# RNAseq Data Analysis

MSU Workshop

Rama Shankar

ramashan@msu.edu

# Outline

I. Introduction of RNAseq

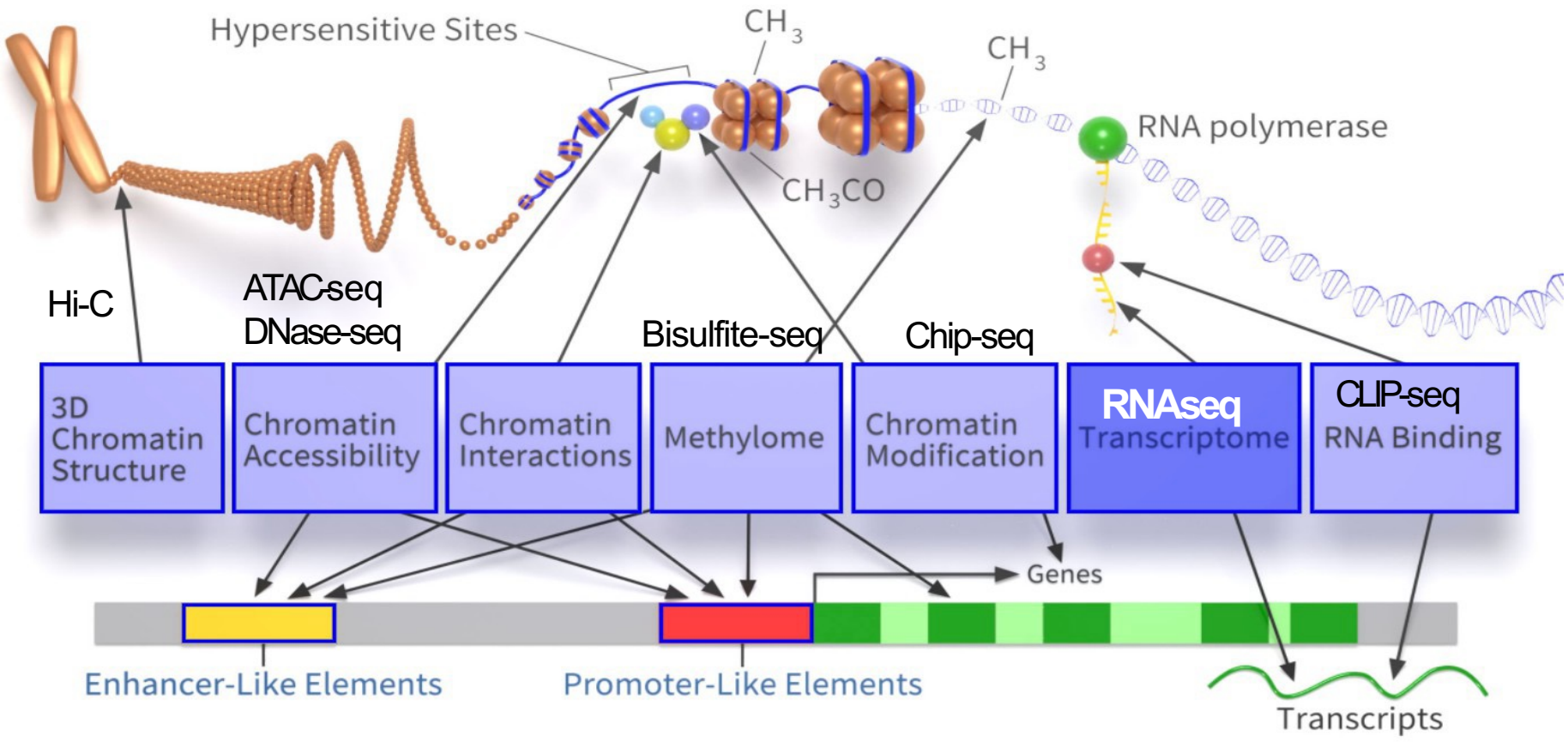
II. RNA isolation and library preparation

III. RNAseq data analysis

IV. Demo of RNAseq data analysis on MSU HPCC

# I. Introduction of RNAseq

# Probing cells with sequencing technologies




Based on an image by Darryl Leja (NHGRI), Ian Dunham (EBI), Michael Pazin (NHGRI)

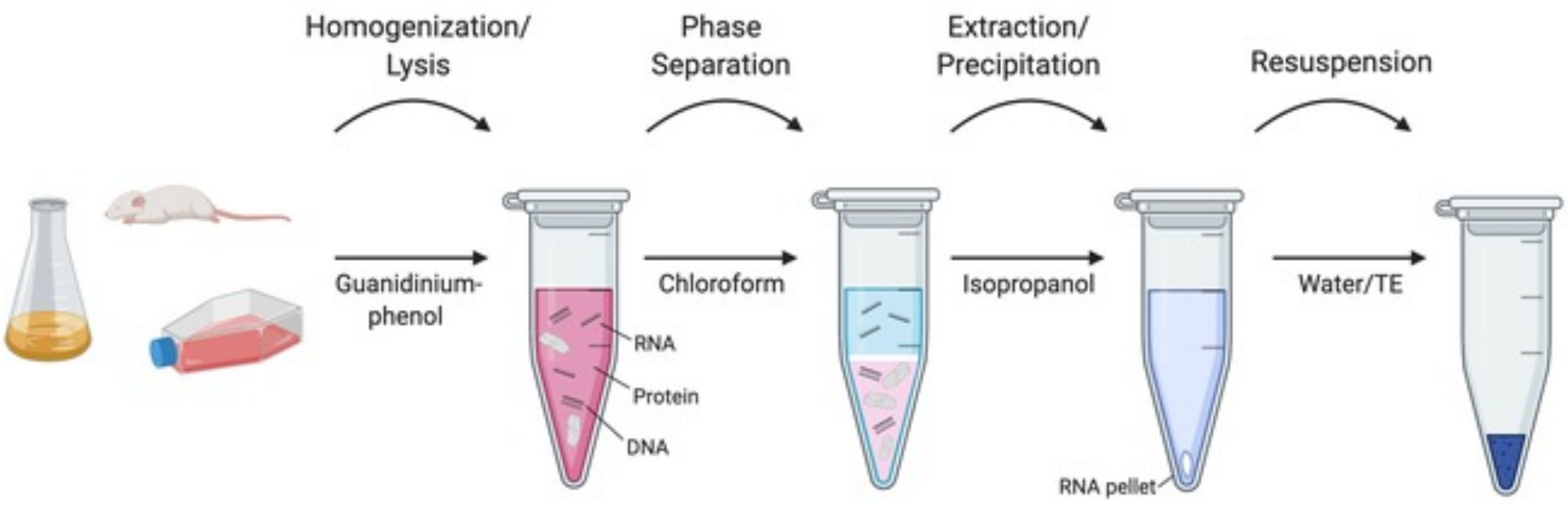
# Types of RNA

mRNA
lncRNA (long non-coding RNA)

microRNA (small RNAseq)
Circular RNA
rRNA
tRNA
snoRNA
piRNA

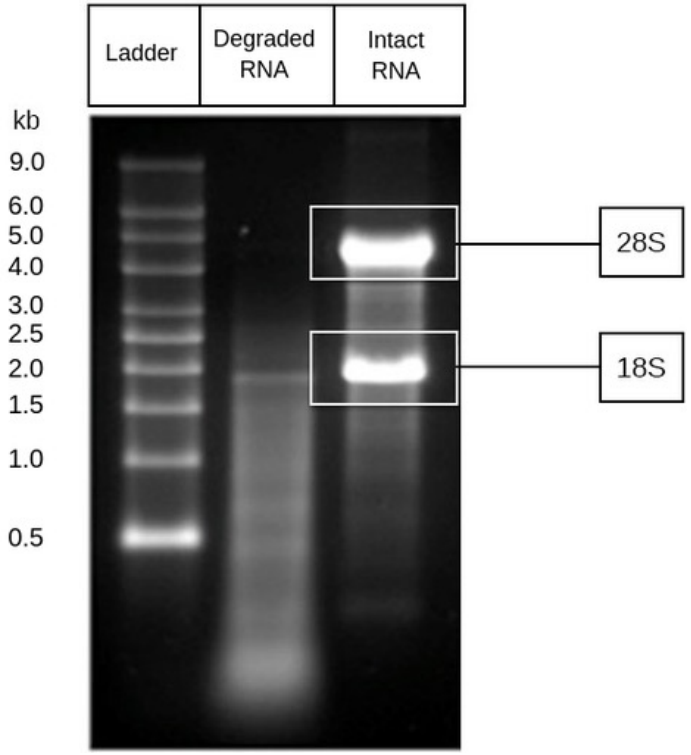
# II. RNA isolation and library preparation

