cleaning_trimming

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1 Cleaning and trimming of fastq files

All data operations in this tutorial are executed from within the jupyter notebooks that were used to generate this documentation. All jupyter notebooks are stored in the folder `docs/notebook` of the `secapr` GitHub project. That means that **all file- and script-paths are in relation to the notebook directory** (`docs/notebook`). When following the tutorial you may have to adjust the paths, either using absolute paths or paths relative to your working directory.

1.0.1 1. Unzip and rename your files

Before starting to process your fastq files with the `secapr` pipeline **unzip all of your fastq files** and make sure they are named properly (the file names coming from the sequencing faciilities are often very long and difficult to work with). A **simple numerical ID is enough followed by `_R1` for the forward reads and `_R2` for the backward reads**. All fastq files should be in the same directory as in this example:

```
In [3]: %%bash
        # example of the input file structure and naming: a plain folder with unzip
        ls ../../data/raw/fastq/ | head -n 20
```

```
1061_R1.fastq
1061_R2.fastq
1063_R1.fastq
1063_R2.fastq
1064_R1.fastq
1064_R2.fastq
1065_R1.fastq
1065_R2.fastq
1068_R1.fastq
1068_R2.fastq
1070_R1.fastq
1070_R2.fastq
1073_R1.fastq
1073_R2.fastq
1074_R1.fastq
1074_R2.fastq
1079_R1.fastq
```

```
1079_R2.fastq
1080_R1.fastq
1080_R2.fastq
```

1.0.2 2. Quality-check your raw (and dirty) reads

To convince yourself that the raw reads are not fit for further processing, it is a good idea to first run some quality tests on the raw fastq files. I'll show you how:

a) Prepare text-file with file-paths Prepare a file that contains the file paths (absolute paths or relative to your work_dir) of all cleaned fastq-files of interest (you can do it manually or use the following commands, after inserting the correct path to your output folder in the bash for-loop)

Adjust path: Replace `../../data/raw/fastq/*/` with the path to your folder containing the raw reads

```
for dir in ../../data/raw/fastq/*R1.fastq; do echo $dir; done > ../../data/processe

for dir in ../../data/raw/fastq/*R2.fastq; do echo $dir; done >> ../../data/process
```

After successfully running these for-loops, the resulting file `raw_fastq_file_list.txt` should contain the path to all samples that you want to quality check:

```
In [15]: %%bash
         head -n 5 ../../data/processed/raw_fastq_file_list.txt
         echo '      ...      '
         tail -n 5 ../../data/processed/raw_fastq_file_list.txt
```

```
../../data/raw/fastq/1061_R1.fastq
../../data/raw/fastq/1063_R1.fastq
../../data/raw/fastq/1064_R1.fastq
../../data/raw/fastq/1065_R1.fastq
../../data/raw/fastq/1068_R1.fastq
      ...
../../data/raw/fastq/1140_R2.fastq
../../data/raw/fastq/1164_R2.fastq
../../data/raw/fastq/1165_R2.fastq
../../data/raw/fastq/1166_R2.fastq
../../data/raw/fastq/1167_R2.fastq
```

b) Run `fastqc` for quality check Now run `fastqc` for all fastq files in order to produce a quality check for all these samples. Make sure to create the output directory manually before running the command (otherwise `fastqc` will return an error).

```
mkdir ../../data/processed/fastqc_results/raw
fastqc -o ../../data/processed/fastqc_results/raw -f fastq $(cat ../../data/process
```

`fastqc` produces two output files per sample: one zip-archive and one .html file. The easiest way to look at the test results of a specific file is to open the html file in your favorite html-reader (e.g. Firefox, Safari, etc.).

