Counting Reads

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Learning Objectives

- Conversion of BAM file to a counts file using `htseq-count`
- Conversion of BAM file to a bigWig file using `deeptools`

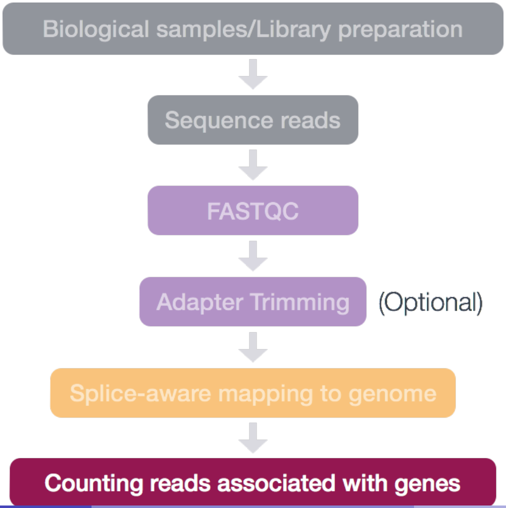
Timeline for next few weeks

Date	Topic
Wed, 19th	Basics in R
Mon, 24th	Advanced DE analysis*
Wed, 26th	Tidyverse part I*
Mon, 31st	Tidyverse part II*
Wed, 2nd	Course catch up day

HW#7 (∼200 pts) due on April 4th - Will be posted tomorrow
*Still deciding on order

Counting reads as a measure of gene expression

Once we have our reads aligned to the genome, the next step is to count how many reads have mapped to each gene.

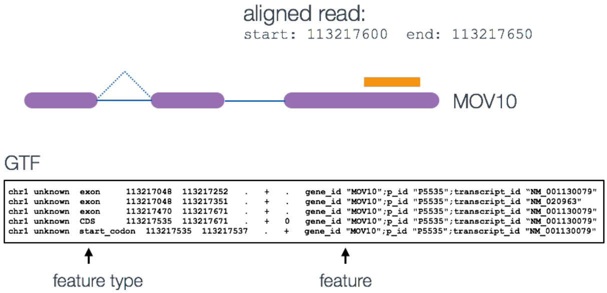

Biological samples/Library preparation
Sequence reads
FASTQC
Adapter Trimming
(Optional)
Splice-aware mapping to genome
Counting reads associated with genes

Gene Level vs Transcript Level Quantification

- In RNA-seq analysis, gene-level quantification summarizes expression by aggregating counts across all transcripts of a gene, while transcript-level quantification considers each transcript (isoform) separately, allowing for the detection of changes in isoform usage
- HTSeq report raw counts; counts are aggregated across all transcripts belonging to a single gene.
- Other tools (RSEM or SALMON) account for multiple transcripts per gene, assigning fractional counts instead of whole numbers. For example, if one read aligns to two transcripts, it may be counted as 0.5 for each transcript rather than a whole number.

Input for counting = BAM files + 1 GTF file

The genomic coordinates of where the read is mapped (BAM) are cross-referenced with the genomic coordinates of whichever feature you are interested in counting expression of (GTF), it can be exons, genes or transcripts.

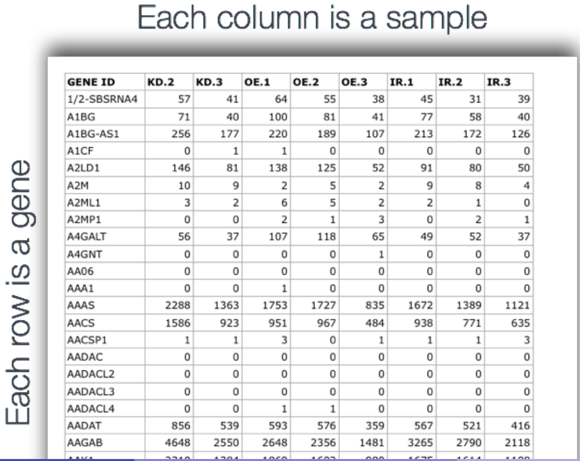


```
chr1 unknown  exon        113217048  113217252   .   +   .   gene_id "MOV10";p_id "P5535";transcript_id "NM_001130079"
chr1 unknown  exon        113217048  113217351   .   +   .   gene_id "MOV10";p_id "P5535";transcript_id "NM_020963"
chr1 unknown  exon        113217470  113217671   .   +   .   gene_id "MOV10";p_id "P5535";transcript_id "NM_001130079"
chr1 unknown  CDS         113217535  113217671   .   +   0   gene_id "MOV10";p_id "P5535";transcript_id "NM_001130079"
chr1 unknown  start_codon   113217535  113217537   .   +   gene_id "MOV10";p_id "P5535";transcript_id "NM_001130079"
```

Figure 2: shells

Output of counting = A count matrix, with genes as rows and samples are columns

These are the “raw” counts and will be used in statistical programs downstream for differential gene expression.