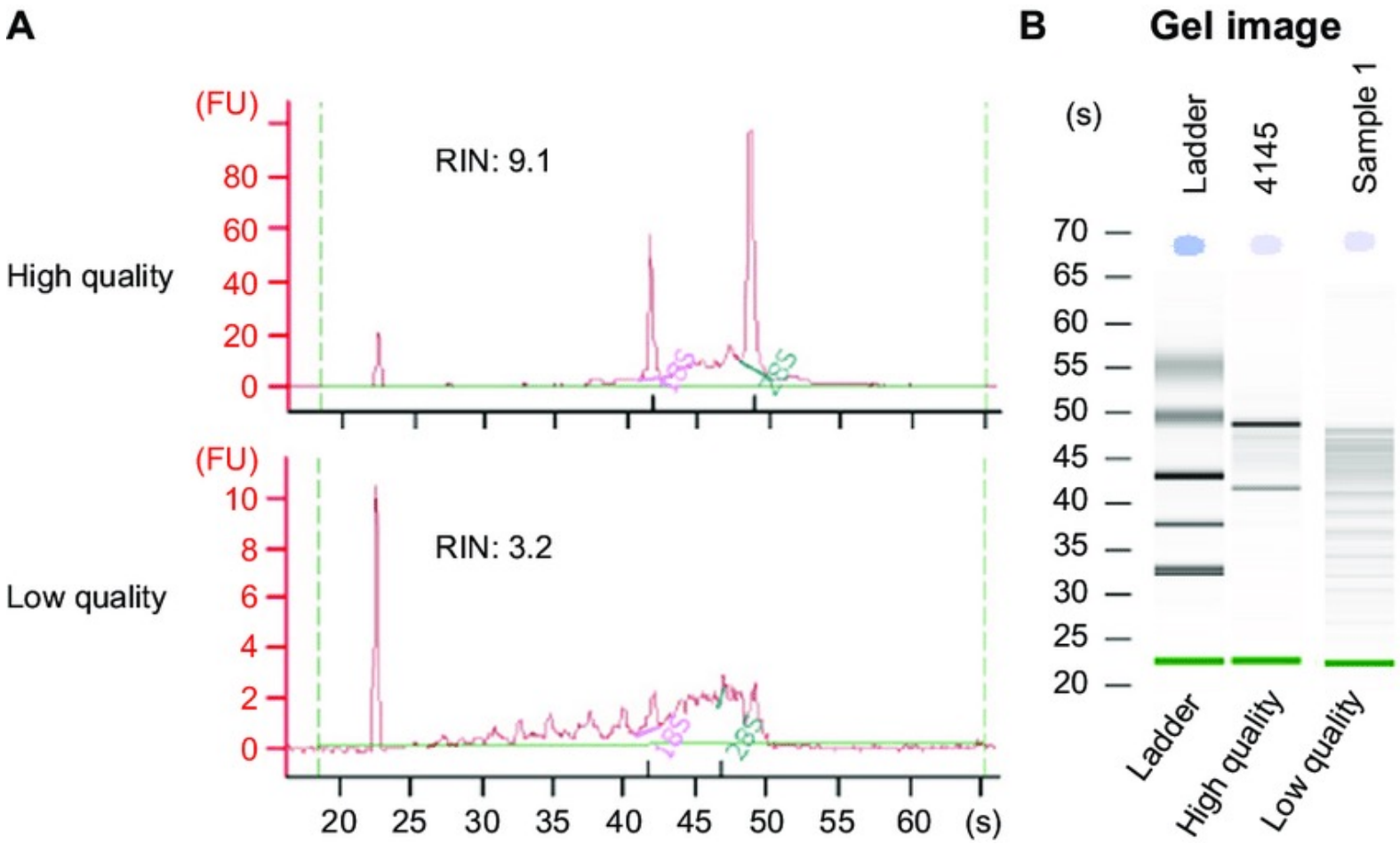
# RNA isolation



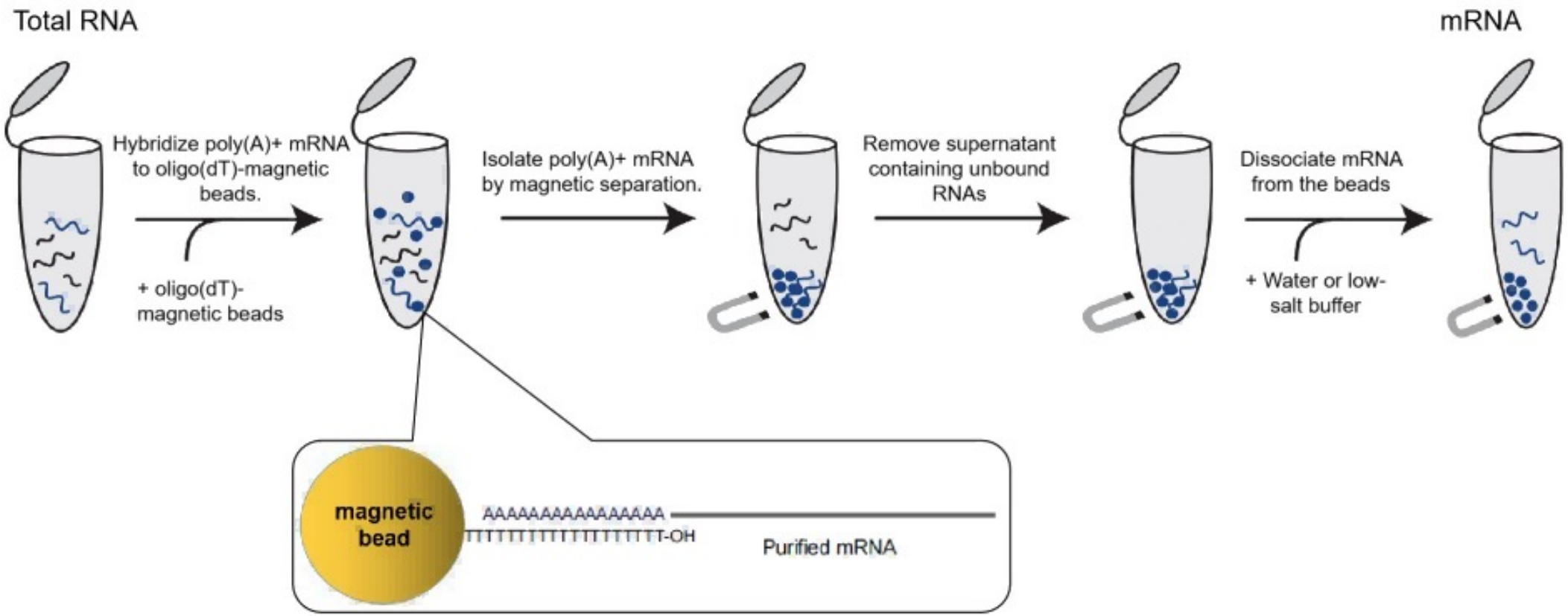

https://www.addgene.org/protocols/kit-free-rna-extraction/

# Quality check of RNA



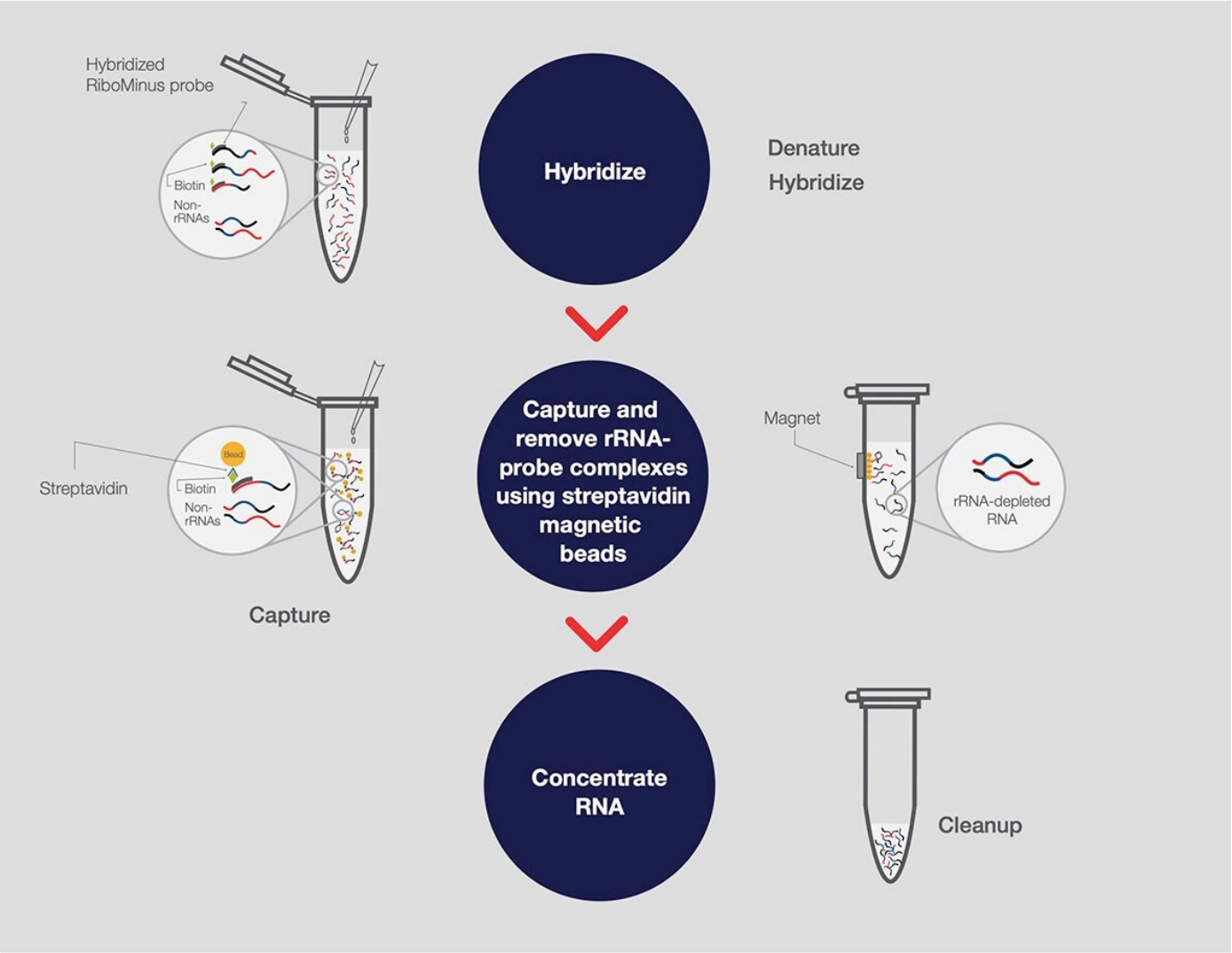



# mRNA enrichment using poly dT beads

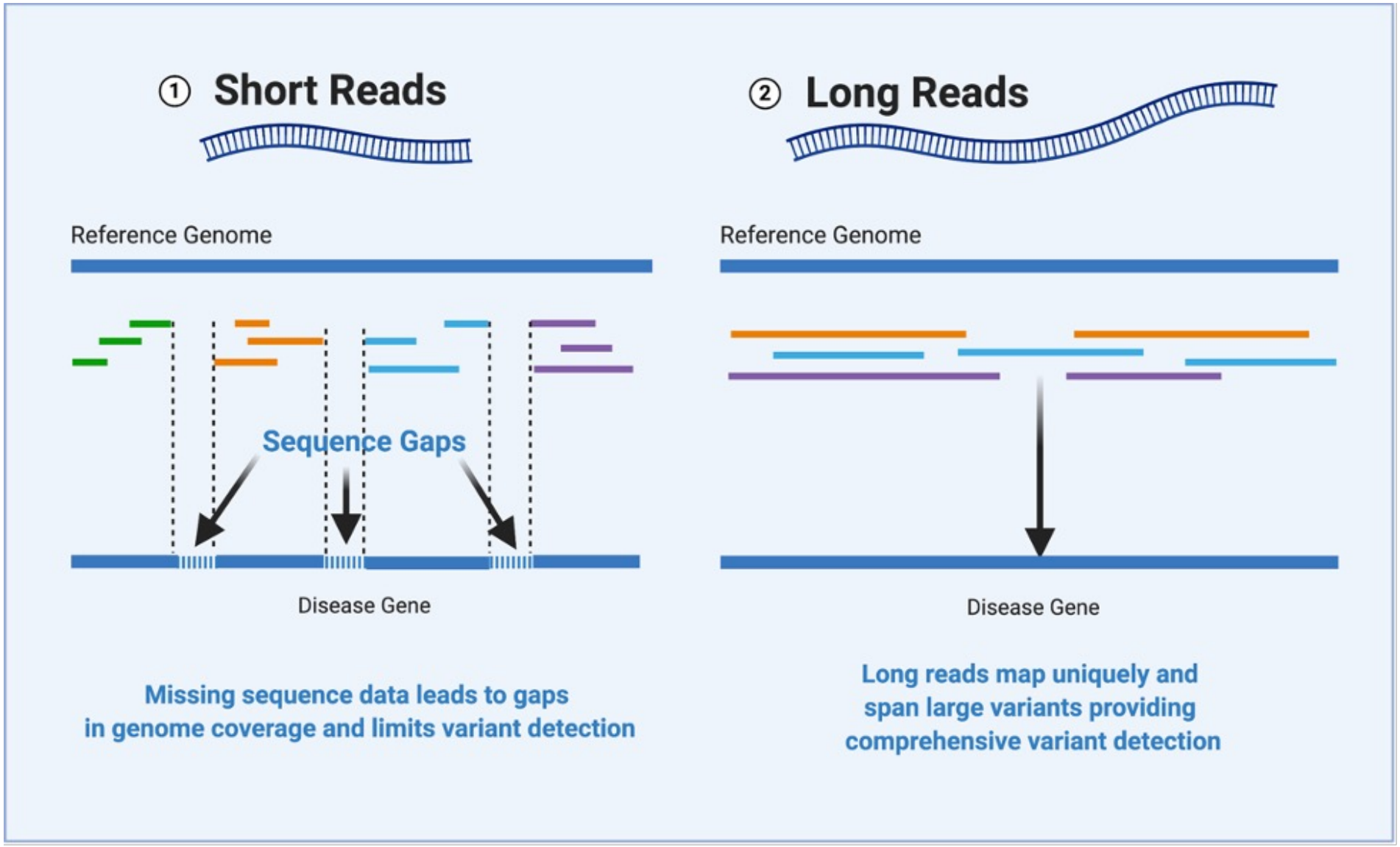



https://www.yeasenbiotech.com/solutiondetail/94

# mRNA enrichment using ribosomal RNA depletion

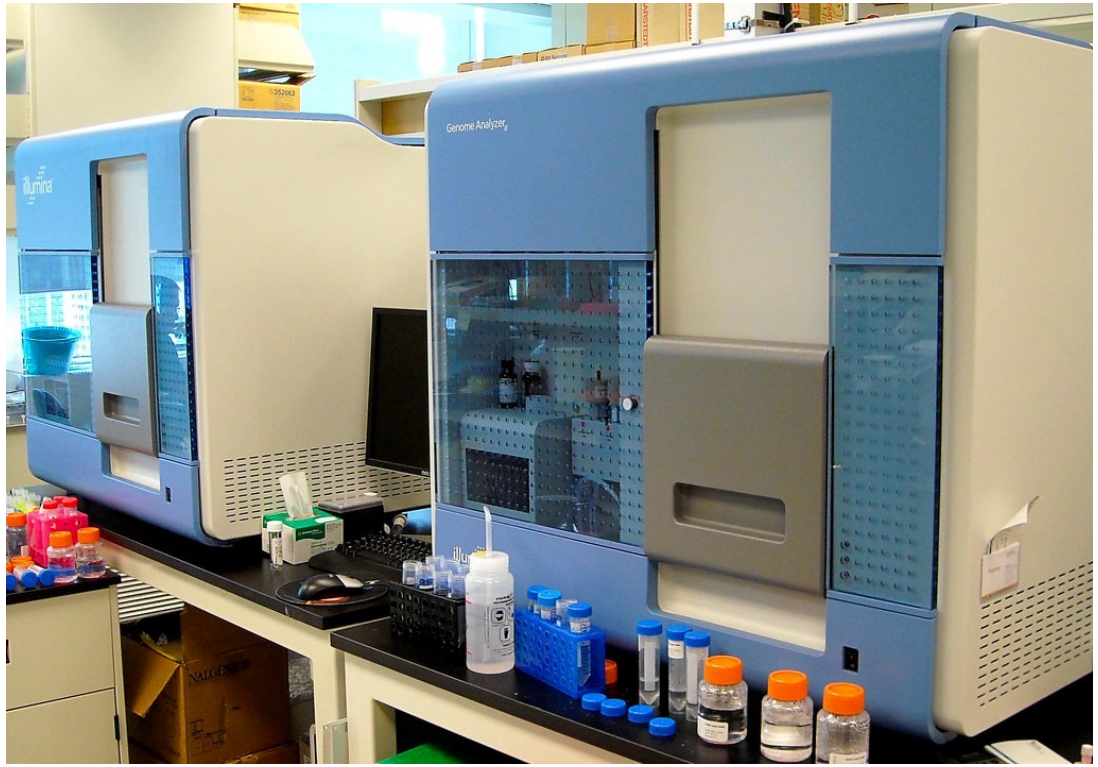


# Short reads vs long reads



# Long read vs short read platforms

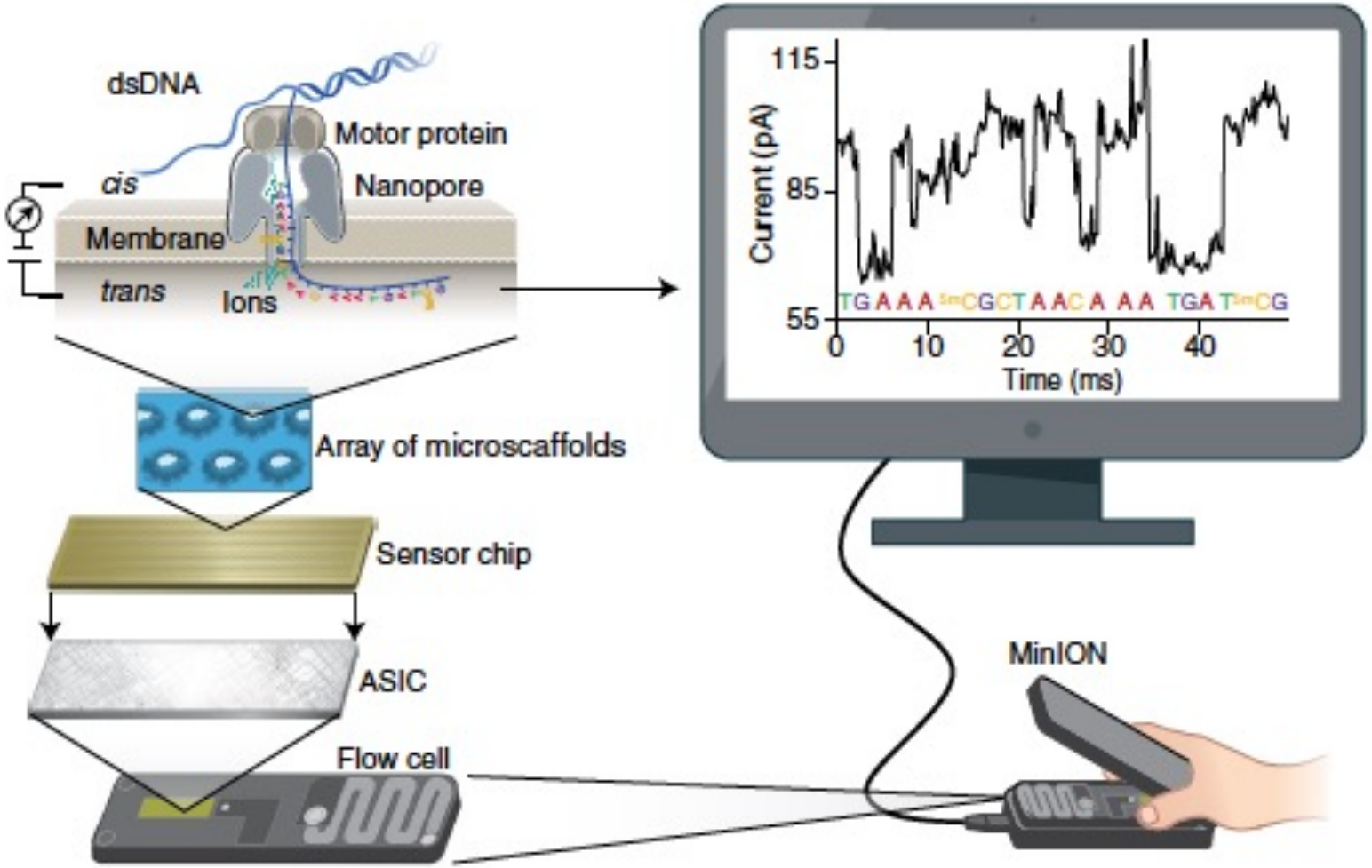

Oxford Nanopore
(long read)