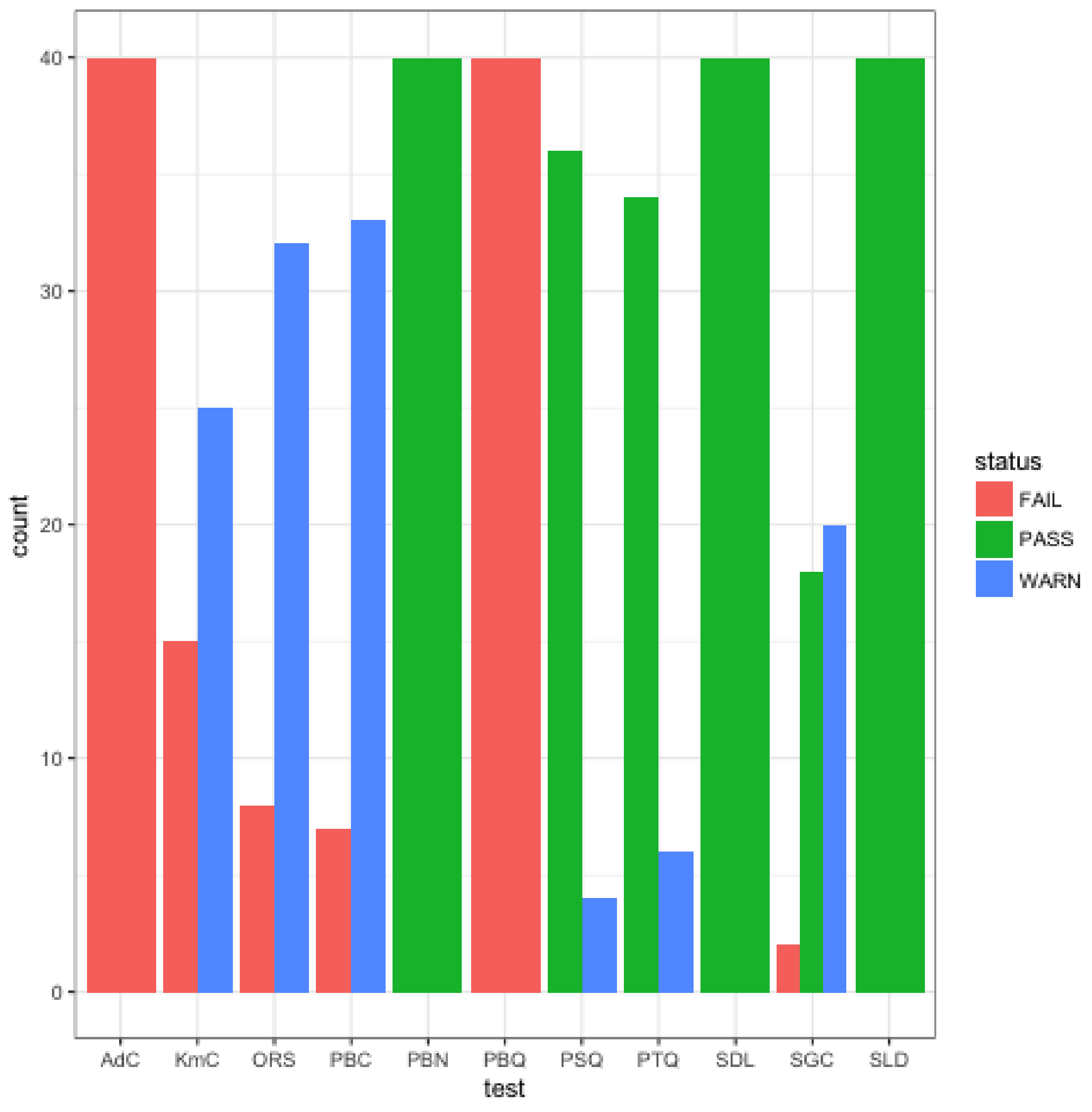
3. Visualize results Since it is somewhat cumbersome to look at all test results for all samples by manually checking all html files, we provide an R-script (in the `src/` folder of the `secapr` GitHub project) which produces a graphical overview of the test results of all samples. This makes it easier to see which samples passed which tests (rather than having to go open each individual report).

