Complete bulk-RNA-seq pipeline. Part 1: fastq to count matrix

Author: Tomàs Montserrat Ayuso

April 6, 2023

Introduction

In the next two posts we will look at how to perform a complete bulk RNA-seq pipeline, from *fastq* files to statistical analysis. In this post we will see all the necessary steps to get the count matrix from the *fastq* files of each sample. In a simplified form we saw how to do this for a single sample in this previous post. Now, however, we'll look at a more realistic scenario where we don't have just one *fastq* file, but 98, counting technical and biological replicates that are paired-end. In the following post we will study how to perform a differential gene expression analysis with DESeq2 of these data.

We will use the data from GEO entry GSE124975, which correspond to the study by Marjanovic et al. (preprint: Salinomycin disturbs Golgi apparatus function and specifically affects cells in epithelial-to-mesenchymal transition).

The authors of this study were interested in studying the effect of the drug salinomycin on the HMLE cell line, used to study breast cancer. They had HMLE-Twist and HMLE-pBp cells, which differ in their ability to carry out epithelial-mesenchymal transition (EMT), which is very important in cancer progression. While HMLE-Twist cells express EMT markers, HMLE-pBp cells do not.

The design of this research has 4 different sample groups: HMLE-Twist and HMLE-pBp cells grown in the presence or absence of salinomycin. For each experimental condition we have 3 biological replicates and, for each sample, sequencing has been carried out in 4 different runs (which can be considered technical replicates). In total we have approximately 690 million reads. In addition, according to the authors' article, the two experimental conditions are paired (cells treated and not treated with salinomycin come from the same cell culture). We will have to keep this in mind when performing the statistical tests for differential gene expression.

Pipeline

The pipeline we will follow to carry out the processing and analysis of the data is as follows:

- Download data from ENA
- Quality control using *fastqc*
- Alignment of the sequences against the reference genome with *hisat2*
- Generation of the count matrix with *featureCounts*
- Combination of technical replicates by adding the counts with *DESeq2*

- Statistical analysis of the results with *DESeq2*

Since we will be generating quite a few intermediate files, it is necessary to be tidy throughout the analysis. So our directory should have a structure similar to this:

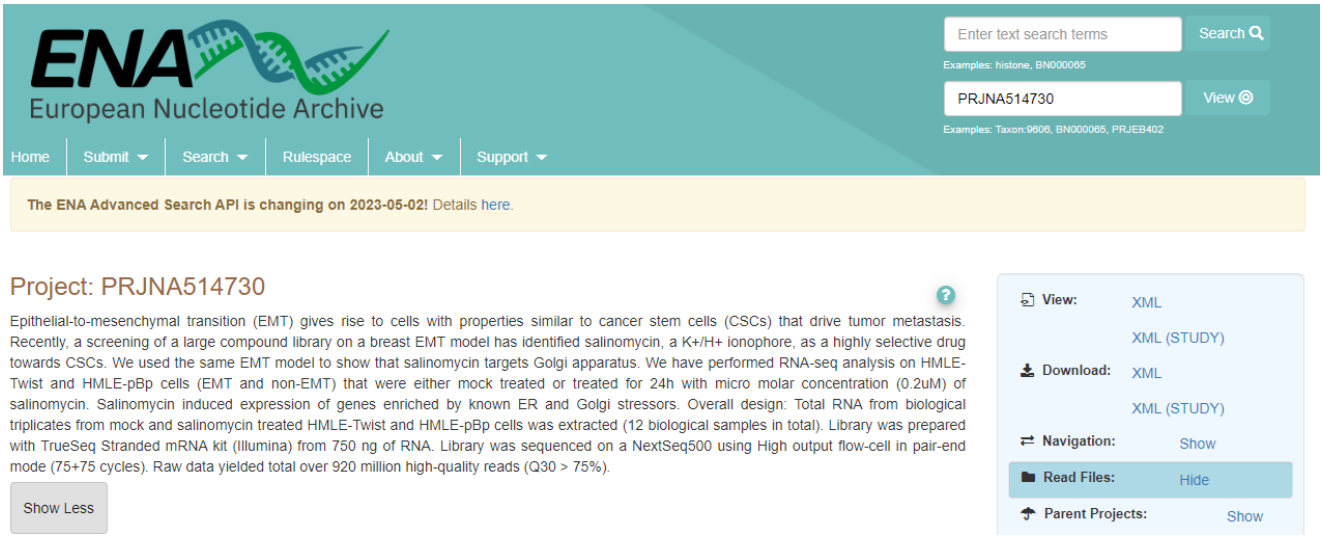
```
.
├── alignment
├── data
├── quality
├── quantification
└── scripts
```

In each directory we will save the different outputs that we will get in each step.