**Illumina genome analyzer**
**(short read)**

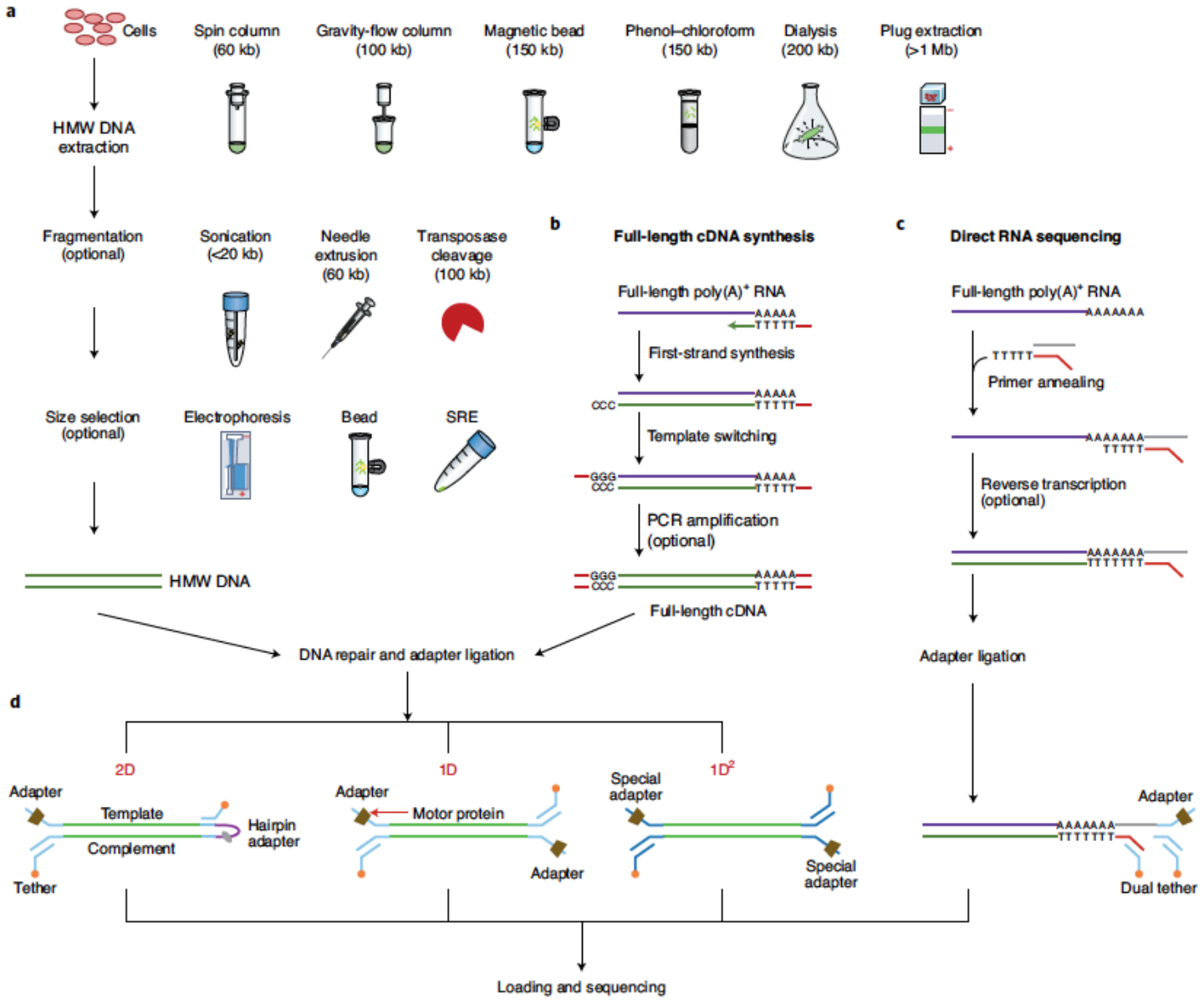
# IIA. From RNA to long reads

# Principle of Nanopore sequencing




https://www.nature.com/articles/s41587-021-01108-x

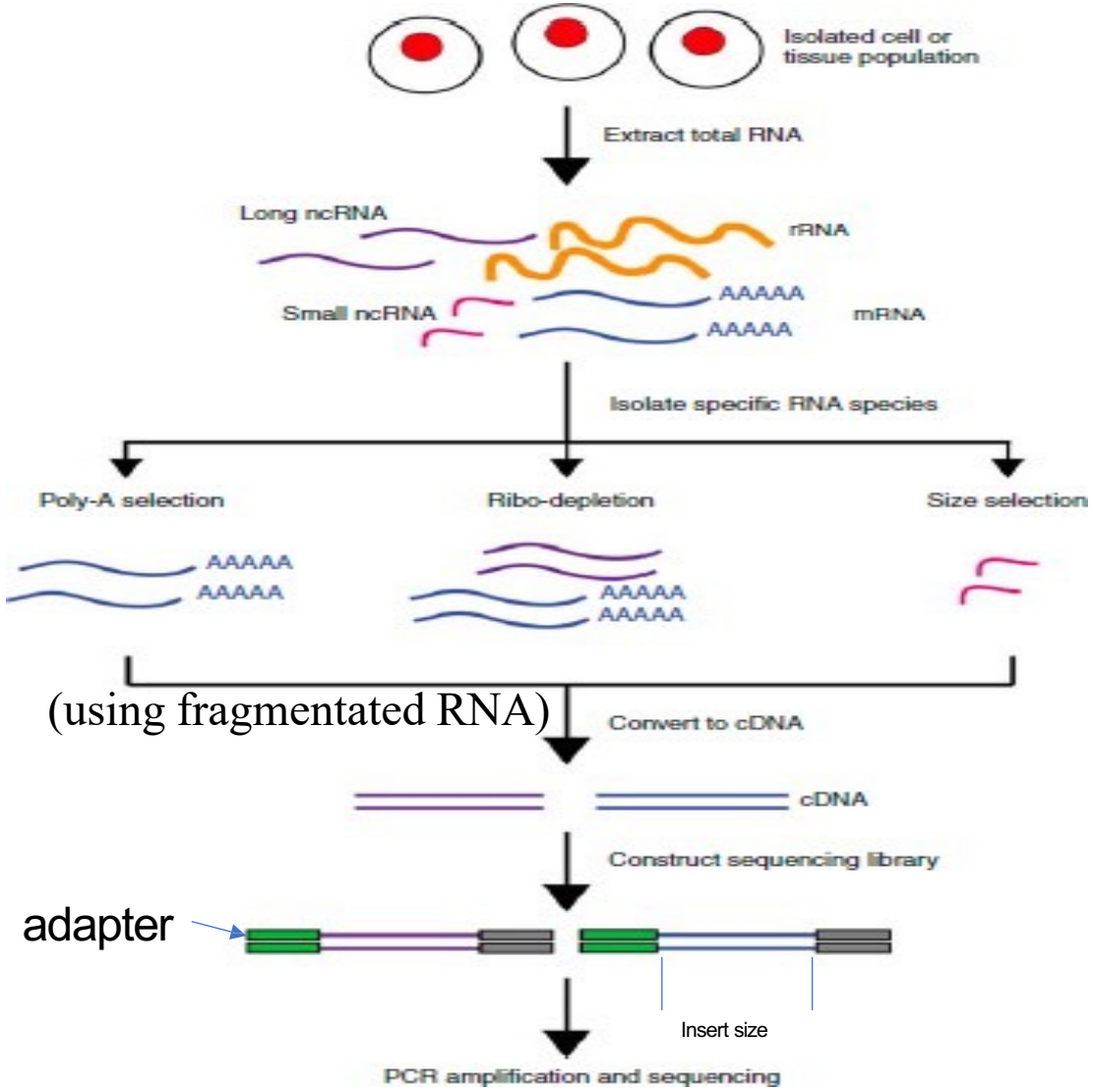
# Library preparation and sequencing workflow for Nanopore




2D (template strand is sequenced), 1D (where each strand is ligated with an adapter and sequenced independently) and $1D^2$ (special adapter ligated to sequence one followed by another one)

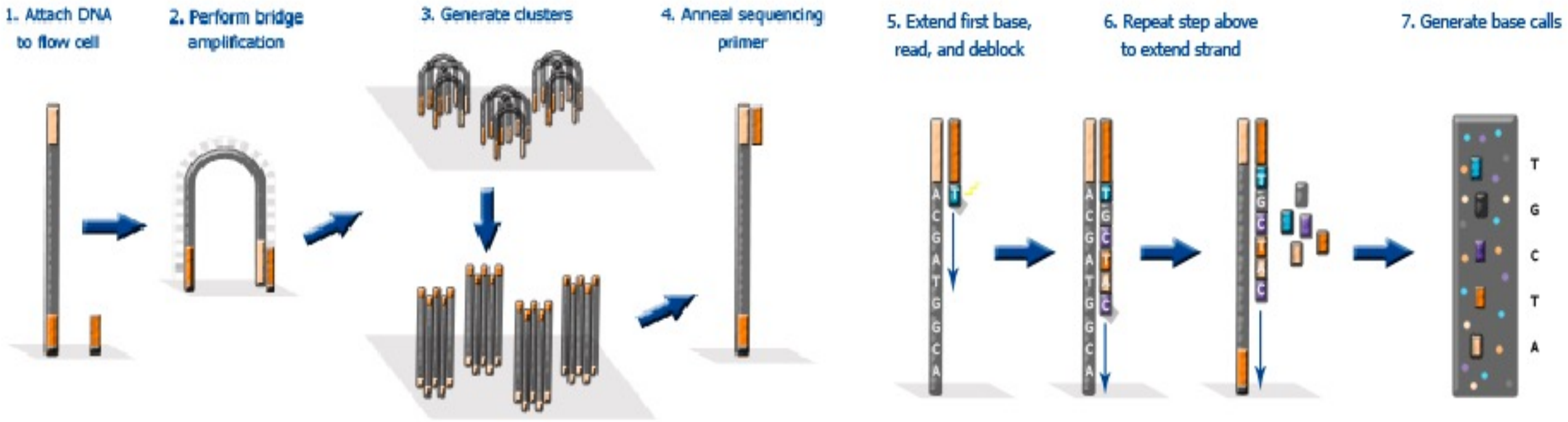
# IIB. From RNA to short reads

# RNAseq library construction for Illumina (short reads)




https://www.rna-seqblog.com/introduction-to-rna-sequencing-and-analysis/

# Overview of Illumina sequencing




Sequencing by Synthesis

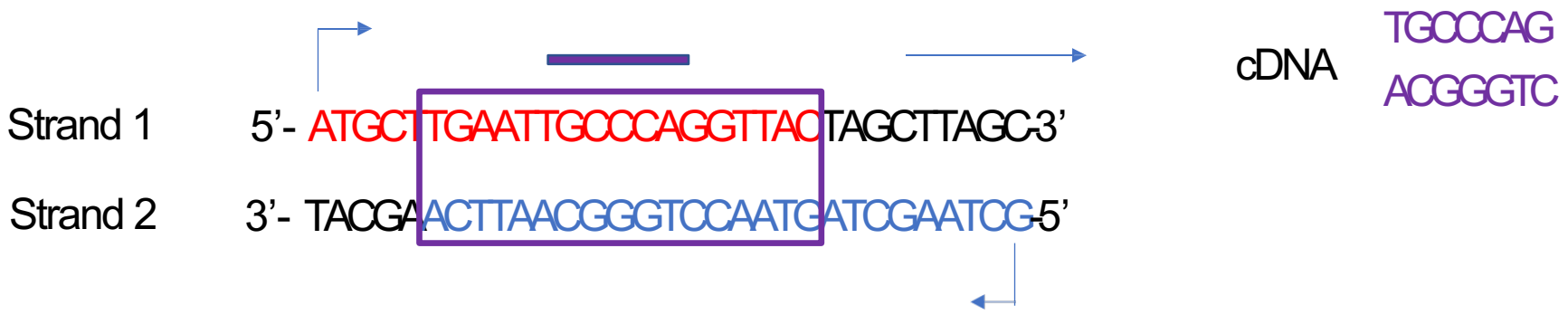
Picture is from https://www.eurofinsgenomics.co.in/en/eurofins-genomics/product-faqs/next-generation-sequencing/general-technical-questions/what-is-the-principal-of-the-illumina-sequencing-technology.aspx

https://www.illumina.com/documents/products/techspotlights/techspotlight_sequencing.pdf (Documentation from illumina)

https://www.youtube.com/watch?v=fCd6B5HRaZ8

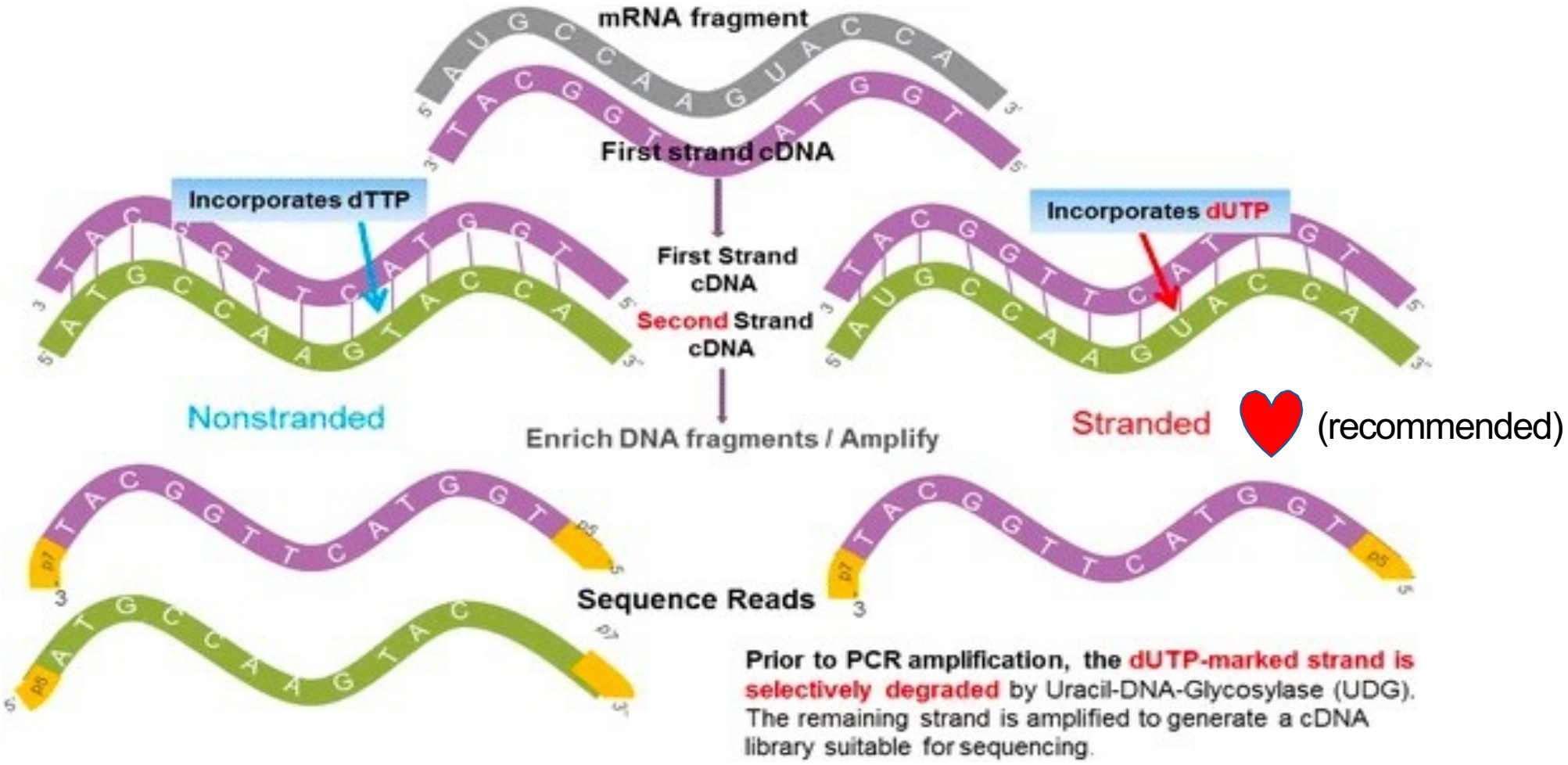
# Strandness matters

DNA is double-stranded, both strand could be transcribed.



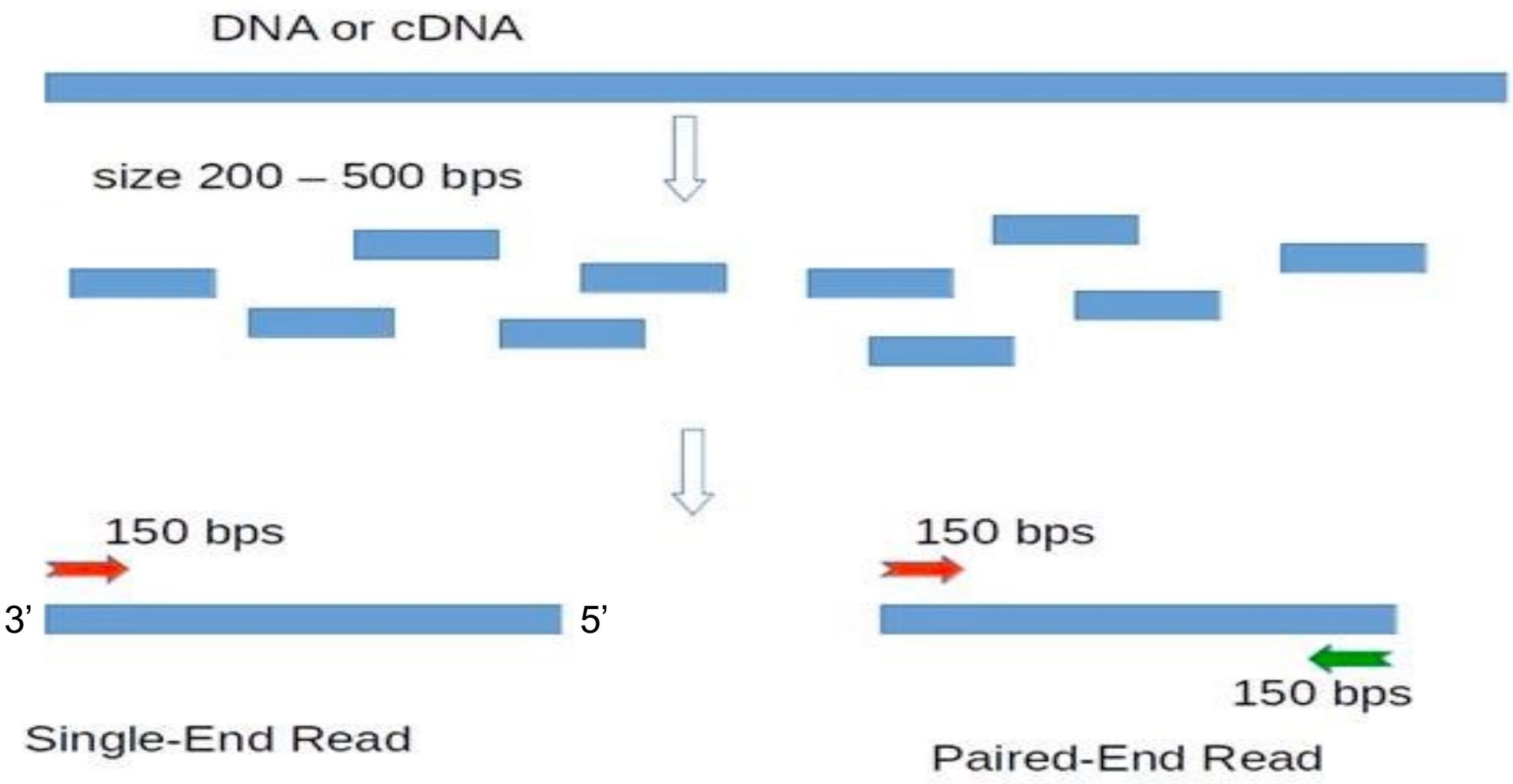

If an RNA fragment comes from the dashed region, it is hard to know its original gene without strand information.