Each column is a sample
Each row is a gene

GENE ID	KD.2	KD.3	OE.1	OE.2	OE.3	IR.1	IR.2	IR.3
1/2-SBSRNA4	57	41	64	55	38	45	31	39
A1BG	71	40	100	81	41	77	58	40
A1BG-AS1	256	177	220	189	107	213	172	126
A1CF	0	1	1	0	0	0	0	0
A2LD1	146	81	138	125	52	91	80	50
A2M	10	9	2	5	2	9	8	4
A2ML1	3	2	6	5	2	2	1	0
A2MP1	0	0	2	1	3	0	2	1
A4GALT	56	37	107	118	65	49	52	37
A4GNT	0	0	0	0	1	0	0	0
AA06	0	0	0	0	0	0	0	0
AAA1	0	0	1	0	0	0	0	0
AAAS	2288	1363	1753	1727	835	1672	1389	1121
AACS	1586	923	951	967	484	938	771	635
AACSP1	1	1	3	0	1	1	1	3
AADAC	0	0	0	0	0	0	0	0
AADACL2	0	0	0	0	0	0	0	0
AADACL3	0	0	0	0	0	0	0	0
AADACL4	0	0	1	1	0	0	0	0
AADAT	856	539	593	576	359	567	521	416
AAGAB	4648	2550	2648	2356	1481	3265	2790	2118
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

HTSeq-count

HTSeq-count is a command-line tool used in RNA-Seq data analysis to count the number of sequencing reads that align to specific genomic features, such as genes. It is part of the HTSeq Python package and is commonly used in differential gene expression analysis pipelines.

What is a feature?

- A common task is to count how many reads map to each feature.
- A feature refers to a specific interval (i.e., a range of positions) on a chromosome or a union of such intervals.
- Since our example data comes from an RNA-Seq experiment, we aim to count how many reads fall within the exonic regions of each gene.

How does `htseq-count` manage reads that align to multiple features?

The htseq-count script offers **three different modes** to handle such cases:

1. **Union (Recommended for most cases):**
   - A read is assigned to a feature if any part of the read overlaps with it.
   - If a read overlaps multiple features, it is not counted at all (to avoid ambiguity).

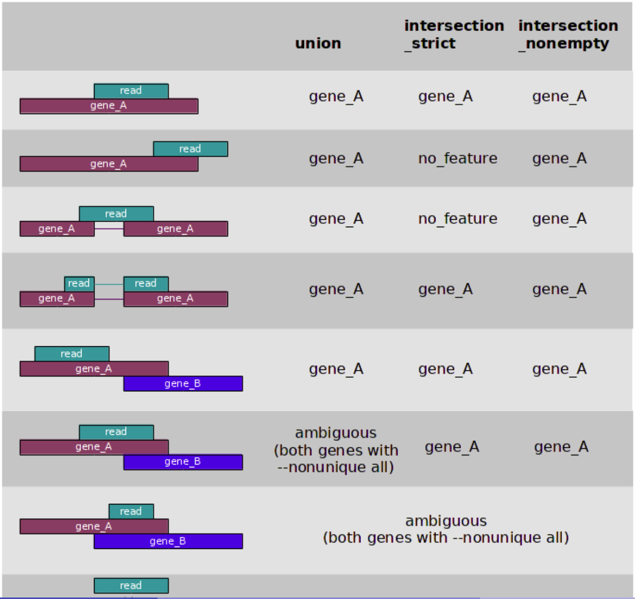
How does `htseq-count` manage reads that align to multiple features?

2. **Intersection-strict:**
   - A read is assigned to a feature only if every position of the read overlaps with that feature.
   - If a read overlaps multiple features but not completely within one, it is not counted.

How does `htseq-count` manage reads that align to multiple features?

3. **Intersection-nonempty:**
   - A read is assigned to a feature only if it overlaps with at least one feature at every position.
   - Unlike intersection-strict, it ignores positions where no features are present.