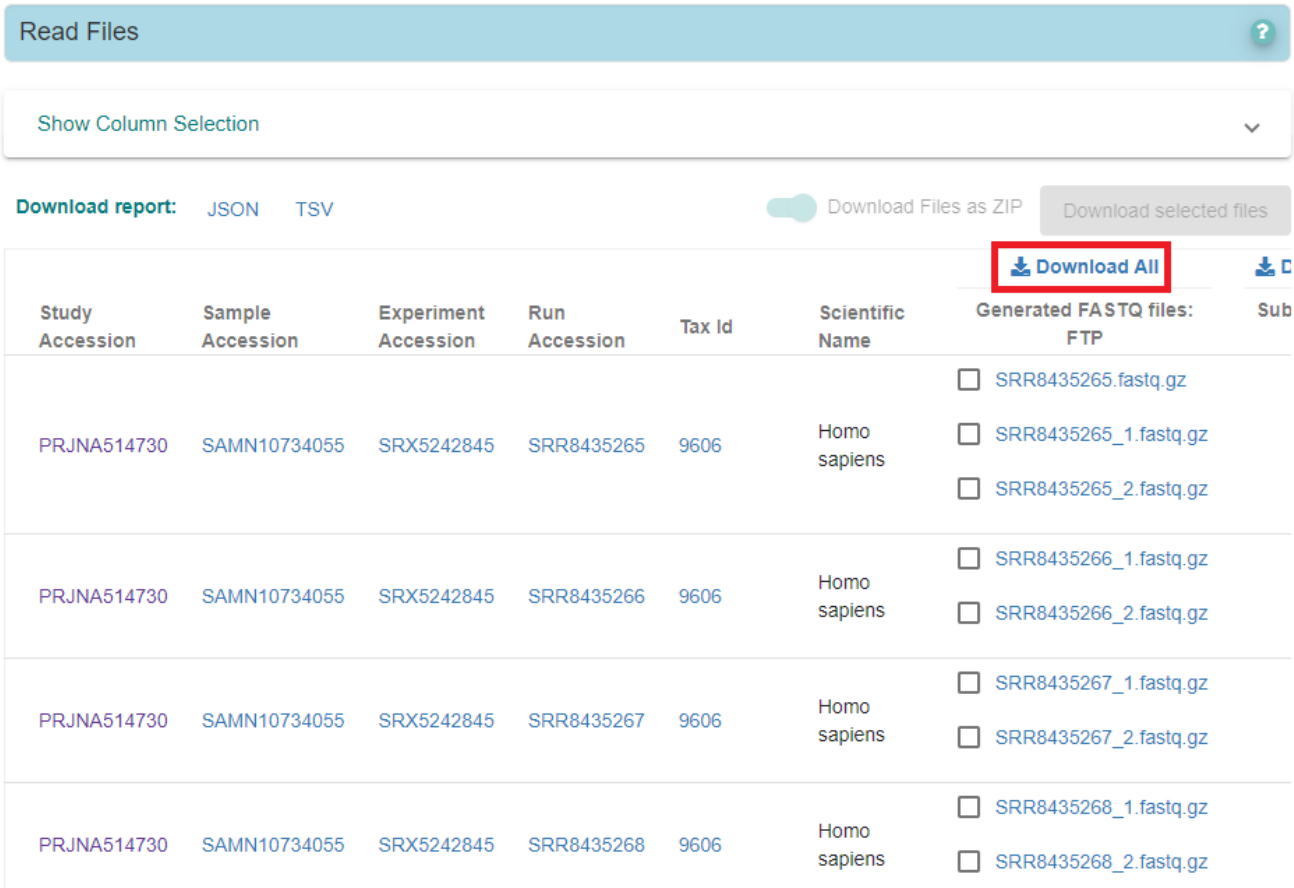
Data download from ENA

To download the raw data, that is, the *fastq* files with the reads of each sample, we will first access Gene Expression Omnibus (GEO) and look for the repository with the accession number provided by the authors in the article: GSE124975.