# Nonstranded vs Stranded library




Ref: Zhao *et al*. Comparison of stranded and non-stranded RNA-seq transcriptome profiling and investigation of gene overlap. (BMC Genomics, 2015)

# Single-end vs Paired-end



# Output of RNAseq

1. According to sequencing mode, single-end or paired-end read.

2. Typical read length: 50bp, 75bp, 100bp.

3. Reads are usually stored in FASTQ format.

```
@SRR4822549.1 UNC13-SN749_0135:5:1101:1389:1958 length=100
NAAAGCACATACCAAGGCCACCACACACCACCTGTCCAAAAAGGCCTTCGATACGGGATAATCCTATTTATTACCTCAGAAGTTTTTTTCTTCGCAGGAT
+SRR4822549.1 UNC13-SN749_0135:5:1101:1389:1958 length=100
#1=B7BDFFFHFDGGEBHGIIGEGCHGIIIIGAG?DFFIGGIIIHG3BGHG@@EDHHEHFFBDFFFEEEDCEDCC;>@CCCD5:AC?B@DDDDDDD@B@C
@SRR4822549.2 UNC13-SN749_0135:5:1101:1498:1960 length=100
NTTCTTCAATTTCTTGCCTTCTTCCTTGGAGGCTGGAAGAATCATGGCAAGGTAGGGCCCATCAACCTCAAAAAAGATGCTGTTCTCTGAGCGGGTGACG
+SRR4822549.2 UNC13-SN749_0135:5:1101:1498:1960 length=100
#1=DFFFFHHHHHJJIJJJJJJJJJJJJJJHIIJJJJIIJJJIJIIIJJJJJJJBGIJJJJJIJJJJJJHHHHHFFDDDDDDDDDCDEDDDDDDDDD<BBDB
```

# Preparation of RNAseq experiments

1. Sequencing depth (library size): deeper is better! Usually > 20 million reads should be OK (from illumina).

2. Stranded vs Non-stranded: Strand-specific is recommended.

3. SE vs PE: consider PE if you have enough budget. For gene expression analysis, SE also works well.

4. Technical replicates, the more the better (at least three) .

5. Longer read length is better.

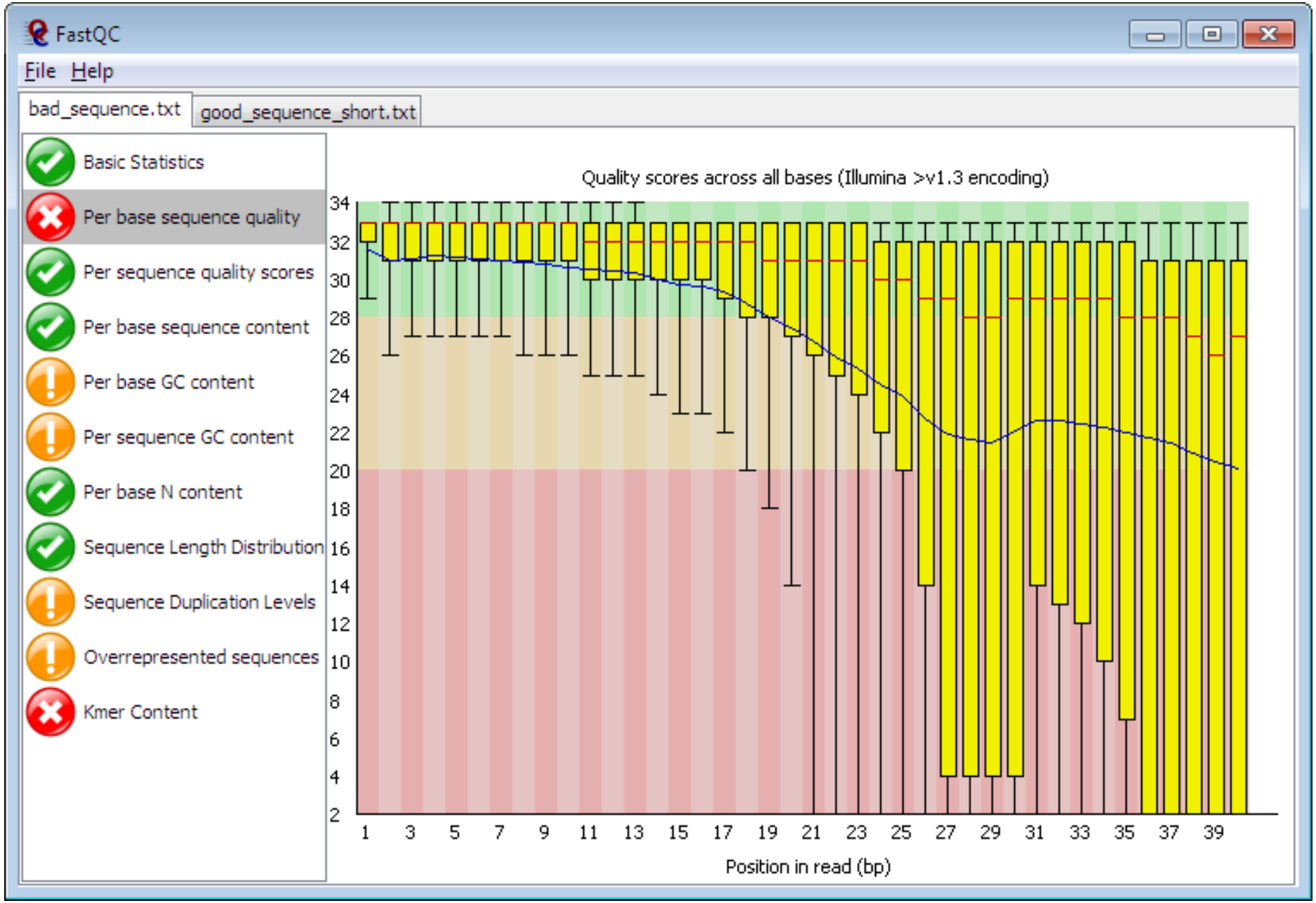
# Q&A

# III. RNAseq data analysis

# RNA-seq data analysis

1. Data quality checking.
2. Read mapping.
3. Quantification of gene expression.
4. Differential gene expression analysis.
5. Interpretation of DE analysis results.

# Quality check of reads using FastQC



# Phred quality score

```
+SEQ_ID
!''*((((***+))%%%++)(%%%%).1**
```

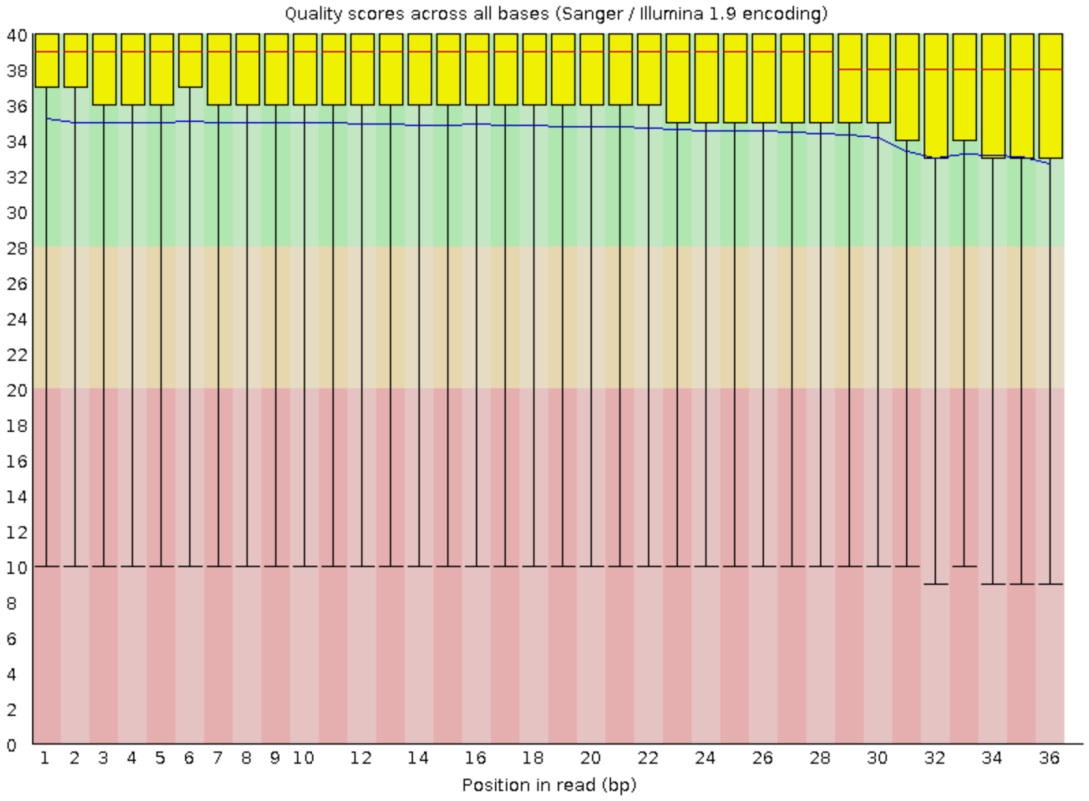
A quality value Q is an integer representation of the probability *p* that the corresponding base call is incorrect.

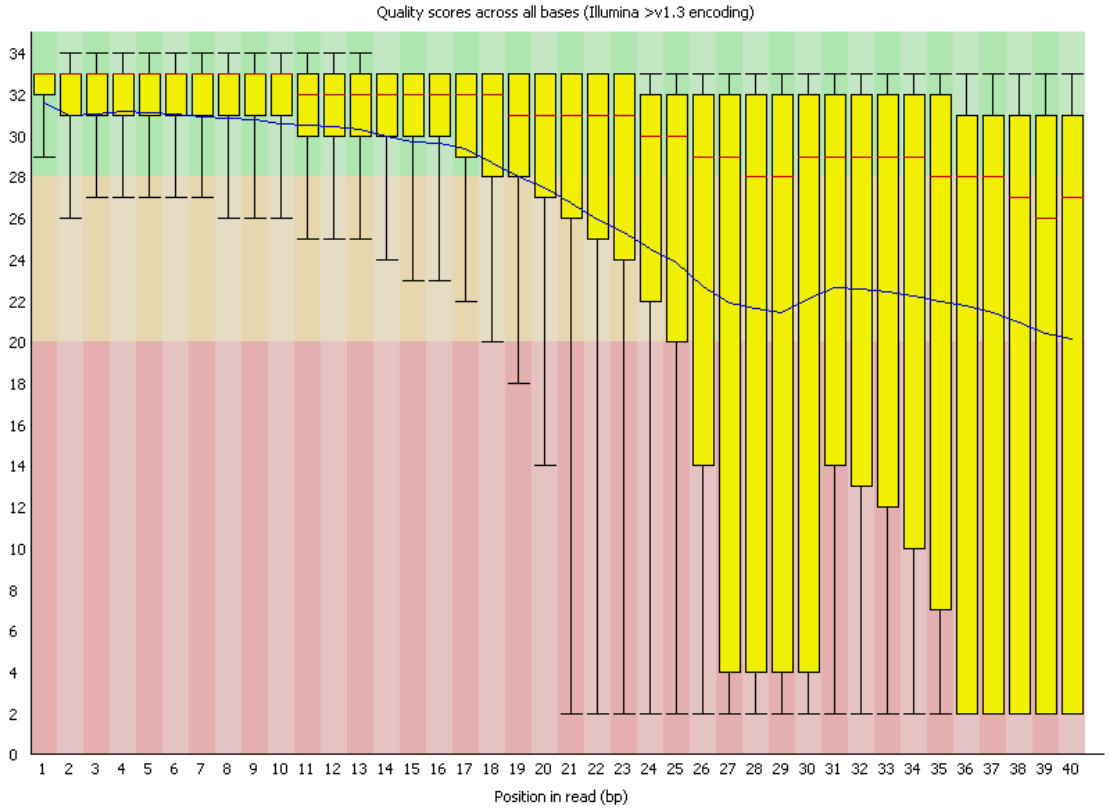
$$Q = -10 \log_{10} P \quad \longrightarrow \quad P = 10^{\frac{-Q}{10}}$$

| Phred Quality Score | Probability of incorrect base call | Base call accuracy |
|---|---|---|
| 10 | 1 in 10 | 90% |
| 20 | 1 in 100 | 99% |
| 30 | 1 in 1000 | 99.9% |
| 40 | 1 in 10000 | 99.99% |
| 50 | 1 in 100000 | 99.999% |

# Good and bad quality of sequencing reads

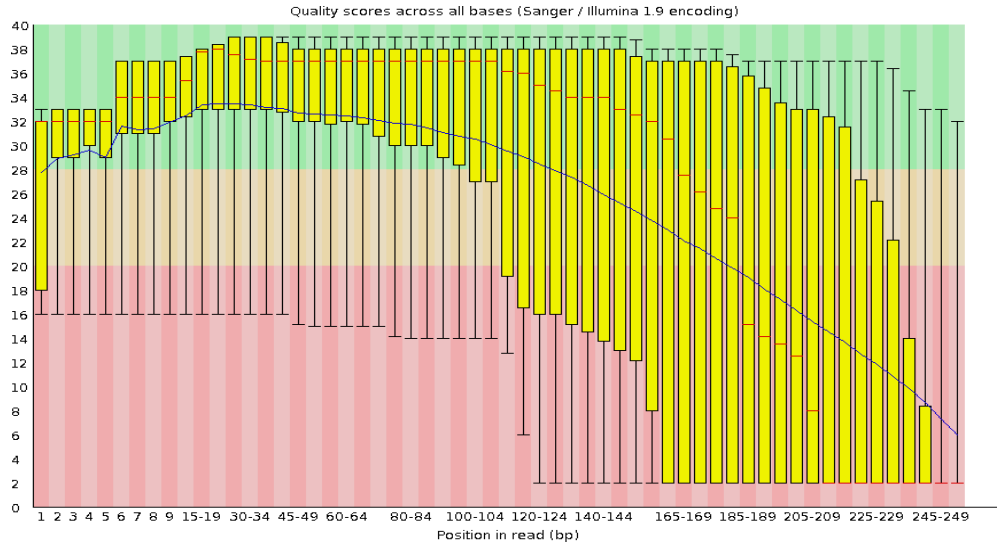
## Per base sequence quality

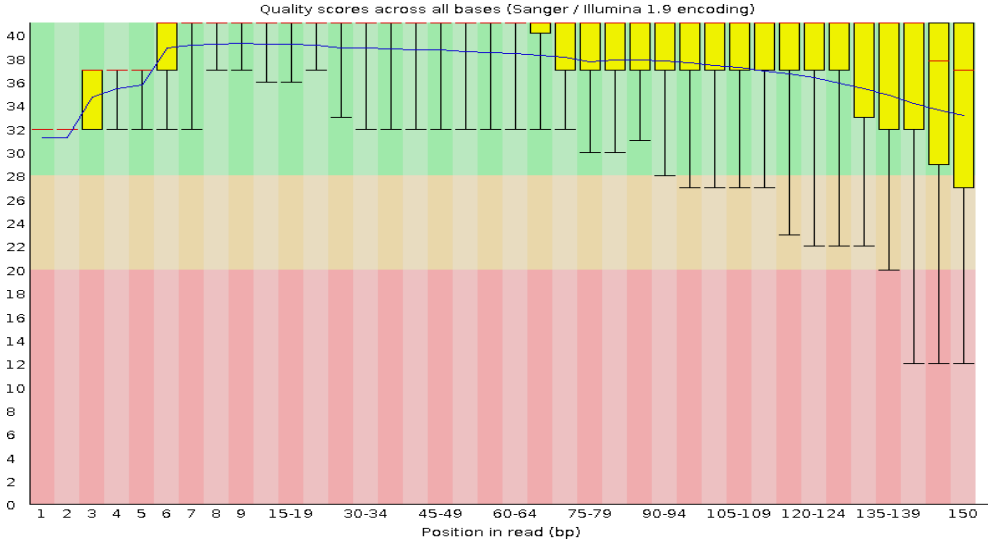


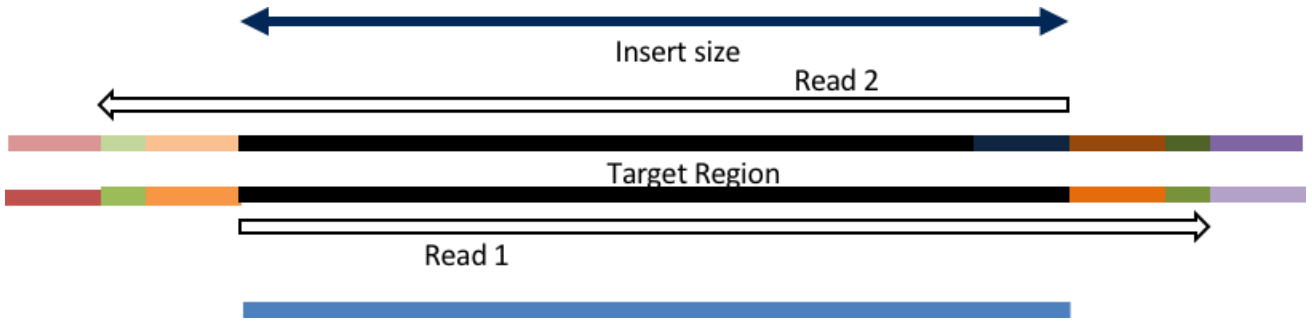
# Data quality checking and removing the contaminations