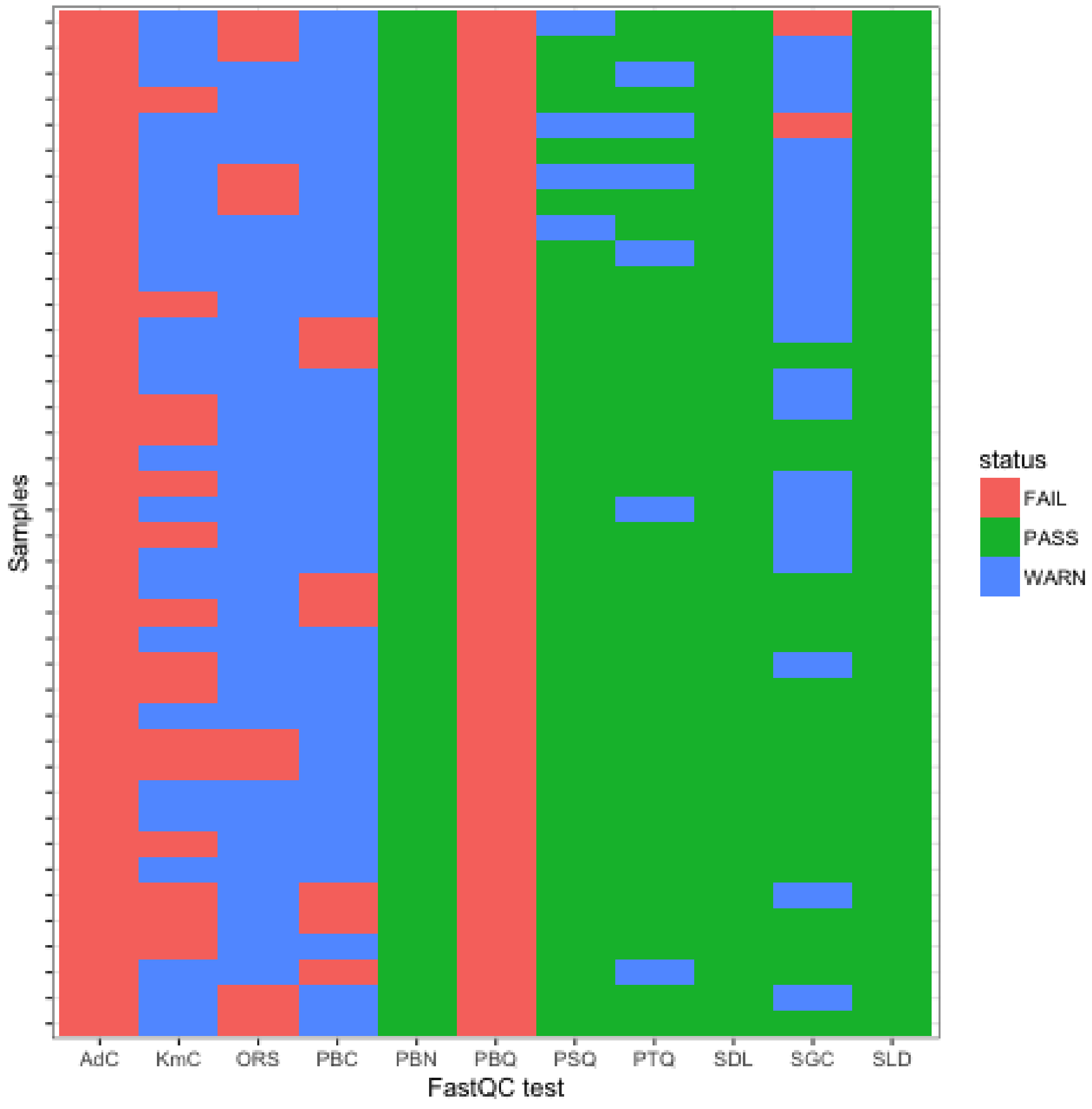
```
Rscript ../../src/fastqc_visualization.r -i ../../data/processed/fastqc_results/raw
```

This is what the quality check results look like for the uncleaned reads:

```
In [20]: from IPython.display import Image, display
         img1 = Image("../../data/processed/fastqc_results/raw/summary_all_samples_
         img2 = Image("../../data/processed/fastqc_results/raw/summary_all_samples_
         print("Fastqc results of uncleaned fastq-files:")
         display(img1,img2)
```

```
Fastqc results of uncleaned fastq-files:
```





The two plots produced by the R-script show summary statistics for each individual test (tests shown on x-axis). The test names carry 3-letter acronyms, and the corresponding full test-name can be found by opening one of the html files. The first plot shows how many occurrences of each test-result (fail,pass,warn) were found for each test among all samples (per-test basis). The second plot shows for each sample (y-axis) which test had which result (per-sample basis). Eventually we want to get rid of all the red in these plots (see below).

