

RNASeq

Efforts to sequence the transcripts expressed in a cell or organism.

Statistics

Measuring gene expression

Resources

- See the [StatQuest Video on RPKM/FPKM/TPM](#) to better understand how the statistics can be used to evaluate gene expression.

Using techniques to extract the Wang et al. Nat Rev Genetics. 2009.

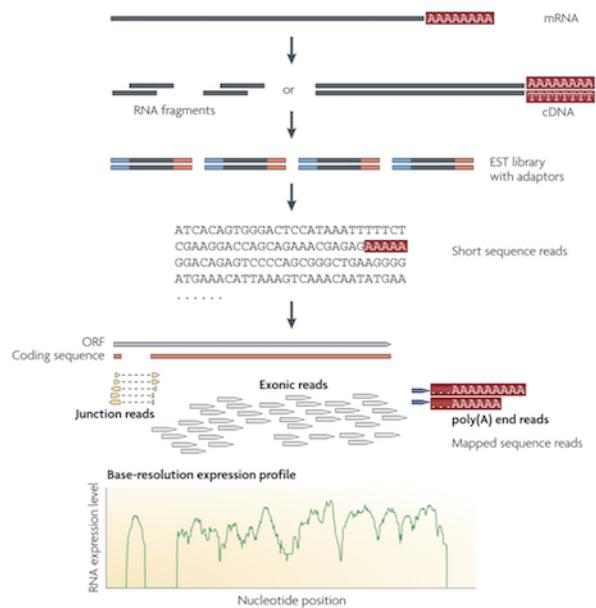


Figure 1. A typical RNA-Seq experiment

doi:[10.1038/nrg2484](https://doi.org/10.1038/nrg2484)

Multiple approaches to understanding the transcriptome

1. Genome sequenced, align RNAseq reads to genome
2. de novo Assembly of mRNA into transcripts

3. Quantify gene expression from reads aligned to genome or transcripts

Reads to Genome mapping

It is important to note that aligning sequences to the genome when there are introns requires dealing with introns. So splice-aware alignments are needed in some cases.

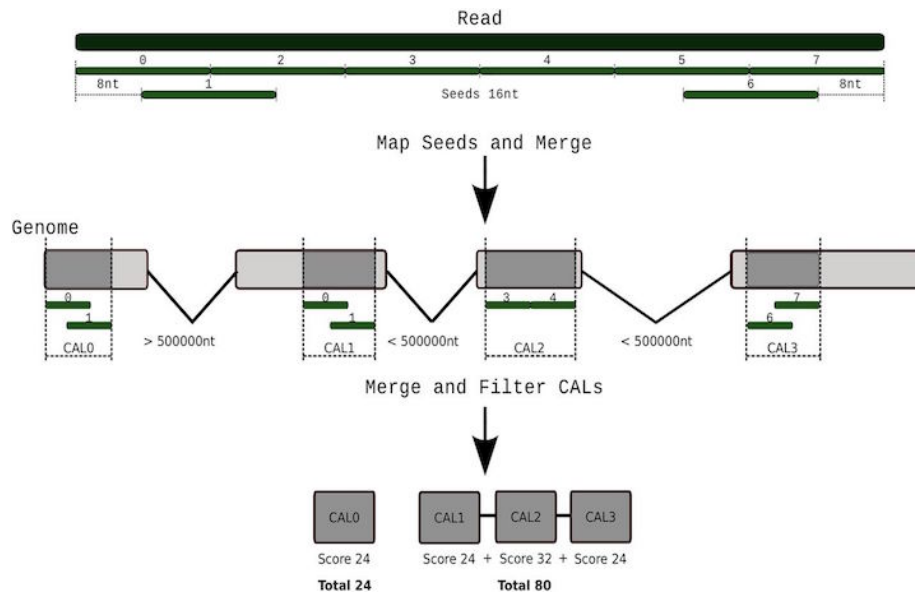


Figure 1: SpliceAlign

Tarraga et al 2017. DNA Research. [10.1093/dnares/dsv039](https://doi.org/10.1093/dnares/dsv039)

#Reads to Genome mapping

Challenges: mRNA is spliced, genome contains introns

Splice-aware short read aligners. Speed and accuracy tradeoffs * Tophat + Bowtie – this is old don't use * [HISAT2](#) * [GMAP/GSNAP](#) * [STAR](#)

Need to Quantify expression

- Count reads overlapping exons
- Table of total read counts per gene
- Normalize counts for gene length and sequencing library depth

- Gene expression then is FPKM - Fragments per Kilobase per Millions of reads
- Tools: htseq-count, stringtie
- [SubRead](#)
- [BEDtools](#)
- R tools with iRanges

Evaluating expression differences

Statistical tools for evaluating gene expression differences

- Ballgown [bioconductor package](#)
- DESeq [bioconductor package](#)
- edgeR [bioconductor package](#)