Once there, look for the *BioProject* code, which is PRJNA514730. With this information we can now go to the European Nucleotide Archive (ENA) and enter the accession number.



We can now download all the *fastq* files. The reads we will work with are paired-end (we have both ends of the fragments sequenced), so for each sample there are two *fastq* files.



We will save all these files in the data folder.

Quality control of the reads

The next step in our analysis is to perform the quality control of the reads. We will do it, as we have done in other posts, with the *fastqc* program. This program works at sample level and generates a report in *html* format for each of them with the results of several quality metrics of the reads:

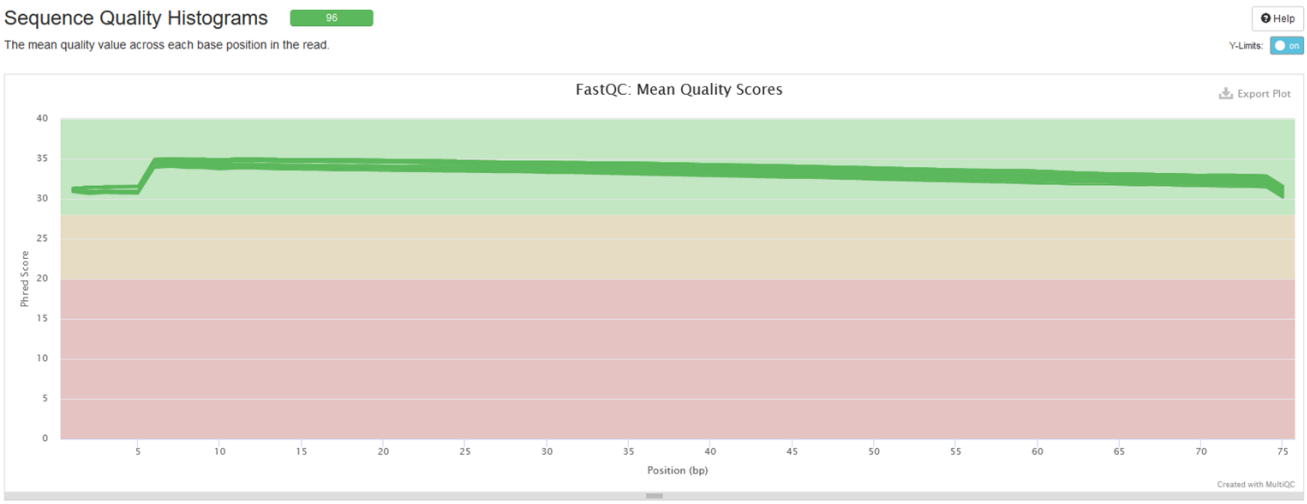
```
for i in SRR8435265 SRR8435266 SRR8435267 (...);
do
    fastqc data/${i}/${i}_1.fastq.gz data/${i}/${i}_2.fastq.gz -o quality/
done
```

fastqc accepts as many arguments as *fastq* files we have, no for loop is needed. Here we have only used it for a matter of space.

Since we have many samples (between biological and technical replicates we have a total of 98 *fastq* files, and thus 98 *fastqc* reports), we will use *multiqc* that automatically groups all reports into one so that we can study them together:

```
multiqc quality/fastqc/ -o multiqc_quality/
```

This program needs only two arguments: the directory where the reports are located and the directory where we want the report. If we open the generated *html* file we can study the quality of our short sequences. The most interesting characteristic of these data is the high quality of the reads, a fact that coincides with what the authors state in the article. There are also no obvious problems in either sample or the presence of adapter sequences:



The *Per Base Sequence Content* section is the worst performing. However, this is normal for RNA-seq data, as explained in the *fastqc* manual.

We will not go into details of the rest of the sections. You can consult the *fastqc* manual for more information.

The question of what to do next arises from the read quality reports: should we remove duplicate sequences and poor quality bases (those with a relatively high probability of being missequenced)?

Regarding duplicated sequences, the current consensus is that they should not be removed for differential expression studies with RNA-seq. As demonstrated in this study, removing duplicate sequences does not improve results, but would only worsen statistical power. On the other hand, removing bad quality bases by trimming (cutting them at the ends until the desired quality is reached) is currently not necessary because aligners such as *hisat2* do soft-clipping of the bases that do not align with the reference genome, as explained in the Harvard Chan Bioinformatics Core training materials. The conclusion of this other article also advises against trimming in RNA-seq studies.