1.0.3 3. Clean reads with `secapr` (default settings)

As we see above, the raw reads that you recevied from the sequencing facility are not fit for further processing, since all files fail several quality tests (red fields). One of the main issues with raw read files is that they contain **low quality reads**. Another issue is that most if not all reads will probably contain parts of the **Illumina adapter sequences**, which are attached on both ends of the read. In order to prepare the reads for further steps, we need to make sure that we properly filter out low-quality reads and that we clip off all remaining adapter contaminations. We can use the **`secapr clean_reads`** function to do exactly that for all of our files. This function applies the cleaning and trimming software *Trimmomatic* (Bolger et al. 2014).

Please check: Is `secapr_env` activated? You can test with `conda info --envs`. Activate the correct environment with `source activate secapr_env`

Here is an overview of all available settings for `secapr clean_reads`:

```
In [21]: %%bash
         source activate secapr_env
         secapr clean_reads -h
```

```
usage: secapr clean_reads [-h] --input INPUT --config CONFIG --output OUTPUT
                          [--read_min READ_MIN] [--index {single,double}]
                          [--seedMismatches SEEDMISMATCHES]
                          [--palindromeClipThreshold PALINDROMECLIPTHRESHOLD]
                          [--simpleClipThreshold SIMPLECLIPTHRESHOLD]
                          [--windowSize WINDOWSIZE]
                          [--requiredQuality REQUIREDQUALITY]
                          [--leadingQuality LEADINGQUALITY]
                          [--trailingQuality TRAILINGQUALITY]
                          [--cropToLength CROPTOLENGTH] [--headCrop HEADCROP]
                          [--minLength MINLENGTH]

Clean and trim raw Illumina read files

optional arguments:
  -h, --help            show this help message and exit
  --input INPUT         The directory containing the unzipped .fastq or .fq
                        files (raw read files)
  --config CONFIG       A configuration file containing the adapter
                        information and the sample names
  --output OUTPUT       The output directory where results will be saved
  --read_min READ_MIN   Set the minimum read count threshold. Any read file
                        containing fewer reads than this minimum threshold
                        will not be processed further. Default: 200000
  --index {single,double}
                        Specify if single- or double-indexed adapters were
                        used for the library preparation (essential
                        information in order to interpret the control-file
                        correctly).
  --seedMismatches SEEDMISMATCHES
                        Specifies the maximum mismatch count which will still
                        allow a full match to be performed. For more
                        information see trimmoatic tutorial. Default: 2
  --palindromeClipThreshold PALINDROMECLIPTHRESHOLD
```

```
                        Specifies how accurate the match between the two
                        "adapter ligated" reads must be for PE palindrome read
                        alignment. Default: 30
  --simpleClipThreshold SIMPLECLIPTHRESHOLD
                        Specifies how accurate the match between any adapter
                        etc. sequence must be against a read. For more
                        information see trimmoatic tutorial. Default: 10
  --windowSize WINDOWSIZE
                        Specifies the number of bases to average across. For
                        more information see trimmoatic tutorial. Default: 4
  --requiredQuality REQUIREDQUALITY
                        Specifies the average quality required. For more
                        information see trimmoatic tutorial. Default: 15
  --leadingQuality LEADINGQUALITY
                        Specifies the minimum quality required to keep a base
                        at the beginning of the read. For more information see
                        trimmoatic tutorial. Default: 20
  --trailingQuality TRAILINGQUALITY
                        Specifies the minimum quality required to keep a base
                        at the end of a read. For more information see
                        trimmoatic tutorial. Default: 20
  --cropToLength CROPTOLENGTH
                        The number of bases to keep, from the start of the
                        read. Everything exceeding this length will be removed
                        from the end of the read. For more information see
                        trimmoatic tutorial. Default: 250
  --headCrop HEADCROP   The number of bases to remove from the start of the
                        read. For more information see trimmoatic tutorial.
                        Default: 0
  --minLength MINLENGTH
                        Specifies the minimum length of reads to be kept. For
                        more information see trimmoatic tutorial. Default: 40
```

a) Prepare config file The script requires a config file which should contain the adapter and barcode information. You see an example of the config file below. The file consists of 3 sections: - [adapters]