## RNAseq reads quality




## Adapter removal




Insert size < read length

## Trimming the reads

**For paired end:** Bbduk, Skewer, HTStream, and FASTP

**For single end:** Cutadapt, HTStream, and BBduk

# Software for data quality checking

1. We use fastqc to run data quality checking.
2. Available at https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

```
fastqc  xx.fastq
```

# Tools used for long reads mapping

- Minimap2
- LAST
- NGMLR
- GraphMap

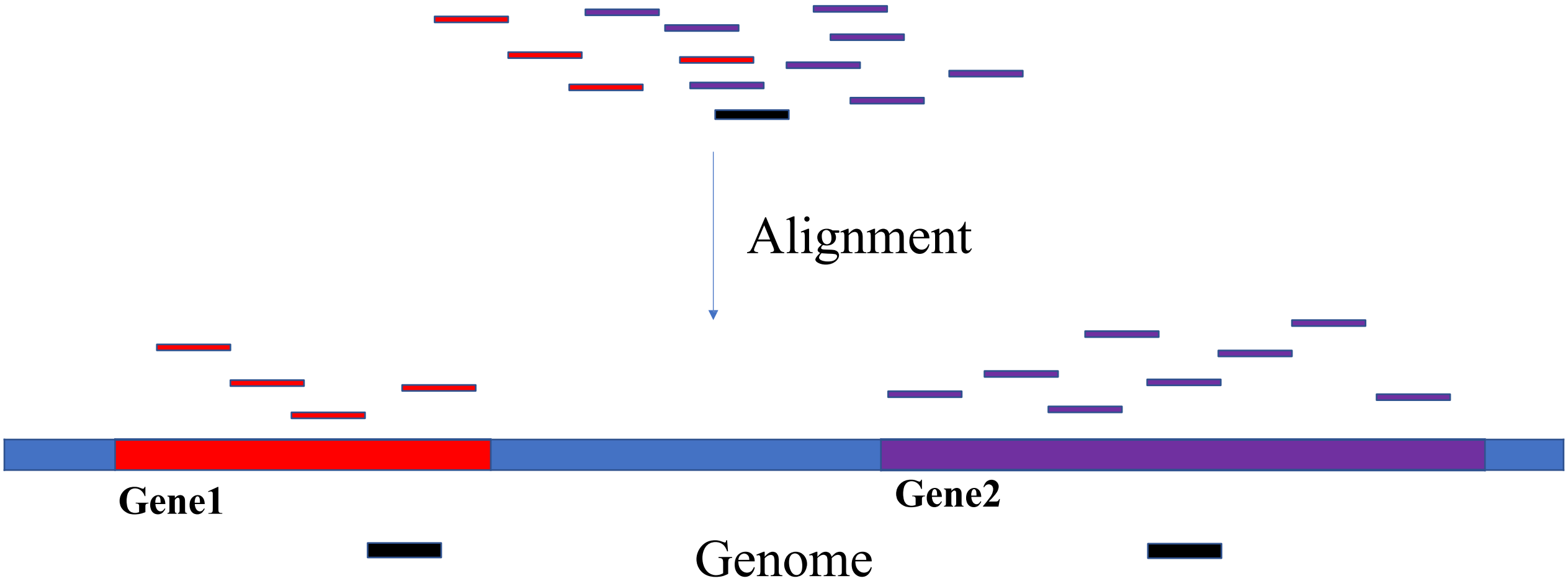
https://www.nature.com/articles/s41587-021-01108-x

# Tools used for short reads mapping

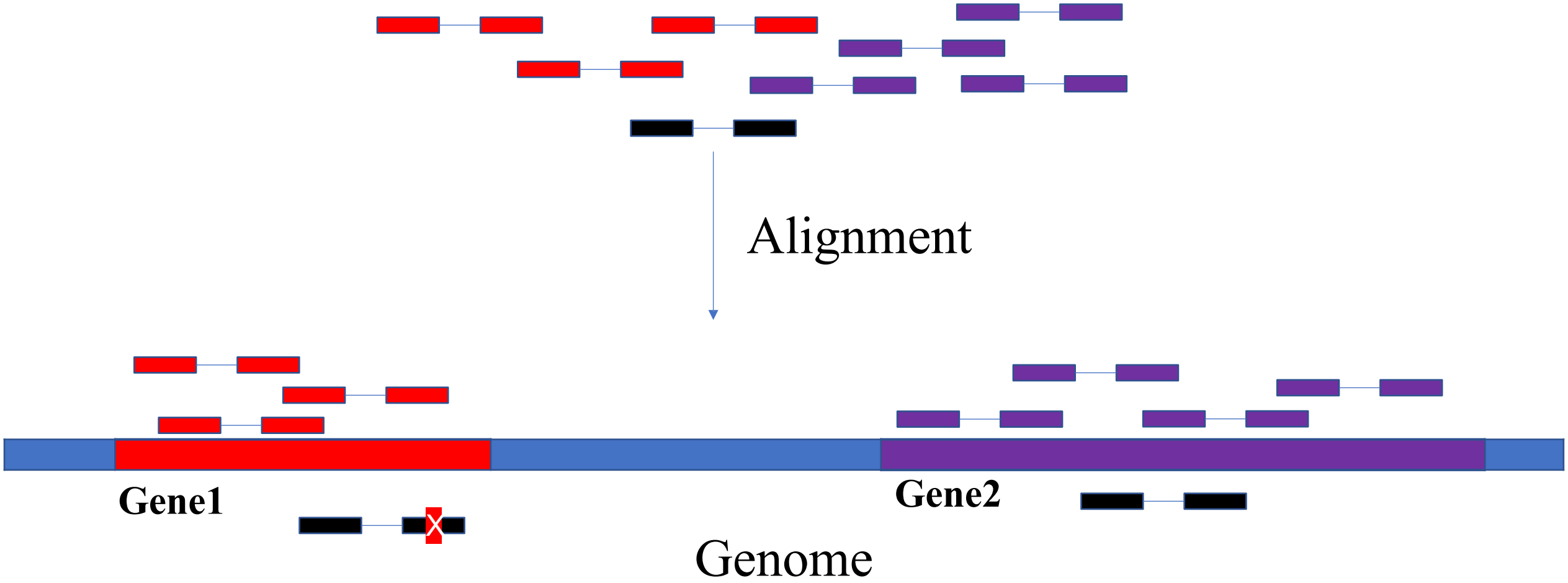
- STAR (Spliced Transcripts Alignment to a Reference)
- Kallisto
- BWA (Burrows-Wheeler Aligner)

# Read mapping (single-end)

The process to align short RNAseq reads with the genome (transcriptome) sequence.



# Read mapping (paired-end)



# Quantification of gene expression (raw read count)


Genome

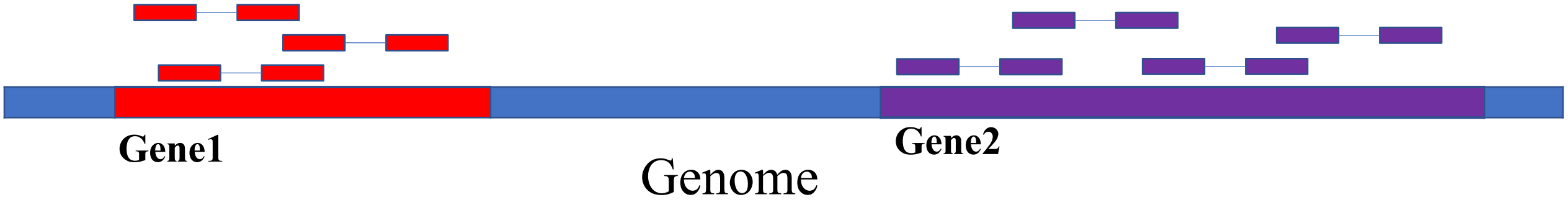
Expression of **Gene1**: 3
Expression of **Gene2**: 4

# Problem of raw read count

1. NOT comparable between different experiments (library size matters).
2. NOT comparable between different genes (gene length matters).

Raw-read-count is proportional to library.size * gene.length,
**normalization** is needed!

Rep1 (library size = 7)




Rep2 (library size = 14)

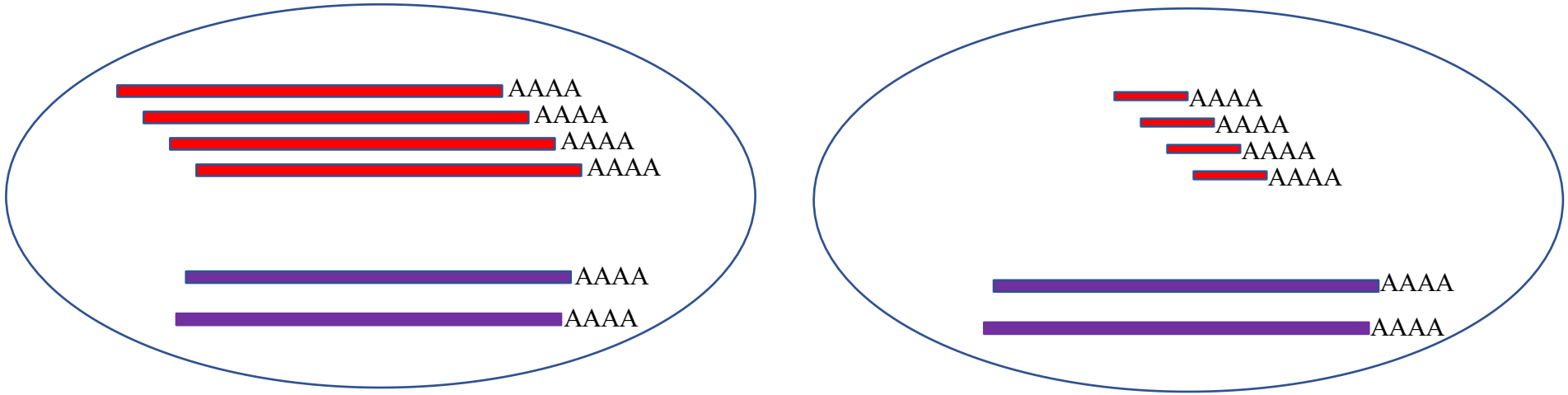
# Quantification of gene expression (RPKM and FPKM)

RPKM: Reads Per Kilobase Per million mapped reads (single-end).
FPKM: Fragments Per Kilobase Per million mapped reads (paired-end).

C = Number of reads (fragments) mapped to a gene
N = Total number of mapped reads (fragments) in the experiment
L = Exon length in base-pairs for a gene

$$RPKM = (10^9 * C) / (\mathbf{N} * \mathbf{L})$$

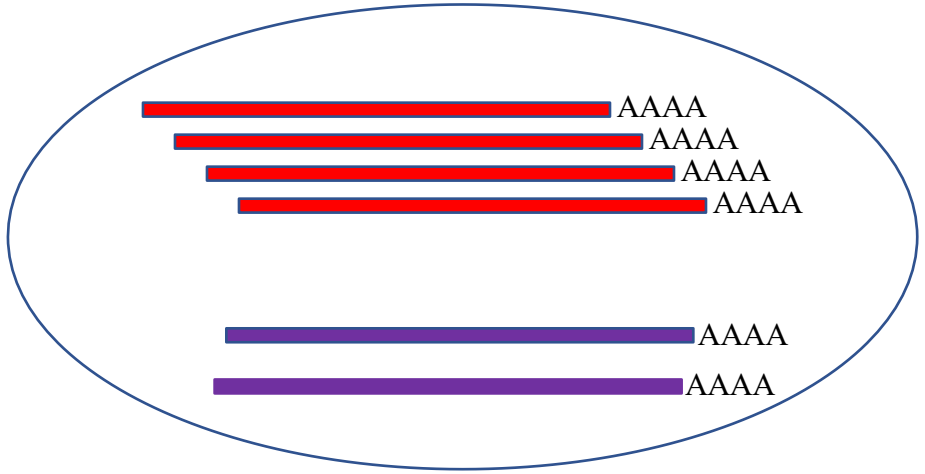
# Problem of RPKM (FPKM)



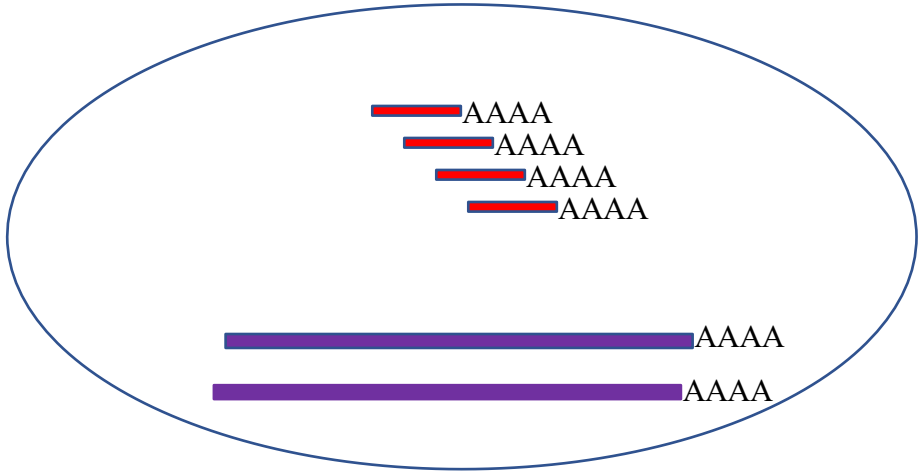

Gene1 $E_1 = (4 / 6)$

Gene2 $E_2 = (2 / 6)$

# Isoform matters




Exp1




Exp2

Suppose we want to compute RPKM for the **purple** gene

RPKM = (10^9 * C)/(**N * L**) = 10^9 * (C / N **) * (1 / L)**

C = Number of reads mapped to a gene
N = Total number of mapped reads in the experiment
L = Exon length in base-pairs for a gene

C / N: proportion of reads coming from a gene

rl: read length RNAseq experiment
Lp: isoform length (purple)
Lr: isoform length (red, exp1)
Lrs: isoform length (red, exp2)

P1 = Lp * 2 / rl  R1 = Lr * 4 / rl
P2 = Lp * 2 / rl  R2 = Lrs * 4 / rl

RPKM1 = 10^9 * ( P1/(P1+R1) ) * 1 / Lp
RPKM2 = 10^9 * ( P2/(P2+R2) ) * 1 / Lp

# Quantification of gene expression (TPM)

TPM: Transcripts Per Million.

Given a Gene Gi, compute $T_i = C_i / L_i$

$C_i$ : Number of reads mapped to the gene
$L_i$: Exon length in base-pairs for the gene

$$TPM_i = 10^6 * T_i / (T_1 + T_2 + \ldots\ldots T_n)$$

Wagner *et* al. **Measurement of mRNA abundance using RNA-seq data: RPKM measure is inconsistent among samples.**

# TPM vs RPKM

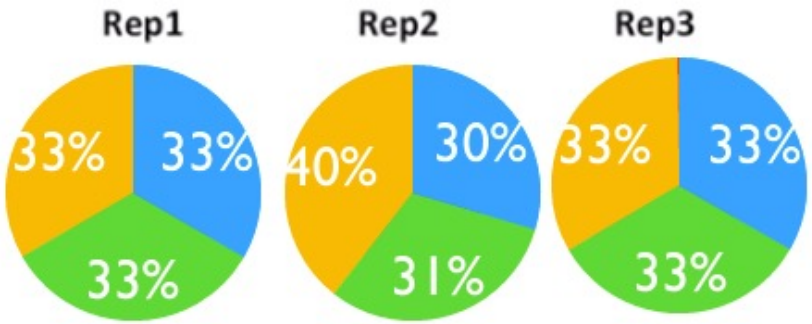
Original data:

| Gene Name | Rep1 Counts | Rep2 Counts | Rep3 Counts |
|---|---|---|---|
| A (2kb) | 10 | 12 | 30 |
| B (4kb) | 20 | 25 | 60 |
| C (1kb) | 5 | 8 | 15 |
| D (10kb) | 0 | 0 | 1 |

Consider 3 pies, each the same size (10).

