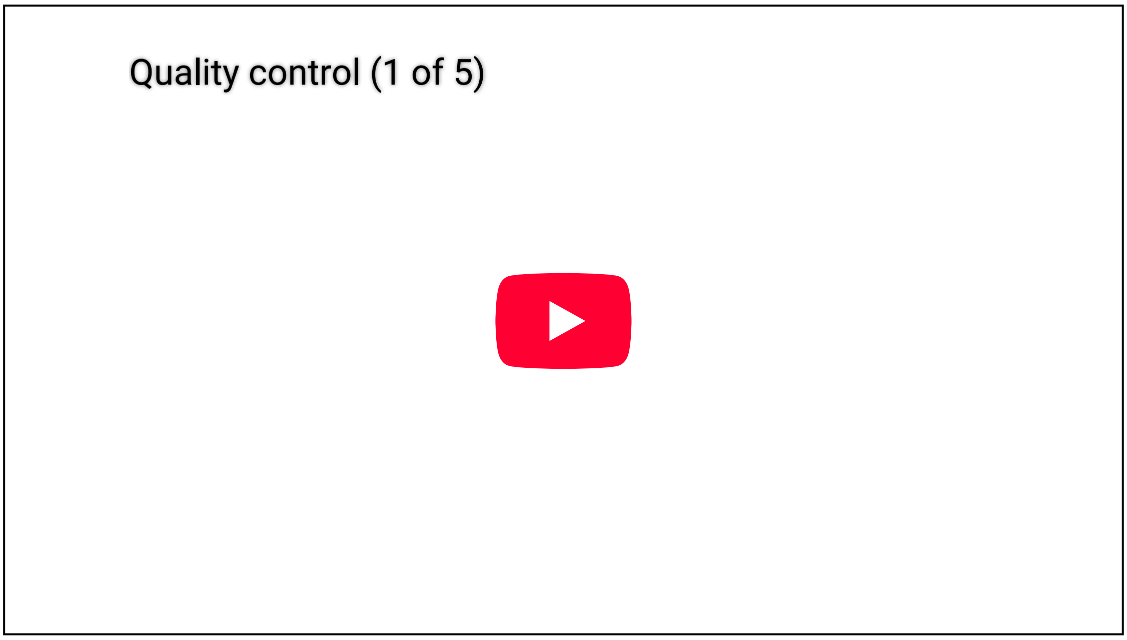
Quality control

Learning outcomes

After having completed this chapter you will be able to:

- Find information about a sequence run on the Sequence Read Archive
- Run `fastqc` on sequence reads and interpret the results
- Trim adapters and low quality bases using `fastp`

Material



Download the presentation

- `fastqc` command line documentation
- `cutadapt` manual
- Unix command line E-utilities documentation

Exercises

Download and evaluate an *E. coli* dataset

Check out the dataset at SRA.

Exercise: Browse around the SRA entry and answer these questions:

A. Is the dataset paired-end or single end?

B. Which instrument was used for sequencing?

C. What is the read length?

D. How many reads do we have?

Answers

A. paired-end

B. Illumina MiSeq

C. 2 x 251 bp

D. 400596

Now we will use some bioinformatics tools to do download reads and perform quality control. The tools are pre-installed in a conda environment called `ngs-tools`. Every time you open a new terminal, you will have to load the environment:

```
conda activate ngs-tools
```

Make a directory `reads` in `~/project` and download the reads from the SRA database using `prefetch` and `fastq-dump` from SRA-Tools into the `reads` directory. Use the code snippet below to create a scripts called `01_download_reads.sh`. Store it in `~/project/scripts/`, and run it.

01_download_reads.sh

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

cd ~/project
mkdir reads
cd reads
prefetch SRR519926
fastq-dump --split-files SRR519926
```

Exercise: Check whether the download was successful by counting the number of reads in the fastq files and compare it to the SRA entry.

Tip

A read in a fastq file consists of four lines (more on that at file types). Use Google to figure out how to count the number of reads in a fastq file.

Answer

e.g. from this thread on Biostars:

```
## forward read
echo $(cat SRR519926_1.fastq | wc -l)/4 | bc

## reverse read
echo $(cat SRR519926_2.fastq | wc -l)/4 | bc
```

Run fastqc

Exercise: Create a script to run `fastqc` and call it `02_run_fastqc.sh`. After that, run it.

Tip

`fastqc` accepts multiple files as input, so you can use a wildcard to run `fastqc` on all the files in one line of code. Use it like this: `*.fastq`.

Answer

Your script `~/project/scripts/02_run_fastqc.sh` should look like:

02_run_fastqc.sh

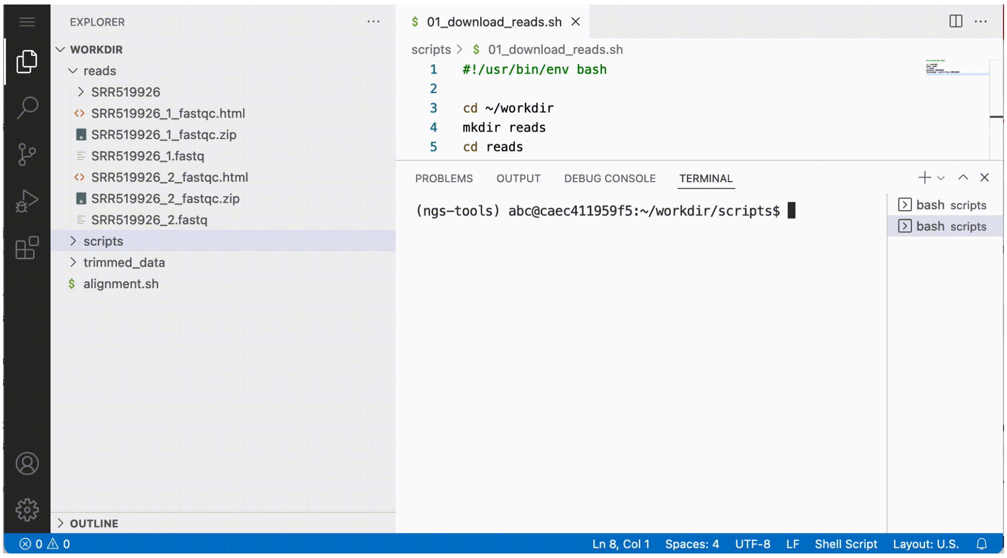
```
#!/usr/bin/env bash
cd ~/project/reads