Here you provide the adapter sequences that were used during library preparation in the lab. Check which Illumina kit was used and find the corresponding sequences either in the Illumina manual or on their webpage. Add a * in the spot where the sample specific barcode will be inserted. - [names]

Here you provide a unique string for each sample, as it occurs in the filename (this is how the program recognizes which files to process). You usually have two file for each sample (forward and backward reads). Make sure that this unique identifier occurs in both of those files. Add ':_' after each identifier (don't ask why). - [barcodes]

Here you specify the sample specific barcode for each sample. First you state which adapter (i7 or i5) the barcode will be inserted in, followed by a dash (-) and the name of the sample, as stated in the section [names].

.

*Note: If you are working with double indexed adapters (both adapters containing barcodes), don't forget to add the * in the position where the barcode is inserted, also for the i5 adapter. In that case make sure in the [barcodes]-section to assign both barcodes for each sample to the correct adapter, e.g.:*

i7-sampleID:AAACCC

i5-sampleID:AATTCC

```
In [16]: %%bash
         cat ../../data/raw/adapter_info.txt
```

```
[adapters]
i7:GATCGGAAGAGCACACGTCTGAACTCCAGTCAC*ATCTCGTATGCCGTCTTCTGCTTG
i5:AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

[names]
1061:_
1063:_
1064:_
1065:_
1068:_
1070:_
1073:_
1074:_
1079:_
1080:_
1082:_
1083:_
1085:_
1086:_
1087:_
1140:_
1164:_
1165:_
1166:_
1167:_

[barcodes]
i7-1061:AGTCAA
i7-1063:ATGTCA
i7-1064:CCGTCC
i7-1065:GTAGAG
i7-1068:GTGGCC
i7-1070:CGTACG
i7-1073:ACTGAT
i7-1074:ATGAGC
i7-1079:CACGAT
i7-1080:CACTCA
```

```
i7-1082:CATGGC
i7-1083:CATTTT
i7-1085:CGGAAT
i7-1086:CTAGCT
i7-1087:CTATAC
i7-1140:TATAAT
i7-1164:GTGGCC
i7-1165:GTTTCG
i7-1166:CGTACG
i7-1167:GAGTGG
```

b) Run `secapr clean_reads` function After preparing your config file in the previous step, you are ready to start with cleaning and trimming of your fastq files. For now let's just run the script with default settings. We will show below how to customize the settings of the script in order to achive the best cleaning results for your dataset. Let's run the script as in this example command:

Please check: Is `secapr_env` activated? You can test with `conda info --envs`. Activate the correct environment with `source activate secapr_env`

```
secapr clean_reads --input data/raw/fastq/ --config data/raw/adapter_info.txt --out
```

`secapr clean_reads` produces a subfolder for each sample in the output directory, containing the cleaned reads for the respective sample.