A 3.33 sized slice is the same in each pie, and is always larger than 3.32.

TPM makes it clear that in Rep1, more of its total reads mapped to gene A than in Rep3.




TPM

| Gene Name | Rep1 TPM | Rep2 TPM | Rep3 TPM |
|---|---|---|---|
| A (2kb) | 3.33 | 2.96 | 3.326 |
| B (4kb) | 3.33 | 3.09 | 3.326 |
| C (1kb) | 3.33 | 3.95 | 3.326 |
| D (10kb) | 0 | 0 | 0.02 |
| Total: | 10 | 10 | 10 |

RPKM

| Gene Name | Rep1 RPKM | Rep2 RPKM | Rep3 RPKM |
|---|---|---|---|
| A (2kb) | 1.43 | 1.33 | 1.42 |
| B (4kb) | 1.43 | 1.39 | 1.42 |
| C (1kb) | 1.43 | 1.78 | 1.42 |
| D (10kb) | 0 | 0 | 0.009 |
| Total: | 4.29 | 4.5 | 4.25 |

With RPKM, it is harder to compare the proportion of total reads because each replicate has different total (each pie has a different size)

A 1.43 size slice represents a different proportion of reads in in different pies.

# Relationship between RPKM (FPKM) and TPM

$$TPM_i = RPKM_i / Sum\ (RPKM)$$

Ref: https://rnajournal.cshlp.org/content/early/2020/04/13/rna.074922.120.full.pdf

# Core analysis

1. Data quality checking.
2. Read mapping.
3. Quantification of gene expression.
4. Differential gene expression analysis (R).
5. Interpretation of DE analysis results.

# Data quality checking

1. We use fastqc to run data quality checking.
2. Available at https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

```
fastqc  xx.fastq
```

# Quantification of gene expression

RNAseq reads
(FASTQ)

↓

Align to genome
(Tophat, STAR, HISAT2)

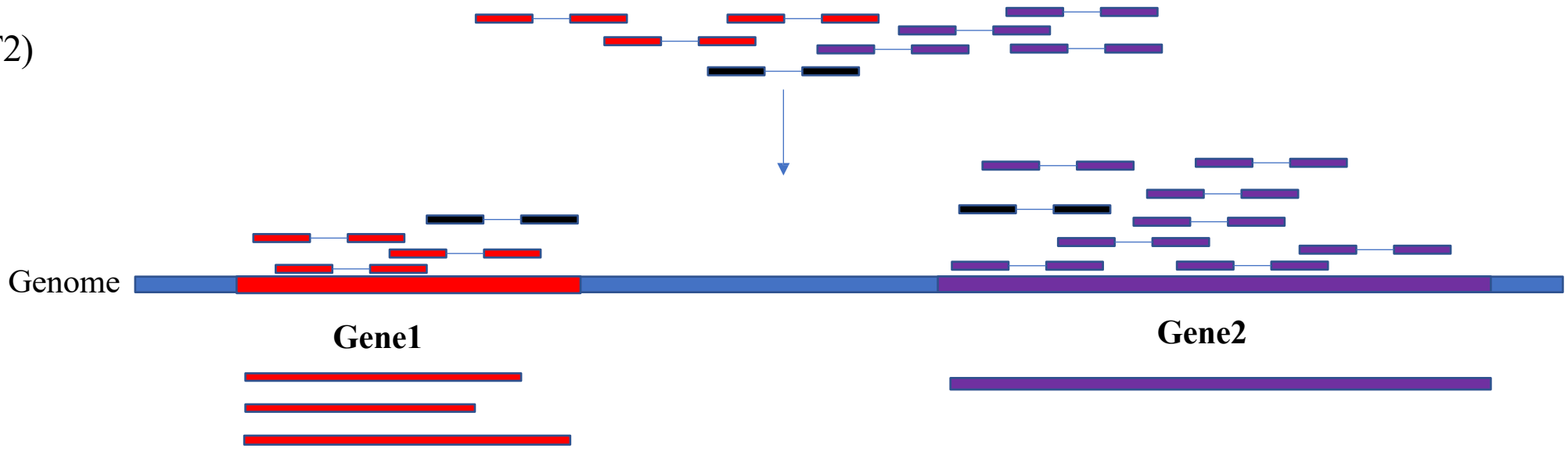
↓

BAM files

↓

read count matrix
(featureCounts, HTSeq)

1. The alignment process is time-consuming!
2. We can get the read count for gene, but NOT for isoforms!
3. Reads ambiguity not solved well.



# Quantification of gene expression

RNAseq reads
(FASTQ)

↓

Align to transcriptome
(**STAR**)

↓

BAM files

↓

RSEM

↓

read count, fpkm, tpm
**(genes and isoforms)**

RNAseq reads
(FASTQ)

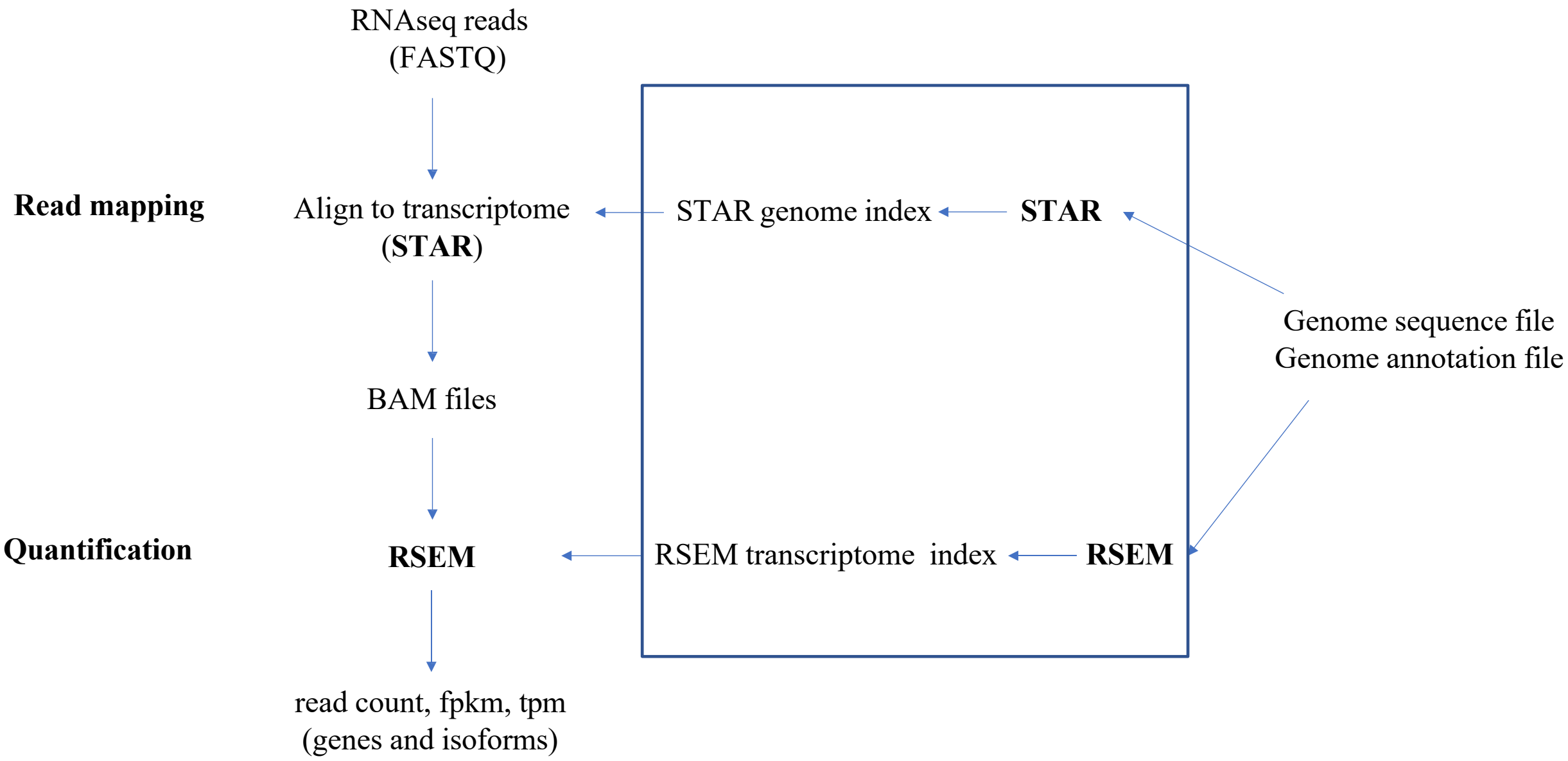
↓

Align to transcriptome
(Kallisto, Salmon)

↓

read count, tpm
**(isoforms)**

# Quantification of gene expression



# Preparation of index files

Genome sequence (from NCBI):
ftp://ftp.ncbi.nlm.nih.gov/genomes/archive/old_genbank/Eukaryotes/vertebrates_mammals/Homo_sapiens/GRCh38/seqs_for_alignment_pipelines/**GCA_000001405.15_GRCh38_no_alt_analysis_set.fna.gz**

Genome annotation file (from GENCODE):
ftp://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_23/**gencode.v23.annotation.gtf.gz**

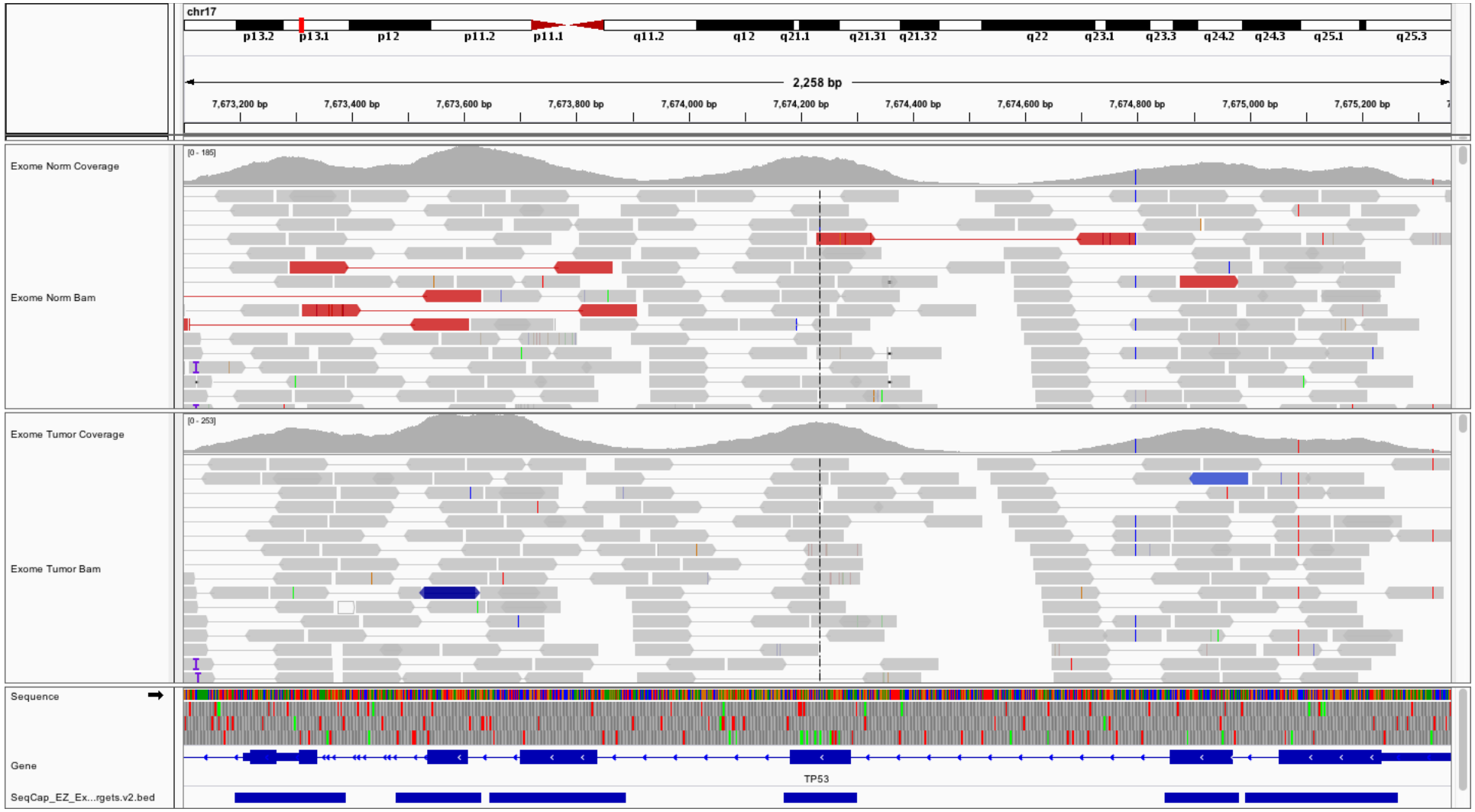
```
STAR  --runThreadN 32     \
          --runMode genomeGenerate    \
          --genomeDir STAR.index \
          --genomeFastaFiles GCA_000001405.15_GRCh38_no_alt_analysis_set.fna \
          --sjdbGTFfile gencode.v23.annotation.gtf
```

```
rsem-prepare-reference -p  4
                          --gtf      gencode.v23.annotation.gtf
                                     GCA_000001405.15_GRCh38_no_alt_analysis_set.fna
                                     RSEM.index/hg38.RSEM.index
```

# Code to run the mapping using STAR aligner

```
STAR --genomeDir /ensembl38_STAR_index/
 --runThreadN 6 \
--readFilesIn Mov10_oe_R1.subset.fq Mov10_oe_R2.subset.fq \
--outFileNamePrefix ../results/STAR/Mov10_oe_1_ \
--outSAMtype BAM SortedByCoordinate \
--outSAMunmapped Within \
--outSAMattributes Standard
```

# STAR aligner output



# Quantify the read count, TPM count and FPKM count

```
Rsem-calculate-expression \
--no-bam-output \
--quiet \
--no-qualities \
-p 8 \
--seed-length 25 \
--bam \
--paired-end  tmp/sample/Aligned.toTranscriptome.out.bam \
  $RSEM_INDEX  \
  RSEM.output/sample.txt
```