fastqc *.fastq
```

Exercise: Download the html files to your local computer, and view the results. How is the quality? Where are the problems?

Downloading files

You can download files by right-click the file and after that select **Download**:



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

cd ~/workdir
mkdir reads
cd reads
```

```
(ngs-tools) abc@caec411959f5:~/workdir/scripts$
```

Answer

There seems to be:

- Low quality towards the 3' end (per base sequence quality)
- Full sequence reads with low quality (per sequence quality scores)
- Adapters in the sequences (adapter content)

We can probably fix most of these issues by trimming.

Trim the reads

We will use fastp for trimming adapters and low quality bases from our reads. The most used adapters for Illumina are TruSeq adapters, and `fastp` will use those by default. A reference for the adapter sequences can be found here.

Exercise: Check out the documentation of fastp, and the option defaults by running `fastp --help`.

- What is the default for the minimum base quality for a qualified base? (option `--qualified_quality_phred`)
- What is the default for the maximum percentage of unqualified bases in a read? (option `--unqualified_percent_limit`)
- What is the default for the minimum required read length? (option `--length_required`)

- What happens if one read in the pair does not meet the required length after trimming? (it can be specified with the options `--unpaired1` and `--unpaired2`)

Answer

- The minimum base quality is 15: `Default 15 means phred quality >=Q15 is qualified. (int [=15])`
- The minimum required length is also 15: `reads shorter than length_required will be discarded, default is 15. (int [=15])`
- If one of the reads does not meet the required length, the pair is discarded if `--unpaired1` and/or `--unpaired2` are not specified: `for PE input, if read1 passed QC but read2 not, it will be written to unpaired1. Default is to discard it. (string [=])`.

Exercise: Complete the script below called `03_trim_reads.sh` (replace everything in between brackets `[]`) to run `fastp` to trim the data. The quality of our dataset is not great, so we will overwrite the defaults. Use a a minimum qualified base quality of 10, set the maximum percentage of unqalified bases to 80% and a minimum read length of 25. Note that a new directory called `~/project/results/trimmed/` is created to write the trimmed reads.

03_trim_reads.sh

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

TRIMMED_DIR=~/project/results/trimmed
READS_DIR=~/project/reads

mkdir -p $TRIMMED_DIR

cd $TRIMMED_DIR

fastp \
-i $READS_DIR/SRR519926_1.fastq \
-I $READS_DIR/SRR519926_2.fastq \
-o $TRIMMED_DIR/trimmed_SRR519926_1.fastq \
-O $TRIMMED_DIR/trimmed_SRR519926_2.fastq \
[QUALIFIED BASE THRESHOLD] \
[MINIMUM LENGTH THRESHOLD] \
[UNQUALIFIED PERCENTAGE LIMIT] \
--cut_front \
--cut_tail \
--detect_adapter_for_pe
```

Additional options

Note that we have set the options `--cut_front` and `--cut_tail` that will ensure low quality bases are trimmed in a sliding window from both the 5’ and 3’ ends. Also `--detect_adapter_for_pe` is set, which ensures that adapters are detected automatically for both R1 and R2.

Answer

Your script (`~/project/scripts/03_trim_reads.sh`) should look like this:

03_trim_reads.sh

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

TRIMMED_DIR=~/project/results/trimmed
READS_DIR=~/project/reads

mkdir -p $TRIMMED_DIR

cd $TRIMMED_DIR

fastp \
-i $READS_DIR/SRR519926_1.fastq \
-I $READS_DIR/SRR519926_2.fastq \
-o $TRIMMED_DIR/trimmed_SRR519926_1.fastq \
-O $TRIMMED_DIR/trimmed_SRR519926_2.fastq \
--qualified_quality_phred 10 \
--length_required 25 \
--unqualified_percent_limit 80 \
--cut_front \
--cut_tail \
--detect_adapter_for_pe
```

The use of `\`

In the script above you see that we’re using `\` at the end of many lines. We use it to tell bash to ignore the newlines. If we would not do it, the `fastp` command would become a very long line, and the script would become very difficult to read. It is in general good practice to put every option of a long command on a newline in your script and use `\` to ignore the newlines when executing.

Exercise: Check out the report in `fastp.html`.

- Has the quality improved?
- How many reads do we have left?

- *Bonus*: Although there were adapters in R2 according to `fastqc`, `fastp` has trouble finding adapters in R2. Also, after running `fastp` there doesn't seem to be much adapter left (you can double check by running `fastqc` on `trimmed_SRR519926_2.fastq`). How could that be?

Answers

- Yes, low quality 3' end, per sequence quality and adapter sequences have improved. Also the percentages >20 and >30 are higher.
- 624724 reads, so 312362 pairs (78.0%)
- The 3' end of R2 has very low quality on average, this means that trimming for low quality removes almost all bases from the original 3' end, including any adapter.