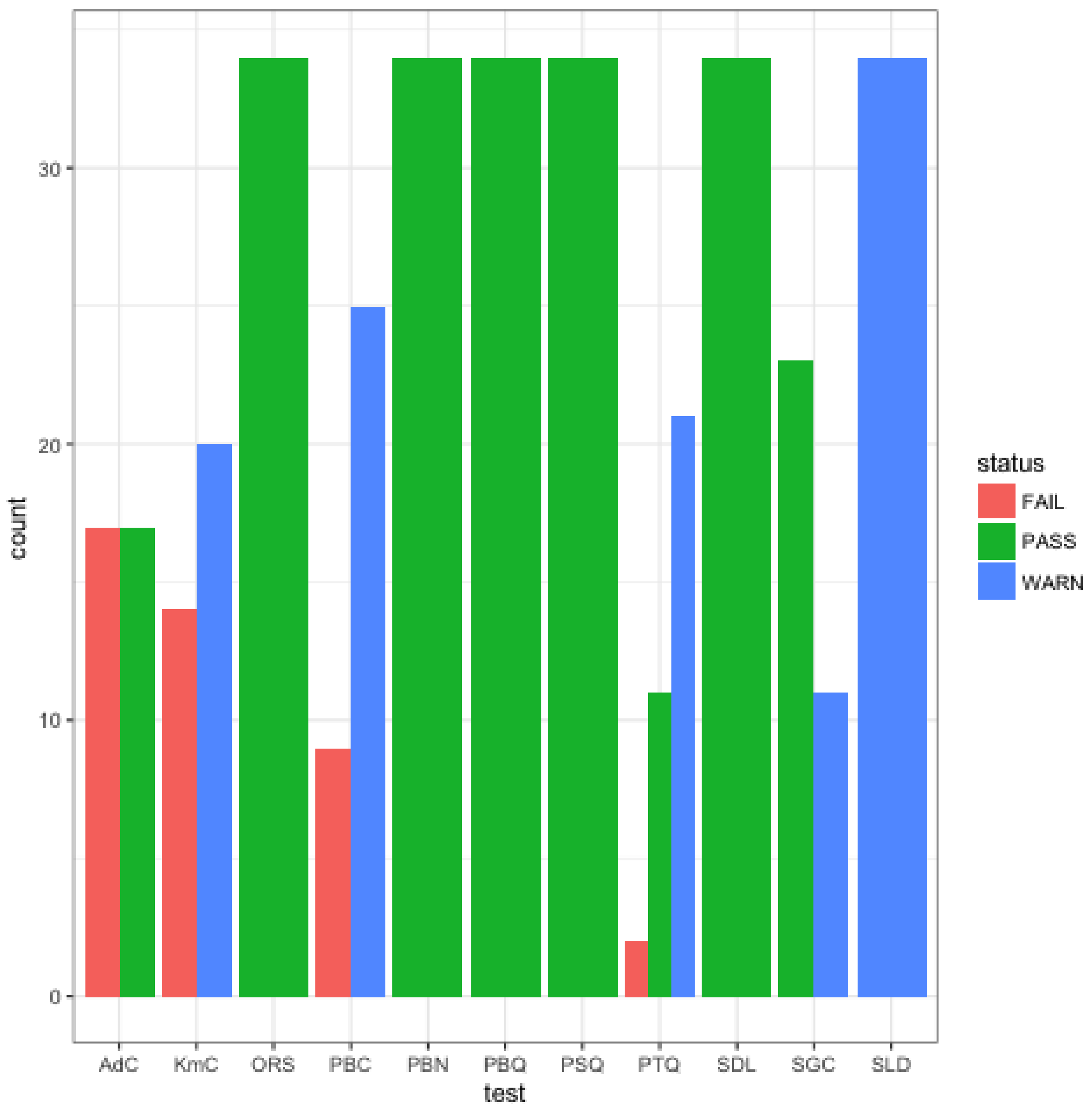
c) Check quality of the results After cleaning the reads with `secapr clean_reads` with default settings we again perform the quality tests on all cleaned files, just as we did above for the raw reads. Therefore we create a text file with the file paths to all cleaned fastq files and run `fastqc` by providing this list of files. Note that the `secapr clean_reads` function named all forward-read files with the tag '*READ1*' and all backward-read files with '*READ2*'.

```
for dir in ../../data/processed/cleaned_trimmed_reads_default/*/*READ1.fastq; do ec

for dir in ../../data/processed/cleaned_trimmed_reads_default/*/*READ2.fastq; do ec

mkdir ../../data/processed/fastqc_results/cleaned_default_settings
fastqc -o ../../data/processed/fastqc_results/cleaned_default_settings -f fastq $(c
```