# RSEM-gene-count output

| gene_id | transcript_id(s) | length | effective_length | expected_count | TPM | FPKM |
|---|---|---|---|---|---|---|
| ENSG00000000003.14 | ENST00000373020.8,ENST00000494424.1,ENST00000496771.5,ENST0000061215 | 2232.08 | 1949.92 | 507 | 21.37 | 16.94 |
| ENSG00000000005.5 | ENST00000373031.4,ENST00000485971.1 | 940.5 | 659.64 | 0 | 0 | 0 |
| ENSG00000000419.12 | ENST00000371582.8,ENST00000371584.8,ENST00000371588.9,ENST0000041308 | 1080.86 | 798.7 | 1030 | 106 | 84.01 |
| ENSG00000000457.13 | ENST00000367770.5,ENST00000367771.10,ENST00000367772.8,ENST000004236 | 3552.99 | 3270.82 | 322.57 | 8.11 | 6.42 |
| ENSG00000000460.16 | ENST00000286031.10,ENST00000359326.8,ENST00000413811.3,ENST000004597 | 2094.04 | 1811.95 | 266.43 | 12.09 | 9.58 |
| ENSG00000000938.12 | ENST00000374003.7,ENST00000374004.5,ENST00000374005.7,ENST0000039917 | 1735.29 | 1453.21 | 0 | 0 | 0 |
| ENSG00000000971.15 | ENST00000359637.2,ENST00000367429.8,ENST00000466229.5,ENST0000047091 | 2516.93 | 2234.77 | 491 | 18.06 | 14.31 |
| ENSG00000001036.13 | ENST00000002165.10,ENST00000367585.1,ENST00000451668.1 | 2310.54 | 2028.39 | 1246.83 | 50.53 | 40.04 |
| ENSG00000001084.10 | ENST00000229416.10,ENST00000504353.1,ENST00000504525.1,ENST000005051 | 2397.67 | 2116.53 | 759 | 29.48 | 23.36 |
| ENSG00000001167.14 | ENST00000341376.10,ENST00000353205.5 | 2873.33 | 2591.16 | 601 | 19.07 | 15.11 |
| ENSG00000001460.17 | ENST00000003583.12,ENST00000337248.8,ENST00000374409.5,ENST000004351 | 2419.78 | 2137.65 | 150 | 5.77 | 4.57 |
| ENSG00000001461.16 | ENST00000003912.7,ENST00000339255.2,ENST00000358028.8,ENST0000037439 | 3891.98 | 3609.82 | 907 | 20.65 | 16.37 |
| ENSG00000001497.16 | ENST00000374804.9,ENST00000374807.9,ENST00000374811.7,ENST0000046909 | 2360.35 | 2078.42 | 1536 | 60.75 | 48.14 |
| ENSG00000001561.6 | ENST00000321037.4 | 4651 | 4368.83 | 200 | 3.76 | 2.98 |
| ENSG00000001617.11 | ENST00000002829.7,ENST00000413852.5,ENST00000414301.5,ENST0000042083 | 3300.24 | 3018.08 | 258 | 7.03 | 5.57 |
| ENSG00000001626.14 | ENST00000003084.10,ENST00000426809.5,ENST00000429014.1,ENST000004468 | 5207.91 | 4925.92 | 2667 | 44.5 | 35.27 |
| ENSG00000001629.9 | ENST00000265742.7,ENST00000413588.1,ENST00000422095.1,ENST0000043988 | 4820.71 | 4538.85 | 1338 | 24.23 | 19.2 |
| ENSG00000001630.15 | ENST00000003100.12,ENST00000422867.1,ENST00000435873.1,ENST000004507 | 2898.46 | 2616.3 | 1178 | 37.01 | 29.33 |
| ENSG00000001631.14 | ENST00000340022.6,ENST00000394503.6,ENST00000394505.6,ENST0000039450 | 2597.49 | 2315.46 | 523.65 | 18.59 | 14.73 |
| ENSG00000002016.16 | ENST00000228345.9,ENST00000358495.7,ENST00000397230.6,ENST0000043009 | 1486.5 | 1207.08 | 75.14 | 5.12 | 4.06 |
| ENSG00000002079.12 | ENST00000413734.1,ENST00000425880.1,ENST00000429079.1,ENST0000043978 | 1368.26 | 1090.4 | 21 | 1.58 | 1.25 |
| ENSG00000002330.13 | ENST00000309032.7,ENST00000394531.3,ENST00000394532.7,ENST0000049214 | 892.39 | 610.71 | 604.95 | 81.42 | 64.53 |
| ENSG00000002549.12 | ENST00000226299.8,ENST00000503467.1,ENST00000504614.5,ENST0000050796 | 1908.17 | 1626 | 1027 | 51.92 | 41.14 |
| ENSG00000002586.17 | ENST00000381177.5,ENST00000381180.7,ENST00000381184.5,ENST0000038118 | 1194.92 | 912.76 | 549 | 49.44 | 39.18 |
| ENSG00000002587.9 | ENST00000002596.5,ENST00000510712.1,ENST00000514690.5 | 6776.5 | 6495.05 | 1076 | 13.62 | 10.79 |
| ENSG00000002726.19 | ENST00000360937.8,ENST00000416793.6,ENST00000460213.1,ENST0000046729 | 2572.25 | 2290.08 | 1888 | 67.77 | 53.7 |

# Q&A

# III. Demo of RNAseq data analysis on MSU HPCC

# Starting the OnDemand on HPCC

MICHIGAN STATE UNIVERSITY

ICER INSTITUTE FOR CYBER-ENABLED RESEARCH

About | System Status | For Users | Research | Education & Events | Contact

## Web Portal to HPCC Resources

Dear HPCC users,

It is now possible for users to access HPCC resources via internet browsers. Many new users will attempt to log into HPCC via the wiki page: docs.icer.msu.edu/. However, HPCC users are not able to login to the HPCC via the wiki pages. Instead, to access HPCC via a web browser, use the following portal:

**HPCC OnDemand, login portal**: https://ondemand.hpcc.msu.edu/
This is our new web access to HPCC resources. To see its features and how to use it, please visit "Open OnDemand" wiki page.

https://ondemand.hpcc.msu.edu/

# Login on OnDemand HPCC

CILogon

Consent to Attribute Release

HPCC OnDemand requests access to the following information. If you do not approve this request, do not proceed.

- Your CILogon user identifier
- Your name
- Your email address
- Your username and affiliation from your identity provider

Selected Identity Provider

Michigan State University

Remember this selection

Log On

Log On

By selecting "Log On", you agree to the privacy policy.

# Request to start Interactive Desktop

Home / My Interactive Sessions / Interactive Desktop (Legacy)

Interactive Apps

Desktops

- Interactive Desktop
- Interactive Desktop (Legacy)

GUIs

- MATLAB
- MATLAB (Legacy)
- ParaView
- ParaView (Legacy)
- RStudio (Legacy)
- Stata
- Stata (Legacy)

Servers

## Interactive Desktop (Legacy)

This app will launch an interactive desktop on one or more compute nodes. You will have full access to the resources these nodes provide. This is analogous to an interactive batch job.

Number of hours

5

Jobs shorter than four hours will schedule much faster

Number of cores per task

5

Amount of memory

50GB

E.g. 100GB or 500MB. 750MB per core if left blank.

☐ I would like to receive an email when the session starts

☐ Advanced Options

Launch

# Lunch Interactive Desktop on HPCC

Interactive Desktop (Legacy) (40114511) 1 node | 5 cores | Running

**Host:** >_css-075

Cancel

**Created at:** 2024-08-02 11:30:22 EDT

**Time Remaining:** 4 hours and 58 minutes

**Session ID:** f69a6fec-9d2c-4b85-a732-7629ab9f3e67

**Problems with this session?** Submit support ticket

Compression

0 (low) to 9 (high)

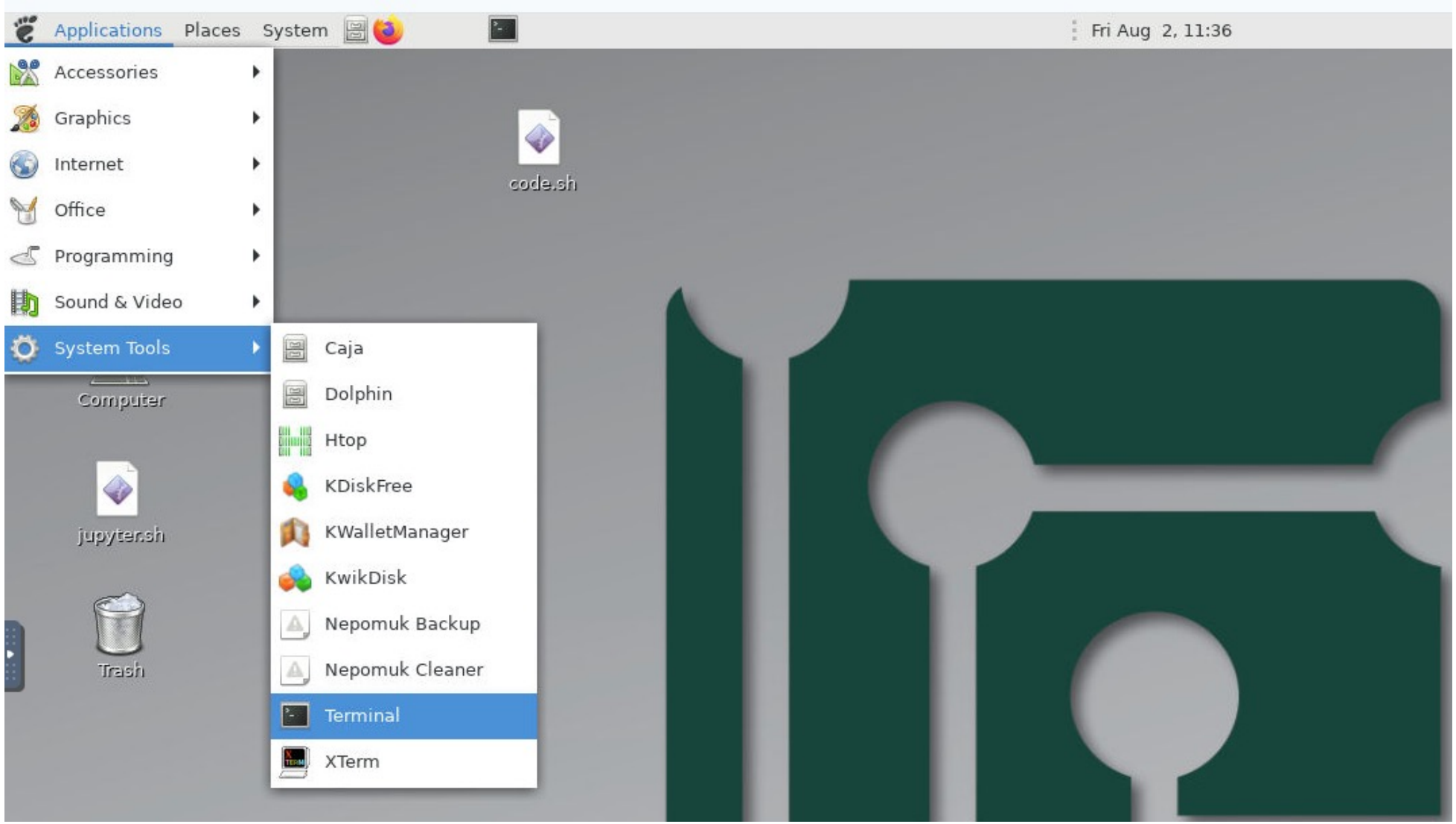
Image Quality

0 (low) to 9 (high)

Launch Interactive Desktop (Legacy)

View Only (Share-able Link)

# Access terminal on Interactive Desktop



# Available versions of STAR package on HPCC

```
[ramashan@css-075 ~]$ module spider STAR

-----------------------------------------------------------------------------------------
  STAR:
-----------------------------------------------------------------------------------------
    Description:
      STAR aligns RNA-seq reads to a reference genome using uncompressed suffix arrays.

     Versions:
        STAR/2.6.0c
        STAR/2.6.1c
        STAR/2.7.2b
        STAR/2.7.3a
        STAR/2.7.9a
        STAR/2.7.10b
     Other possible modules matches:
        stars

-----------------------------------------------------------------------------------------
  To find other possible module matches execute:

      $ module -r spider '.*STAR.*'

-----------------------------------------------------------------------------------------
  For detailed information about a specific "STAR" package (including how to load the modules) u
se the module's full name.
  Note that names that have a trailing (E) are extensions provided by other modules.
  For example:

     $ module spider STAR/2.7.10b
-----------------------------------------------------------------------------------------
```

# Loading of STAR package on HPCC

```
[ramashan@css-075 ~]$ module spider STAR/2.7.10b

----------------------------------------------------------------------------------------
  STAR: STAR/2.7.10b
----------------------------------------------------------------------------------------
    Description:
      STAR aligns RNA-seq reads to a reference genome using uncompressed suffix arrays.


    You will need to load all module(s) on any one of the lines below before the "STAR/2.7.10b"
module is available to load.

      GCC/11.3.0

    Help:
      Description
      ===========
      STAR aligns RNA-seq reads to a reference genome using uncompressed suffix arrays.


      More information
      ================
       - Homepage: https://github.com/alexdobin/STAR
```

```
[ramashan@css-075 ~]$ module purge
[ramashan@css-075 ~]$ module load GCC/11.3.0 STAR/2.7.10b
```

# Loading of RSEM package on HPCC

```
[ramashan@css-075 ~]$ module spider RSEM

-----------------------------------------------------------------------------------------
  RSEM:
-----------------------------------------------------------------------------------------
    Description:
      RNA-Seq by Expectation-Maximization

     Versions:
        RSEM/1.3.0
        RSEM/1.3.1
        RSEM/1.3.3

[ramashan@css-075 ~]$ module spider RSEM/1.3.3

-----------------------------------------------------------------------------------------
  RSEM: RSEM/1.3.3
-----------------------------------------------------------------------------------------
    Description:
      RNA-Seq by Expectation-Maximization


    You will need to load all module(s) on any one of the lines below before the "RSEM/1.3.3" mo
dule is available to load.

      GCC/11.2.0  OpenMPI/4.1.1
      GCC/11.3.0  OpenMPI/4.1.4
      GCC/8.3.0  OpenMPI/3.1.4
```

[ramashan@css-075 ~]$ module load GCC/11.3.0  OpenMPI/4.1.4 RSEM/1.3.3

# Loading R on HPCC

[ramashan@css-075 ~]$ module spider R

```
Versions:
   R/3.3.1
   R/3.4.3-X11-20160819
   R/3.4.3-X11-20171023
   R/3.4.3xF
   R/3.4.3xS
   R/3.4.4-X11-20180131
   R/3.5.0-X11-20180131
   R/3.5.1-X11-20180131
   R/3.5.1-X11-20180604
   R/3.6.0-X11-20180604
   R/3.6.2-X11-20180604
   R/3.6.2
   R/3.6.3
   R/4.0.0-X11-20180604
   R/4.0.0
   R/4.0.2.bak
   R/4.0.2.test
   R/4.0.2-X11-20180604
   R/4.0.2
   R/4.0.3
   R/4.1.0
   R/4.1.2
   R/4.2.1
   R/4.2.2
   R/4.3.1
Other possible modules matches:
   ADMIXTURE  AMDuProf  APR  APR-util  Abaqus_parallel  AdapterRemoval  Advisor  ...
```

[ramashan@css-075 ~]$ module spider R/4.2.1

```
-----------------------------------------------------------------------------------------
  R: R/4.2.1
-----------------------------------------------------------------------------------------
    Description:
      R is a free software environment for statistical computing and graphics.


    You will need to load all module(s) on any one of the lines below before the "R/4.2.1" modul
e is available to load.

      GCC/11.3.0  OpenMPI/4.1.4
```

[ramashan@css-075 ~]$ module load GCC/11.3.0  OpenMPI/4.1.4 R/4.2.1

# Test the loaded module

```
ramashan@dev-amd20-v100:~/data/RNASeq_test$ STAR
Usage: STAR  [options]... --genomeDir /path/to/genome/index/  \
--readFilesIn R1.fq R2.fq

Spliced Transcripts Alignment to a Reference (c) Alexander Dobin, 2009-2022


STAR version=2.7.11b
STAR compilation time,server,dir=2024-07-18T13:18:20-04:00 dev-intel14:/tmp/
panchyni/easybuild/easybuild/build/STAR/2.7.11b/GCC-13.2.0/STAR-2.7.11b/source

For more details see:
<https://github.com/alexdobin/STAR>
<https://github.com/alexdobin/STAR/blob/master/doc/STARmanual.pdf>


To list all parameters, run STAR --help
```

# Create reference index file for STAR and RSEM

```
[ramashan@skl-162 RNASeq_test]$ STAR --runThreadN 32 --runMode genomeGenerate --genomeDir Gen
ome/ --genomeFastaFiles Genome/fasta/genome.fa --sjdbGTFfile Genome/genes/genes.gtf
        STAR --runThreadN 32 --runMode genomeGenerate --genomeDir Genome/ --genomeFastaFiles
Genome/fasta/genome.fa --sjdbGTFfile Genome/genes/genes.gtf
        STAR version: 2.7.10b   compiled: 2023-09-22T17:53:50-0400 dev-intel18:/tmp/panchyni/
EASYBUILD/STAR/2.7.10b/GCC-11.3.0/STAR-2.7.10b/source
Aug 01 15:34:04 ..... started STAR run
Aug 01 15:34:04 ... starting to generate Genome files
Aug 01 15:34:48 ..... processing annotations GTF
Aug 01 15:35:15 ... starting to sort Suffix Array. This may take a long time...
Aug 01 15:35:30 ... sorting Suffix Array chunks and saving them to disk...
Aug 01 16:24:53 ... loading chunks from disk, packing SA...
Aug 01 16:25:56 ... finished generating suffix array
Aug 01 16:25:56 ... generating Suffix Array index
Aug 01 16:28:56 ... completed Suffix Array index
Aug 01 16:28:57 ..... inserting junctions into the genome indices
Aug 01 16:32:13 ... writing Genome to disk ...
Aug 01 16:32:14 ... writing Suffix Array to disk ...
Aug 01 16:32:23 ... writing SAindex to disk
Aug 01 16:32:24 ..... finished successfully
```