Alignment with the reference genome

Once we are satisfied with the quality of the data, we can now align the reads with the reference genome to know which part of it they come from. To do this, we need to use a splice-aware aligner to take into account those sequences that have an unsequenced intron in the middle. The one we will use is *hisat2*. If we have not downloaded the indexed reference genome yet, we can do so with this command:

```
wget https://genome-idx.s3.amazonaws.com/hisat/grch38_genome.tar.gz  \
-P hisat2/
tar -xvzf grch38_genome.tar.gz
```

Now we have everything to perform the alignment of the sequences:

```
for i in SRR8435265 SRR8435266 SRR8435267 (...);
do
    echo "Aligning ${i}."
    hisat2 -x ../hisat2/grch38/genome \
    -1 data/${i}/${i}_1.fastq.gz -2 data/${i}/${i}_2.fastq.gz \
    -t \
    -p 6 \
    --rna-strandness RF \
    | samtools sort -o alignment/${i}.bam
done
```

The `-t` option is used to display the time required for the operations, while the `-p` option specifies the number of processors that the program can use. The `-1` and `-2` options are for specifying the two reads of the fragment.

Finally, the `--rna-strandness` option allows us to specify the orientation of each read in the fragment if the protocol used maintains this information. The cDNA libraries for obtaining the data were prepared with the Illumina TrueSeq Stranded mRNA kit, in which the second read has the same orientation as the transcript and the first read is the reverse complement. As we see in the code, we pass the alignment result directly to *samtools sort* to sort and convert it into bam format.

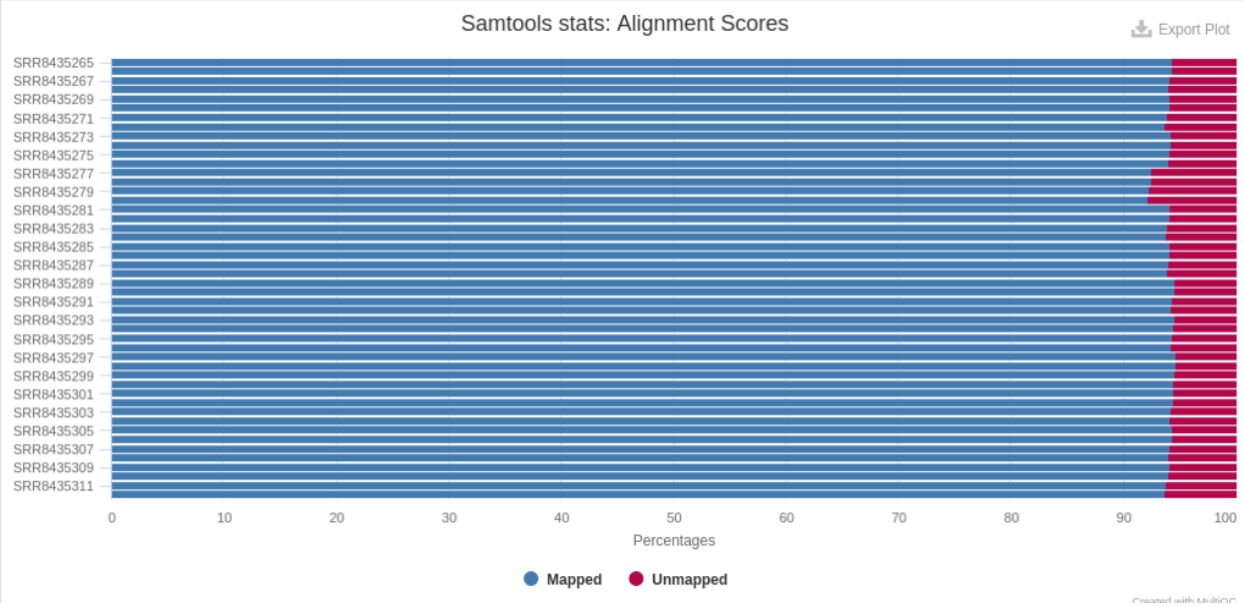
Once we have the bam files of the alignment, it is convenient to run *samtools stats*. The generated files (one for each sample) can also be processed by *multiqc*, adding them to the quality report:

```
for i in SRR8435265 SRR8435266 SRR8435267 (...);

do

    samtools stats alignment/${i}.bam > quality/alignment/${i}.txt

done
```