The figure below illustrates the effect of these three modes:



	union	intersection _strict	intersection _nonempty
read / gene_A	gene_A	gene_A	gene_A
read / gene_A	gene_A	no_feature	gene_A
read / gene_A gene_A	gene_A	no_feature	gene_A
read read / gene_A gene_A	gene_A	gene_A	gene_A
read / gene_A / gene_B	gene_A	gene_A	gene_A
read / gene_A / gene_B	ambiguous (both genes with --nonunique all)	gene_A	gene_A
read / gene_A / gene_B	ambiguous (both genes with --nonunique all)		
read			

Running htseq-count

Begin by loading the `htseq-count` module with `module load`

```
module load gcc/13.3.0-xp3epyt
py-htseq/2.0.3-mb7ap7s
```

Run the following to check the module is loaded:

```
htseq-count --help
```

Basic Command Syntax

```
htseq-count -f bam -s no -i gene_id sample1.bam genes.gtf
```

- `-f bam` → Specifies input format (can be `sam` or `bam`).
- `-s no` → Defines strandedness (`yes`, `no`, or `reverse`).
- `-i gene_id` → used to identify the GTF feature attribute (`gene_name` or `gene_id`)
- `aligned_reads.bam` → Input file with mapped reads.
- `genes.gtf` → Reference annotation file.
- `> gene_counts.txt` → Redirects output to a file.

GFF/GTF attribute

```
-i <id attribute>, --idattr=<id attribute>
```

- The feature ID is used to identity the counts in the output table.
- The default, suitable for RNA-Seq analysis using an Ensembl GTF file, is `gene_id`.

Limitations & Alternatives

- HTSeq-count is a gene-level summarization tool; it does not handle transcript-level quantification.
- It requires sorted BAM/SAM files.
- Alternative tools like featureCounts (..which is faster) or Salmon/RSEM (for transcript-level quantification) may be preferred in some cases.

Review Class Exercise Folder Content

Class Exercise: Running RSeQC”

1. **Copy Folder:** Make a copy of the following folder into your home directory:

```
/gpfs1/cl/mmg3320/course_materials/htseq_2025_demo
```

2. **Determine Strandedness:** You will need to run RSeQC to determine strandedness of the demo data. There are three options to select from:
   - `-s yes`, reads are mapped to the same strand as the sense strand
   - `-s no`, reads can map to either strand (unstranded)
   - `-s reverse`, reads are mapped to the opposite strand (anti-sense)
3. **Interpret the multiqc output**

How To Run RSeQC Section Provided

- Activate conda environment
- Test that `RSeQC` is loaded
- The `rseqc-loop.sh` script. Make a copy and modify the path for variables `BAM_DIR` and `BED_FILE`.
- Submit the script and then check your outputs are the same size. This script will take ~5 minutes to run.

```
-rw-r--r-- 1 pdrodrig pi-jdragon  165 Mar 16 11:38 KO_hg1
-rw-r--r-- 1 pdrodrig pi-jdragon 1.1K Mar 16 11:39 KO_hg1
```

How To Run RSeQC Section Provided

- Deactivate the conda environment **before** running multiqc.
- Run multiqc in the `rseqc_results/` folder to determine strandedness of the FASTQ files.
- **Note:** I tried running the multiqc-rseqc module yesterday and continued to get the issue: `The 'rseqc' MultiQC module broke...`

Class Exercise 2: Running HTSeq-count

1. **Modify `htseq-count.sh` script:**
2. **Submit the `htseq-count.sh` script** after modifying it. This should only take a few minutes.
3. **Look inside** of the `htseq-count_XXXXXX.out` file after the job is completed. It should appear identical as below:

Class Exercise 3: MultiQC

1. Read the section below titled `htseq-count` output
2. Generate a final multiqc output; this time do so inside of the `bams\` folder. *This should work with no issue, it was only the RSeQC Module which is broken*

htseq-count output

The output of htseq-count consists of two main files for each sample:

- A summary/log file
- A counts file that lists the number of reads mapped to each gene or feature. This is a tab-delimited file with gene IDs and their associated read counts.

```
head KO_hg19_rep2_sorted.gene_id.count.txt
```

```
AADACL3 0
AADACL4 0
ABCA4   0
ABCB10  0
ABCD3   1
```