Alternative approach for Quantifying

Compare reads to **Transcripts** instead of Genome * Kalisto and Sailfish are common tools * Bray et al 2016 “Near-optimal probabilistic RNA-seq quantification” doi:[10.1038/nbt.3519](#) * Patro et al 2014 “Sailfish enables alignment-free isoform quantification from RNA-seq reads using lightweight algorithms” doi:[10.1038/nbt.2862](#)

Alignment free quantification

Usage: kallisto quant [arguments] FASTQ-files

Required arguments:

-i, --index=STRING	Filename for the kallisto index to be used for quantification
-o, --output-dir=STRING	Directory to write output to

Optional arguments:

--bias	Perform sequence based bias correction
-b, --bootstrap-samples=INT	Number of bootstrap samples (default: 0)
--seed=INT	Seed for the bootstrap sampling (default: 42)
--plaintext	Output plaintext instead of HDF5
--fusion	Search for fusions for Pizzly
--single	Quantify single-end reads
--single-overhang	Include reads where unobserved rest of fragment is predicted to lie outside a transcript

<code>--fr-stranded</code>	Strand specific reads, first read forward
<code>--rf-stranded</code>	Strand specific reads, first read reverse
<code>-l, --fragment-length=DOUBLE</code>	Estimated average fragment length
<code>-s, --sd=DOUBLE</code>	Estimated standard deviation of fragment length (default: -l, -s values are estimated from paired end data, but are required when using --single)
<code>-t, --threads=INT</code>	Number of threads to use (default: 1)
<code>--pseudobam</code>	Save pseudoalignments to transcriptome to BAM file
<code>--genomebam</code>	Project pseudoalignments to genome sorted BAM file
<code>-g, --gtf</code>	GTF file for transcriptome information (required for --genomebam)
<code>-c, --chromosomes</code>	Tab separated file with chromosome names and lengths (optional for --genomebam, but recommended)

Note this won't quite work to copy and paste.

```
#!/usr/bin/bash
```

```
module load kallisto
ln -s /bigdata/gen220/shared/data-examples/rnaseq/kallisto/S_cerevisiae_ORFs.fasta
ln -s
kallisto index -i Scer.idx S_cerevisiae_ORFs.fasta
cat samples.tsv | while read ACC COND REP
do
    OUT=output/$COND.$REP
    kallisto quant -t 8 --single -l 300 -s 20 -i Scer.idx -o $OUT data/${ACC}_1.fastq.gz
done
```

Go see /bigdata/gen220/shared/data-examples/rnaseq/kallisto

See also https://github.com/stajichlab/C_lusitaniae_DHED1_RNAseq/blob/master/Rscripts/kallisto_profile_rf_stranded.R

Denovo assembly

[Trinity Assembler](#) for RNASeq

```
$ module load trinity-rnaseq
$ module switch perl/5.22.0
$ Trinity --seqType fq --left reads_1.fq --right reads_2.fq --CPU 8 --max_memory 20G
```

ORF identification

Once we have assembled the transcriptome, want to find genes in there.

TransDecoder

- Finds Open Reading Frames in mRNA transcripts

```
$ module load transdecoder
$ TransDecoder.LongOrfs -t target_transcripts.fasta
```

RNAseq read mapping

Using HISAT2 for RNAseq read mapping * [S_cerevisiae.fasta.gz](#) *

[S_cerevisiae.gff3.gz](#)

Download those files.

```
# start an interactive session
srun -N 1 -n 4 -p short --mem 16gb --pty bash -l

module load hisat2
# uncompress
gunzip S_cerevisiae.gff3.gz S_cerevisiae.fasta.gz
# build index
hisat2-build S_cerevisiae.fasta yeast
# run search
ln -s /bigdata/gen220/shared/data-examples/rnaseq/yeast_rnaseq/*.gz .
hisat2 -x yeast -1 SRR3396381_1.fastq.gz -2 SRR3396381_2.fastq.gz -S SRR3396381.sam -p 4

module load samtools
samtools view -Ob -o SRR3396381.bam SRR3396381.sam

samtools sort -o SRR3396381.sort.bam SRR3396381.bam
samtools index SRR3396381.sort.bam SRR3396381.bam
samtools flagstat SRR3396381.sort.bam
```

Get counts

Subread - <http://subread.sourceforge.net/>

```
module load subread
GENOME=S_cerevisiae.fasta
GFF=S_cerevisiae.gff3
OUTFILE=SRR3396381.tab
INFILE=SRR3396381.sort.bam
featureCounts -g gene_id -T $CPUS -G $GENOME -s 0 -a $GFF -o $OUTFILE \
-F GTF $INFILE
```

Template for Projects

Here's a template for RNASeq analyses

https://github.com/biodataprog/RNASeq_template Click on 'Use this template'
- you can create your own version of this.

It will prompt you give it a name.

Go to the command line to download.

```
git clone yourname/YourRNASeqAnalysis.git
```

Edit `samples.csv` to describe names of some experiments SRR3396381.

Download data in `input` folder.

Download or us download script to get genome files (need to put a genome FASTA file in the folder). If want to do kallisto will need a mRNA file of transcriptome.