If we now use *multiqc* again, we can see the data from these new files in the quality report:

```
multiqc quality/fastqc/ quality/alignment/ -o multiqc_quality/
```

Perhaps the most important part of *bam* file statistics is the percentage of mapped reads for each sample. Ideally, we hope to obtain a percentage of mapped reads greater than 90% for each sample. The percentages of our data are around 95%, so this is very good news.



Quantification of gene expression

Now we have everything ready to obtain a matrix with the gene expression of each sample. The *bam* files contain the reads with the coordinates of the reference genome they come from. We will download the *gtf* file with the

annotations. It is very important that the version of the reference genome in this file and the version of the reference genome used in the alignment are the same, otherwise the coordinates will not match.

It is also important to check that the names of the chromosomes are the same in the *gtf* file and the *bam* files. We can quickly check this with the following commands in the terminal:

```
head ../gtf/Homo_sapiens.GRCh38.107.gtf | grep -v "#" | cut -f1,3,4,5

1	gene	1471765	1497848

1	transcript	1471765	1497848

1	exon	1471765	1472089

1	CDS	1471885	1472089

1	start_codon	1471885	1471887


samtools index alignment/SRR8435265.bam

samtools idxstats alignment/SRR8435265.bam | cut -f1-3 | head -n5

1	248956422	908435

10	133797422	462794

11	135086622	554481

12	133275309	764466

13	114364328	139946
```

The first column of the above outputs is the name of the chromosomes. So the name encoding is the same.

We will use *featureCounts* to quantify gene expression. This program basically needs two inputs: the *gtf* file with the genome annotations (the genes and the coordinates of each gene) and the *bam* files. The program will count, for each sample, how many reads overlap and return an array with the genes in the rows and the samples in the columns:

```
featureCounts \

-T 6 \

-p \

-a ../gtf/Homo_sapiens.GRCh38.107.gtf \

-o quantification/count_matrix.txt \

-t exon \
```

```
-s 2 \

alignment/*.bam
```

The `-t` option specifies that we are interested in counting reads that fall into exons, while the `-s` option informs the program of the orientation of the reads.

The program generates two text files: the count matrix (*txt*) and the summary. We can take a look at the header of the count matrix:

```
Geneid Chr    Start  End    Strand Length alignment/SRR8435265.bam
alignment/SRR8435266.bam  alignment/SRR8435267.bam  alignment/SRR8435268.bam
alignment/SRR8435269.bam  alignment/SRR8435270.bam  alignment/SRR8435271.bam
alignment/SRR8435272.bam  alignment/SRR8435273.bam  alignment/SRR8435274.bam
alignment/SRR8435275.bam  alignment/SRR8435276.bam  alignment/SRR8435277.bam
alignment/SRR8435278.bam  alignment/SRR8435279.bam  alignment/SRR8435280.bam
alignment/SRR8435281.bam  alignment/SRR8435282.bam  alignment/SRR8435283.bam
alignment/SRR8435284.bam  alignment/SRR8435285.bam  alignment/SRR8435286.bam
alignment/SRR8435287.bam  alignment/SRR8435288.bam  alignment/SRR8435289.bam
alignment/SRR8435290.bam  alignment/SRR8435291.bam  alignment/SRR8435292.bam
alignment/SRR8435293.bam  alignment/SRR8435294.bam  alignment/SRR8435295.bam
alignment/SRR8435296.bam  alignment/SRR8435297.bam  alignment/SRR8435298.bam
alignment/SRR8435299.bam  alignment/SRR8435300.bam  alignment/SRR8435301.bam
alignment/SRR8435302.bam  alignment/SRR8435303.bam  alignment/SRR8435304.bam
alignment/SRR8435305.bam  alignment/SRR8435306.bam  alignment/SRR8435307.bam
alignment/SRR8435308.bam  alignment/SRR8435309.bam  alignment/SRR8435310.bam
alignment/SRR8435311.bam  alignment/SRR8435312.bam
```

