https://umi-tools.readthedocs.io/en/latest/#

STAR

Spliced Transcripts Alignment to a Reference (STAR) is a program which aligns high-throughput RNA-seq data to a reference file. This tool will allow us to map the CLIP-seq reads.
https://github.com/alexdobin/STAR

Samtools

Sequence Alignment/Map tools (Samtools) will be used to filter the reads, leaving only reads that map against the main chromosomes. It will then be used to merge the preprocessed alignments of the replicates.
http://www.htslib.org/

FastQC

FastQC is used to assess the quality of the preprocessed data. This pipeline will generate the output file, but the quality assessment is done visually by the user.
https://www.illumina.com/products/by-type/informatics-products/basespace-sequence-hub/apps/fastqc.html
https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

CLAM

CLIP-seq Analysis of Multi-mapped reads (CLAM) is a tool for re-aligning multi-mapped reads in CLIP-seq or RIP-seq data and for calling peaks.

https://github.com/Xinglab/CLAM#section1

CLIPper (Suggested)

CLIPper is another peak-caller tool for CLIP-seq dataset. It is also the peak-caller in ENCODE's CLIP-seq analysis protocol.
https://github.com/YeoLab/clipper/wiki/CLIPper-Home

Bedtools

We will be using bedtools to merge the peak-caller outputs (.bed format) by the *intersect* function.
https://bedtools.readthedocs.io/en/latest/

PRAS

Protein-RNA Association Strength (PRAS) takes in the peak data and annotation file to predict function targets for the RBP, scoring the genes based on binding intensity and position information.
https://github.com/ouyang-lab/PRAS

III. Using the Pipeline

Running the Pipeline

Please follow the instructions below or in the README.md at:
https://github.com/shuangerli/bio_practicum

- Clone the repo: `git clone https://github.com/shuangerli/bio_practicum.git`
- Make the script executable (if necessary): `chmod 755 run.sh` or `chmod 755 run`
- Run the script with: `run [argument]` or `slurm run [argument]`
- Please use `run -h` or `./run -h` to see instructions
- To run the pipeline with example data (will download the necessary files), use `run -example`. *Note: You do not need to provide your own input files here*.

Input Files

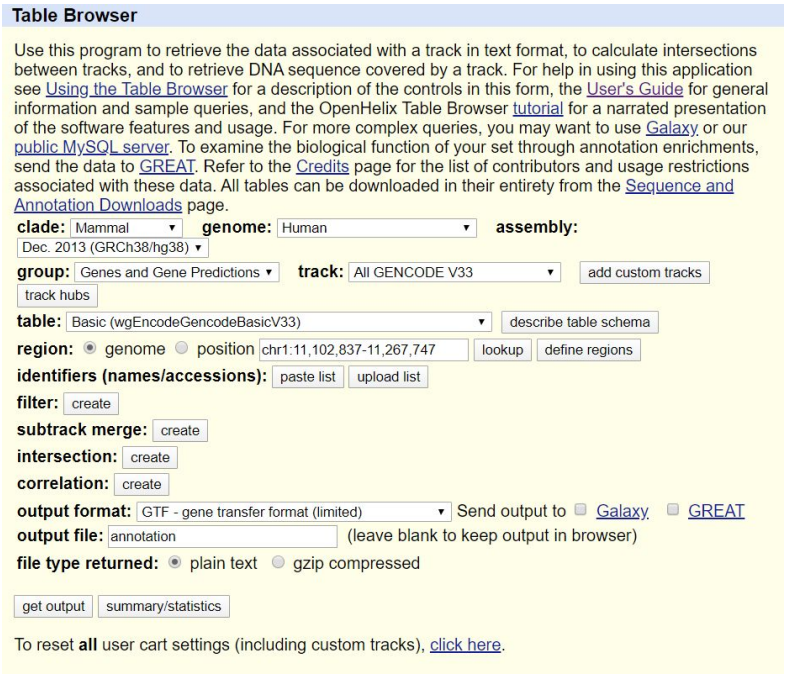
Human Reference Genome (fasta)

- https://www.gencodegenes.org/human/

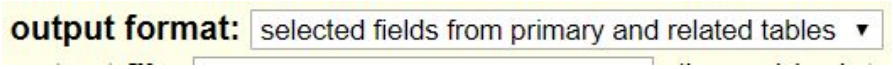
Annotation (gtf) & ID file (txt)