[ramashan@css-075 ~]$ rsem-prepare-reference -p 4 --gtf Genome/genes/genes.gtf Genome/fasta/Genome.fa hg38

# Running bulk.rna.seq.pipeline.R to process the samples

```
[ramashan@skl-162 HUMAN_JUL24]$ Rscript chenlab.bulk.rna.seq.pipeline.R sample.name.txt
Loading required package: data.table
Loading required package: plyr
Loading required package: dplyr

Attaching package: ‘dplyr’

The following objects are masked from ‘package:plyr’:

    arrange, count, desc, failwith, id, mutate, rename, summarise,
    summarize

The following objects are masked from ‘package:data.table’:

    between, first, last

The following objects are masked from ‘package:stats’:

    filter, lag

The following objects are masked from ‘package:base’:

    intersect, setdiff, setequal, union

Loading required package: foreach
Loading required package: parallel
Loading required package: doParallel
Loading required package: iterators
[1] "sample HT29.Ctr1 finished!"
[1] "sample HT29.Ctr2 finished!"
[1] "sample HT29.Ctr3 finished!"
[[1]]
[1] "sample HT29.Ctr1 finished!"

[[2]]
[1] "sample HT29.Ctr2 finished!"

[[3]]
[1] "sample HT29.Ctr3 finished!"
```

# Raw read count in processed samples

| | HT29_CPN_OE1 | HT29_CPN_OE2 | HT29_CPN_OE3 | HT29_Ctr1 | HT29_Ctr2 | HT29_Ctr3 |
|---|---|---|---|---|---|---|
| ENSG00000000003 | 9.192293 | 9.269127 | 9.330917 | 8.988685 | 8.965784 | 9.157347 |
| ENSG00000000005 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 |
| ENSG00000000419 | 10.051209 | 10.317413 | 10.323055 | 10.009829 | 10.125413 | 10.317413 |
| ENSG00000000457 | 8.463851 | 8.578826 | 8.552439 | 8.337934 | 8.354602 | 8.283551 |
| ENSG00000000460 | 8.128871 | 8.236636 | 7.[illegible] | 8.063018 | 8.122414 | 8.310158 |
| ENSG00000000938 | 0.000000 | 1.000000 | 0.000000 | 0.000000 | 1.000000 | 0.000000 |
| ENSG00000000971 | 9.330917 | 9.560333 | 9.467606 | 8.942515 | 9.019591 | 8.960002 |
| ENSG00000001036 | 10.278275 | 10.410271 | 10.383564 | 10.285206 | 10.229816 | 10.310567 |
| ENSG00000001084 | 9.479780 | 9.622052 | 9.632995 | 9.569856 | 9.567956 | 9.471391 |
| ENSG00000001167 | 9.105909 | 9.385862 | 9.442943 | 9.233620 | 9.182394 | 9.038919 |
| ENSG00000001460 | 7.011227 | 7.383704 | 6.930737 | 7.238405 | 7.139551 | 7.228819 |
| ENSG00000001461 | 10.080818 | 10.166163 | 9.981567 | 9.826548 | 9.823367 | 9.829723 |
| ENSG00000001497 | 10.634811 | 10.760720 | 10.676839 | 10.585901 | 10.544964 | 10.502832 |
| ENSG00000001561 | 7.451211 | 7.807355 | 7.918863 | 7.651052 | 8.055282 | 7.584963 |
| ENSG00000001617 | 7.189825 | 7.383704 | 7.118941 | 8.016808 | 7.826548 | 8.027906 |
| ENSG00000001626 | 11.611025 | 11.811776 | 11.706496 | 11.381543 | 11.442943 | 11.123475 |
| ENSG00000001629 | 10.590587 | 10.716819 | 10.568906 | 10.386940 | 10.454299 | 10.090112 |
| ENSG00000001630 | 10.592457 | 10.827343 | 10.743151 | 10.203348 | 10.197217 | 10.286558 |

# FPKM values in processed samples

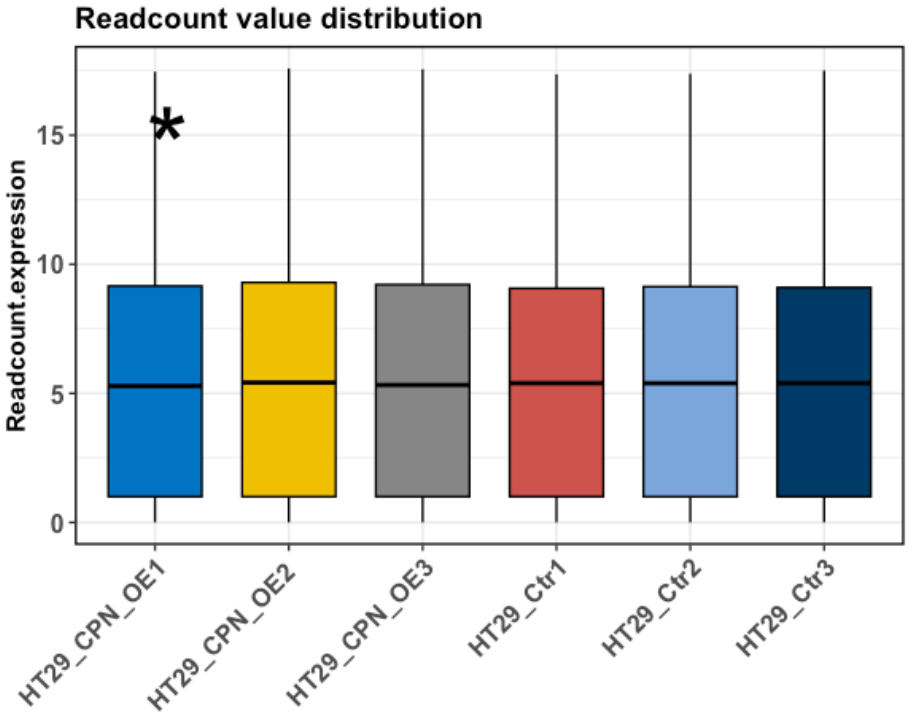
| | HT29_CPN_OE1 | HT29_CPN_OE2 | HT29_CPN_OE3 | HT29_Ctr1 | HT29_Ctr2 | HT29_Ctr3 |
|---|---|---|---|---|---|---|
| ENSG00000000003 | 4.38474059 | 4.29278175 | 4.40190347 | 4.16510799 | 4.14812063 | 4.33199178 |
| ENSG00000000005 | 0.00000000 | 0.00000000 | 0.00000000 | 0.00000000 | 0.00000000 | 0.00000000 |
| ENSG00000000419 | 6.36457243 | 6.50937915 | 6.59155975 | 6.40956065 | 6.49441561 | 6.64745843 |
| ENSG00000000457 | 2.86987141 | 3.00719550 | 2.92219785 | 2.89141919 | 2.88557436 | 2.75060650 |
| ENSG00000000460 | 3.28540222 | 3.38818954 | 3.11935618 | 3.40326772 | 3.35614381 | 3.51222689 |
| ENSG00000000938 | 0.00000000 | 0.04264434 | 0.00000000 | 0.00000000 | 0.05658353 | 0.00000000 |
| ENSG00000000971 | 4.22650853 | 4.24031433 | 4.27575205 | 3.93640238 | 3.91838623 | 4.02236781 |
| ENSG00000001036 | 5.28429203 | 5.28798935 | 5.33378150 | 5.35895883 | 5.26190686 | 5.33449677 |
| ENSG00000001084 | 4.64558639 | 4.78816366 | 5.03033608 | 4.60644223 | 4.85199884 | 4.79233481 |
| ENSG00000001167 | 3.82171022 | 3.89239103 | 4.12928302 | 4.00988459 | 3.96347412 | 3.64616266 |
| ENSG00000001460 | 2.33342373 | 2.76765480 | 2.15380534 | 2.47767733 | 2.36176836 | 2.63691458 |
| ENSG00000001461 | 4.39574833 | 4.34411833 | 4.27127626 | 4.11852585 | 4.19298317 | 4.00898878 |
| ENSG00000001497 | 5.56955185 | 5.61470984 | 5.56437817 | 5.61882595 | 5.49952702 | 5.48284828 |
| ENSG00000001561 | 1.79077204 | 1.95977016 | 2.09761080 | 1.99276843 | 2.28688115 | 1.90303827 |
| ENSG00000001617 | 1.94860085 | 1.99276843 | 2.30742853 | 2.71589337 | 2.58255600 | 2.69599381 |
| ENSG00000001626 | 5.25247621 | 5.32624970 | 5.40803247 | 5.18070484 | 5.13996057 | 4.75381844 |
| ENSG00000001629 | 4.78920758 | 4.36946648 | 4.85399565 | 4.33628339 | 4.59514557 | 3.97361128 |
| ENSG00000001630 | 5.25738784 | 5.31542132 | 5.35473424 | 4.92267359 | 4.93545975 | 4.90400232 |
| ENSG00000001631 | 4.45549162 | 4.25851892 | 4.26077843 | 3.97544677 | 4.23496109 | 4.02768488 |

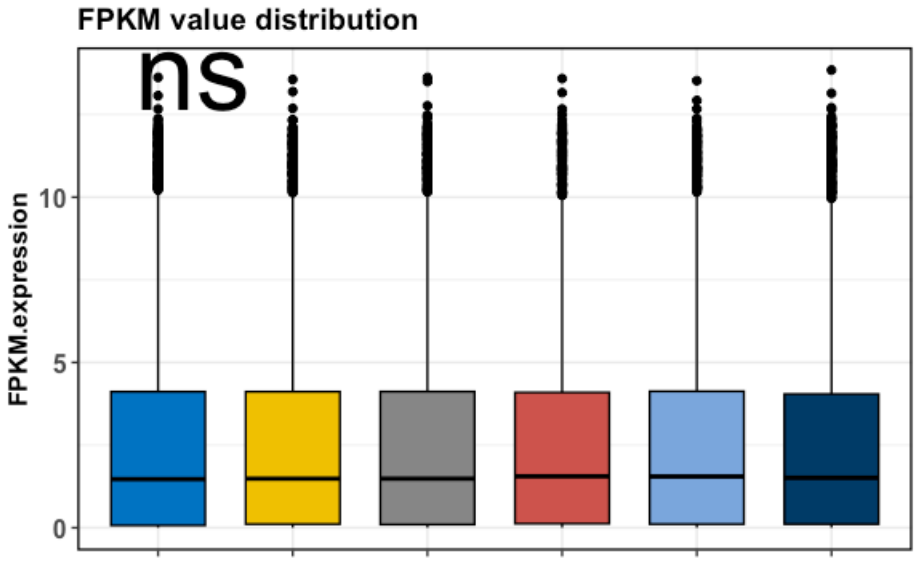
* Data was paired end

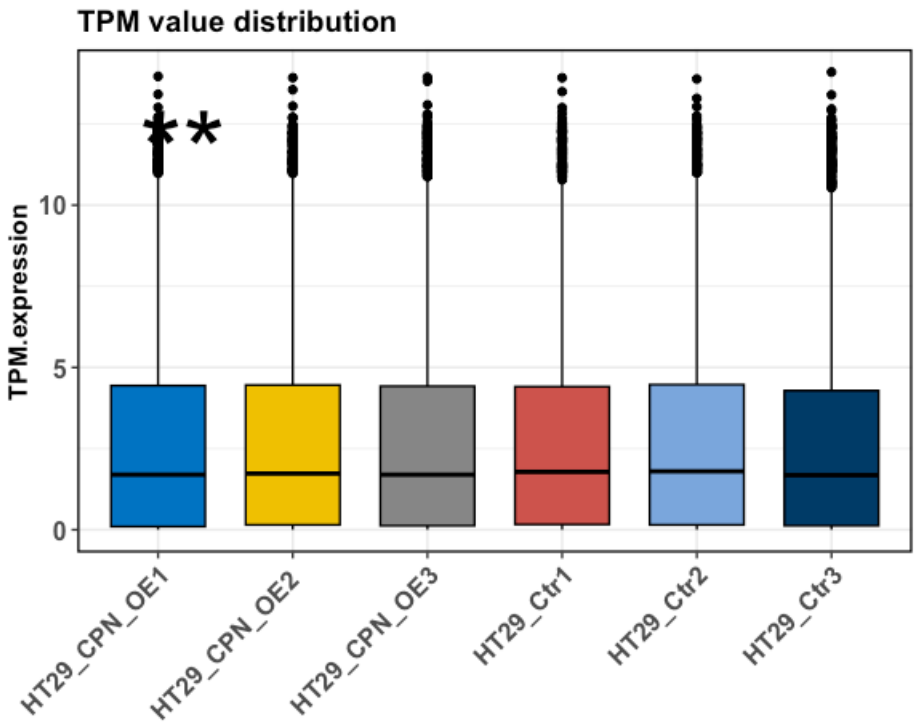
# TPM values in processed samples

| | HT29_CPN_OE1 | HT29_CPN_OE2 | HT29_CPN_OE3 | HT29_Ctr1 | HT29_Ctr2 | HT29_Ctr3 |
|---|---|---|---|---|---|---|
| ENSG[illegible]3 | 4.71314590 | 4.63981098 | 4.70984202 | 4.48349335 | 4.49313492 | 4.57470705 |
| ENSG00000000005 | 0.00000000 | 0.00000000 | 0.00000000 | 0.00000000 | 0.00000000 | 0.00000000 |
| ENSG00000000419 | 6.70431868 | 6.86888427 | 6.90977310 | 6.74146699 | 6.85436974 | 6.89953819 |
| ENSG00000000457 | 3.17152711 | 3.33055840 | 3.20633065 | 3.18745105 | 3.20476675 | 2.97085365 |
| ENSG00000000460 | 3.59693514 | 3.71918344 | 3.40599236 | 3.71039319 | 3.68818036 | 3.74631277 |
| ENSG00000000938 | 0.00000000 | 0.05658353 | 0.00000000 | 0.00000000 | 0.07038933 | 0.00000000 |
| ENSG00000000971 | 4.55397481 | 4.58616425 | 4.58255600 | 4.25247621 | 4.26077843 | 4.26228281 |
| ENSG00000001036 | 5.61970645 | 5.64299031 | 5.64789009 | 5.68734069 | 5.61676937 | 5.58315800 |
| ENSG00000001084 | 4.97682185 | 5.13955135 | 5.34269696 | 4.92979100 | 5.20437551 | 5.03826058 |
| ENSG00000001167 | 4.14323013 | 4.23342794 | 4.43362717 | 4.32696871 | 4.30597052 | 3.88166462 |
| ENSG00000001460 | 2.61353165 | 3.08406426 | 2.40871186 | 2.75915583 | 2.66220550 | 2.85199884 |
| ENSG00000001461 | 4.72410452 | 4.69097574 | 4.57773093 | 4.43629512 | 4.53853816 | 4.24868663 |
| ENSG00000001497 | 5.90665013 | 5.97108366 | 5.87946072 | 5.94836723 | 5.85574059 | 5.73199779 |
| ENSG00000001561 | 2.04614178 | 2.23878686 | 2.35049725 | 2.25096157 | 2.58255600 | 2.09423607 |
| ENSG00000001617 | 2.21101219 | 2.27202319 | 2.56803210 | 3.00539999 | 2.89141919 | 2.91264986 |
| ENSG00000001626 | 5.58796499 | 5.68116812 | 5.72246602 | 5.50779464 | 5.49441561 | 4.99954909 |
| ENSG00000001629 | 5.12142991 | 4.71644224 | 5.16551002 | 4.65706830 | 4.94532678 | 4.21256934 |
| ENSG00000001630 | 5.59305492 | 5.67044381 | 5.66902677 | 5.24830712 | 5.28835856 | 5.15015346 |
| ENSG00000001631 | 4.78502736 | 4.60466442 | 4.56681515 | 4.29204549 | 4.58135125 | 4.26753580 |

# Visualization of Raw read count, FPKM, and TPM



# Take-home message

1. Know your RNAseq library clearly (SE or PE, Stranded or Non-stranded).

2. Three gene expression quantification measurements (raw-read-count, RPKM (FPKM), TPM).

# Resources

# Resources

1. Wang *et al*. RNA-Seq: a revolutionary tool for transcriptomics. (*Nature Reviews Genetics*).

2. Ana *et al*. A survey of best practices for RNA-seq data analysis (*Genome Biology*).

3. Lior Pachter's blog (https://liorpachter.wordpress.com/).

4. RNASeq blog (https://www.rna-seqblog.com/).

# Thank you

# Q & A