Columns 2 to 6 are not of interest to us for differential gene expression analysis, so we will eliminate them:

```
cat quantification/count_matrix.txt | cut -f1,7-55 | \

grep -v "#" > quantification/genes_count_matrix.txt
```

Now we can take a look at the count matrix and see that it has the format we expect (gens in the rows and samples in the columns):

```
head genes_count_matrix.txt | cut -f1-4

Geneid alignment/SRR8435265.bam  alignment/SRR8435266.bam
alignment/SRR8435267.bam

ENSG00000160072     120    196    206

ENSG00000234396     2      1      0

ENSG00000225972     0      0      0
```

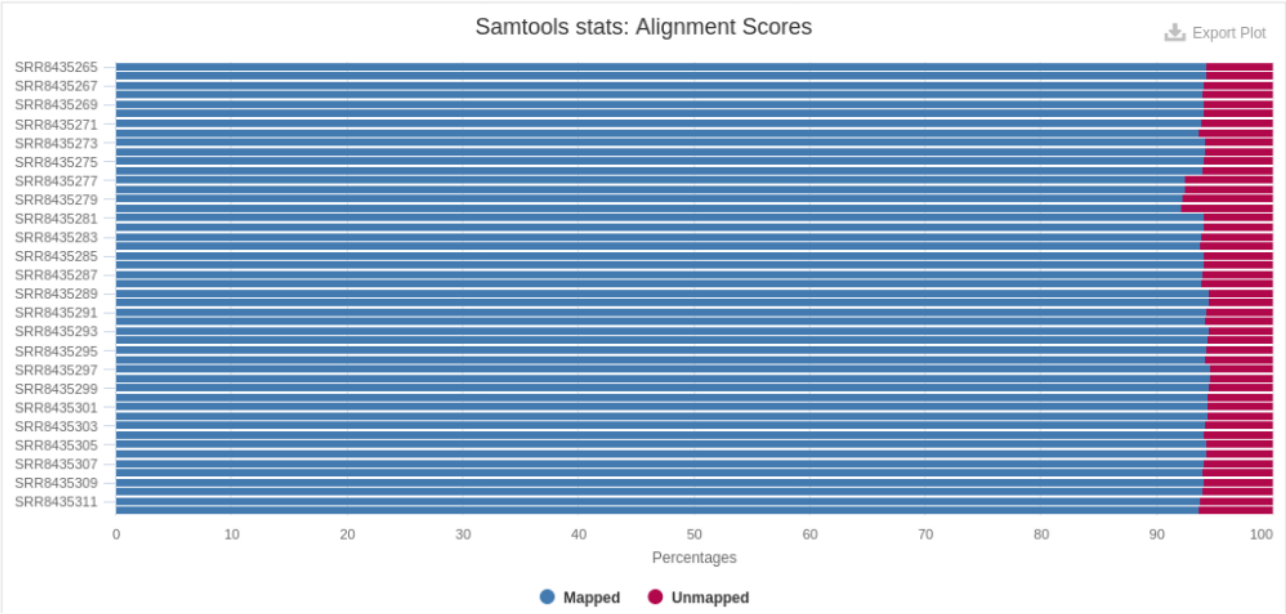
```
ENSG00000224315     0      0      0
ENSG00000198744     2      8      4
ENSG00000279928     1      0      2
ENSG00000228037     0      0      0
ENSG00000142611     0      0      0
ENSG00000225630     385    413    427
```

Although the samples name contains the full path to the file, this is a minor problem that we will easily fix when we have the data in R.

Now, the last thing left for us to do is to incorporate the information from the summary file into the report of *multiqc*:

```
multiqc quality/fastqc/ quality/alignment/ quantification/ -o multiqc_quality/
```

Between 75% and 80% of the reads have been assigned to a gene, this numbers seem high enough to proceed with the statistical analysis of the data:



Conclusion

In this first part of the complete RNA-seq bulk data analysis pipeline we have seen how to do the processing of all *fastq* files at once. First, we did the quality control of the reads, where we verified that the data is of very good quality. We then aligned the reads against the reference genome to determine the origin of each of them. With this information in the *bam* files, we were able to quantify gene expression by generating the count matrix that we will use in the next post to perform the differential gene expression analysis. The *multiqc* program has helped us

to visualize, jointly for all the samples and in a single report, the statistics of the quality measures of the reads, the alignments and the quantification of gene expression.