- https://genome.ucsc.edu/cgi-bin/hgTables

- To get the annotation file, set the parameters of the table to be as follows:



- To get the ID files, use the same parameters, but change the output format to be also specify the fields to only contain:



bin	
name	Name of gene (usually transcript_id from GTF)
chrom	Reference sequence chromosome or scaffold
strand	+ or - for strand
txStart	Transcription start position (or end position for minus strand item)
txEnd	Transcription end position (or start position for minus strand item)
cdsStart	Coding region start (or end position for minus strand item)
cdsEnd	Coding region end (or start position for minus strand item)
exonCount	Number of exons
exonStarts	Exon start positions (or end positions for minus strand item)
exonEnds	Exon end positions (or start positions for minus strand item)
score	score
name2	Alternate name (e.g. gene_id from GTF)
cdsStartStat	enum('none','unk','incmpl','cmpl')
cdsEndStat	enum('none','unk','incmpl','cmpl')
exonFrames	Exon frame {0,1,2}, or -1 if no frame for exon

Raw CLIP-seq Data (fastq)

- This pipeline requires the user to use two replicates, each with read1 and read2 (four total files).

Peak Data (bed)

- This pipeline requires the user to provide additional peak data (at most 2) in bed format. We have tested and suggest utilizing *CLIPper*. The workflow (with its own preprocessing steps using *GSNAP*) can be found at: https://github.com/YeoLab/clipper/wiki/CLIPper-Home.

- Another option is to download the *CLIPper* data directly from ENCODE (narrow_peak)

Output

- To manually assess the quality of preprocessed reads, we included FastQC output in the /fastqc_result directory.
- The final output of the pipeline is binding_PRAS.txt, which will contain the predicted functional targets of the RBP with scores.

IV. Limitations and Pitfalls

1. In the example run, downloading the files sometimes encounter issues that the downloaded file got broken. If that happens, you will see downloading and cutadapt complete extremely fast (under 1min). In this case, please download the files manually by the following commands (available on the README.txt too):

```
mkdir rep1 rep2
wget -O rep1/reads.R1.fastq.gz https://www.encodeproject.org/files/ENCFF173ODI/@@download/ENCFF173ODI.fastq.gz
wget -O rep1/reads.R2.fastq.gz https://www.encodeproject.org/files/ENCFF507EGU/@@download/ENCFF507EGU.fastq.gz
wget -O rep2/reads.R1.fastq.gz https://www.encodeproject.org/files/ENCFF578XQC/@@download/ENCFF578XQC.fastq.gz
wget -O rep2/reads.R2.fastq.gz https://www.encodeproject.org/files/ENCFF735QHY/@@download/ENCFF735QHY.fastq.gz
wget -O annotation.gtf.gz ftp://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_33/gencode.v33.primary_assembly.annotation.gtf.gz
gunzip annotation.gtf.gz
wget -O ref.GRCh38.fa.gz ftp://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_33/GRCh38.primary_assembly.genome.fa.gz
gunzip ref.GRCh38.fa.gz
wget -O clipper.bed.gz https://www.encodeproject.org/files/ENCFF862EKD/@@download/ENCFF862EKD.bed.gz
wget -O control.bam https://www.encodeproject.org/files/ENCFF608WNY/@@download/ENCFF608WNY.bam
```

2. Using peak caller output files in different orders currently yields unique output files. This is to say that inputting *CLIPper* data and then *CLAM* data yields results which are not identical to those seen when first inputting *CLAM* data and then *CLIPper* data. The reason for this believed to be a result of the *intersect* function. For more information, please consult the following link: https://bedtools.readthedocs.io/en/latest/content/tools/intersect.html
3. A maximum of three peak callers may be utilized in this pipeline.
4. Certain combinations of peak callers will yield unsuccessful *PRAS* data, where the scores of the predicted genes will all be 0. For example, data generated by *PureCLIP*, *CLIPper*, and *CLAM* all run successfully with *PRAS* by themselves. However, all combinations with *PureCLIP* will end with scores of 0, while the combination of *CLAM* and *CLIPper* is successful. We believe this is possibly due to differing file formats, where *PureCLIP* generates BED6, and *CLAM* and *CLIPper* are in BED10 (narrow_peak) format.