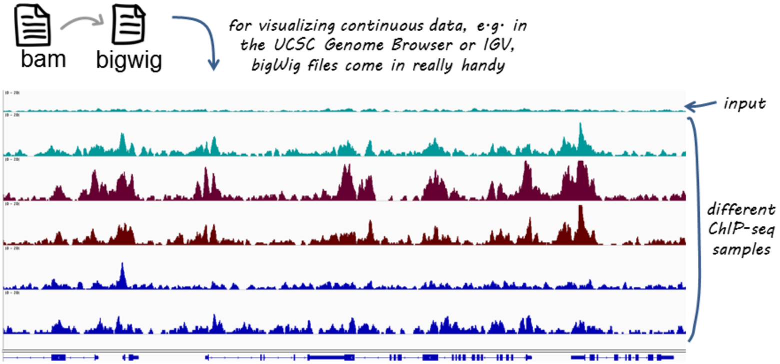
htseq-count output (tail)

```
tail KO_hg19_rep2_sorted.gene_id.count.txt
```

```
__no_feature    32813
__ambiguous     4372
__too_low_aQual 0
__not_aligned   15209
__alignment_not_unique  3667
```

Creating bigWig files

- We will now take our BAM files and convert them into bigWig files. The bigWig format is an indexed binary format useful for dense, continuous data that can be displayed in a genome browser as a graph/track.
- To create bigWig files we will use `deepTools`, a suite of Python tools developed for the efficient analysis of high-throughput sequencing data, such as ChIP-seq, RNA-seq or MNase-seq. `deepTools` has a wide variety of commands that go beyond what we will cover today.



remember that there are 2 deepTools for bam → bigWig conversion:

- ***bamCoverage:*** *for individual files (like those shown here)*
- ***bamCompare:*** *to normalize two files to each other*

Figure 5: BigWig Tracks

Checking/Creating index file for the BAM file

- Often, when working with BAM files you will find that many tools require an index (an associated `.bai` file). Indexing the BAM file aims to achieve fast retrieval of alignments overlapping a specified region without going through the whole alignment file. Essentially, a `bai` file along with the `bam` ensures that downstream applications are able to use the information with the `bam` file much more speedily.
- As a reminder, we used SAMtools, specifically the **`samtools index`** command, to index the BAM files.

bamCoverage from deepTools

This command takes a **BAM file as input** and evaluates which areas of the genome have reads associated with them, i.e. how much of the genome is "covered" with reads. The coverage is calculated as the number of reads per bin, where bins are short consecutive sections of the genome (bins) that can be defined by the user. The **output of this command is a bigWig file**.

These are some parameters of bamCoverage that are worth considering:

- `normalizeUsing`: Possible choices: RPKM, CPM, BPM, RPGC. By default, no normalization is applied. More on this below.
- `binSize`: size of bins in bases (default is 50)
- `--effectiveGenomeSize`: the portion of the genome that is mappable. It is useful to consider this when computing your scaling factor..

DeepTools offers different **methods of normalization**

- Reads Per Kilobase per Million mapped reads (RPKM)
  - number of reads per bin / (number of mapped reads (in millions) * bin length (kb))
- Counts per million (CPM); this is similar to CPM in RNA-seq
  - number of reads per bin / number of mapped reads (in millions)
- Bins Per Million mapped reads (BPM); same as TPM in RNA-seq
  - number of reads per bin / sum of all reads per bin (in millions)

Class Exercise: Running deeptools/bamCoverage

We will be using the bare minimum of parameters as shown in the code below.
Create a bigWig file for `KO_hg19_rep2_sorted.bam` and `WT_hg19_rep2_sorted.bam`:

```
module load deeptools/3.5.5
```

```
bamCoverage -b KO_hg19_rep2.bam -o KO_hg19_rep2.bw
```

Note: Normally, this command can take up to 10 minutes to complete.

Visualize with IGV:

- Start IGV, You may have this previously installed on your laptop. If not no worries, use the IGV Web App.
- Load the Human genome (hg19) into IGV using the dropdown menu at the top left of your screen.
- Load the .bw file using the “Load from File. . . “ or”Tracks” option.
- Type MOV10 into the search bar.

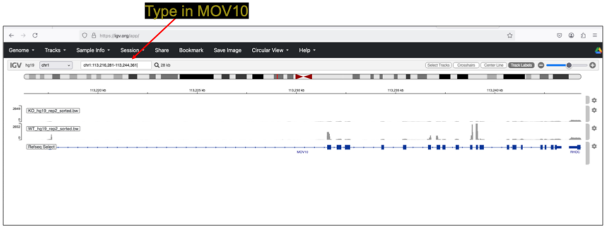


Figure 6: shells

Citation