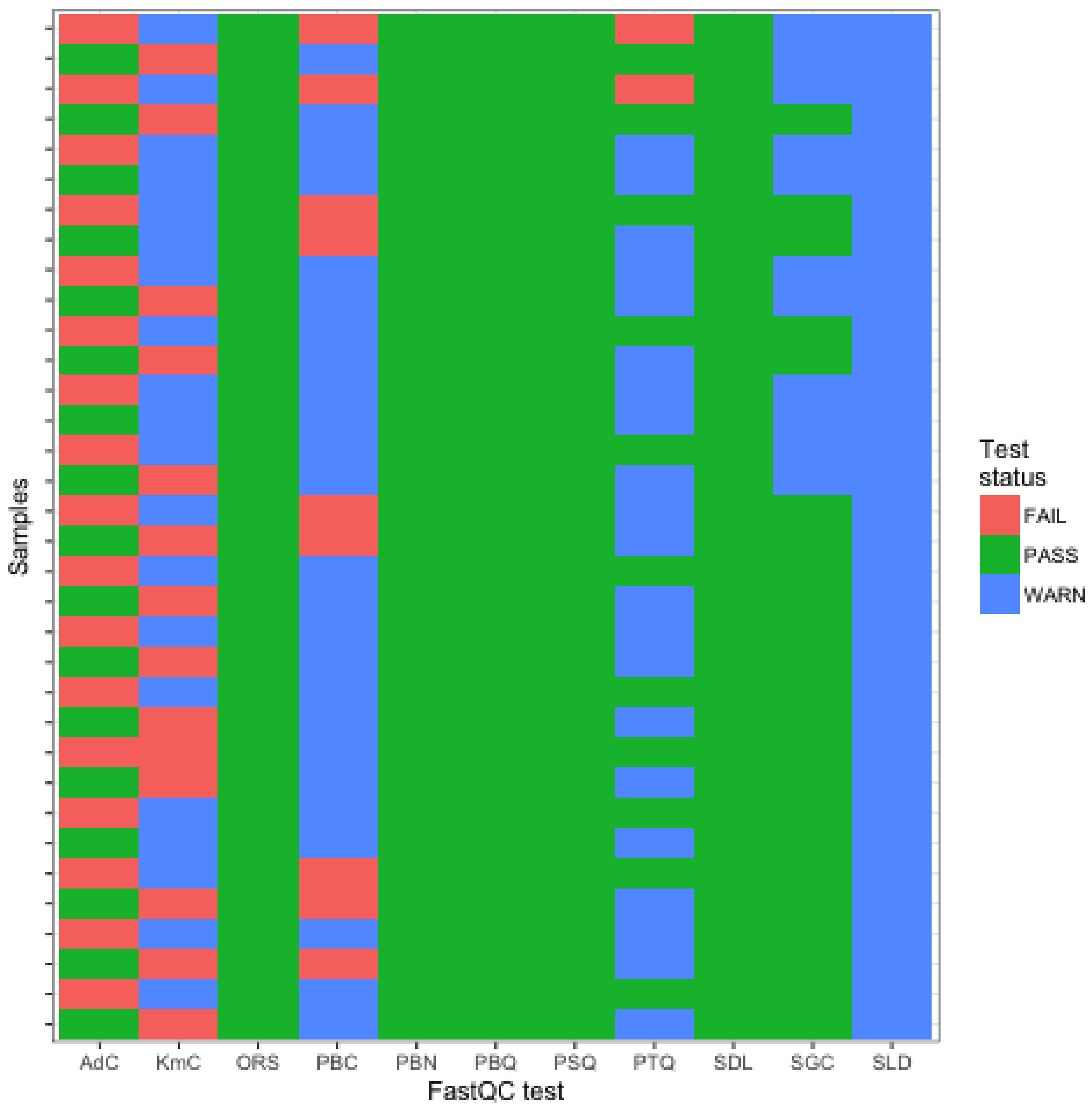
Finally we run our R-script which plots the results:

```
In [30]: from IPython.display import Image, display
         img1 = Image("../../data/processed/fastqc_results_default/summary_all_samp
         img2 = Image("../../data/processed/fastqc_results_default/summary_all_samp
         print("Fastqc results of fastq-files cleaned with default settings:")
         display(img1,img2)
```

```
Fastqc results of fastq-files cleaned with default settings:
```



count
30
20
10
0
AdC
KmC
ORS
PBC
PBN
PBQ
PSQ
PTQ
SDL
SGC
SLD
test
status
FAIL
PASS
WARN



We ran `secapr clean_reads` with default settings and we see a clear improve in comparison to the quality test results of the raw reads (see plots further up in this document). However, there are still quite a few failed tests and I'm convinced we can do better than that. Check the `secapr clean_reads` documentation (by adding `-h` to the command) in order to see the available options and try some different settings in order to see if and how the results improve. It helps to check out in one of the html files what the different tests mean and try to find a settings in `secapr clean_reads` that could be taking care of the specific problem. Preferably all samples should pass all tests (there may still be some warnings) before you continue with further processing of the reads. Below we show an example of how the results can be further improved:

1.0.4 4. Fine-tune cleaning settings

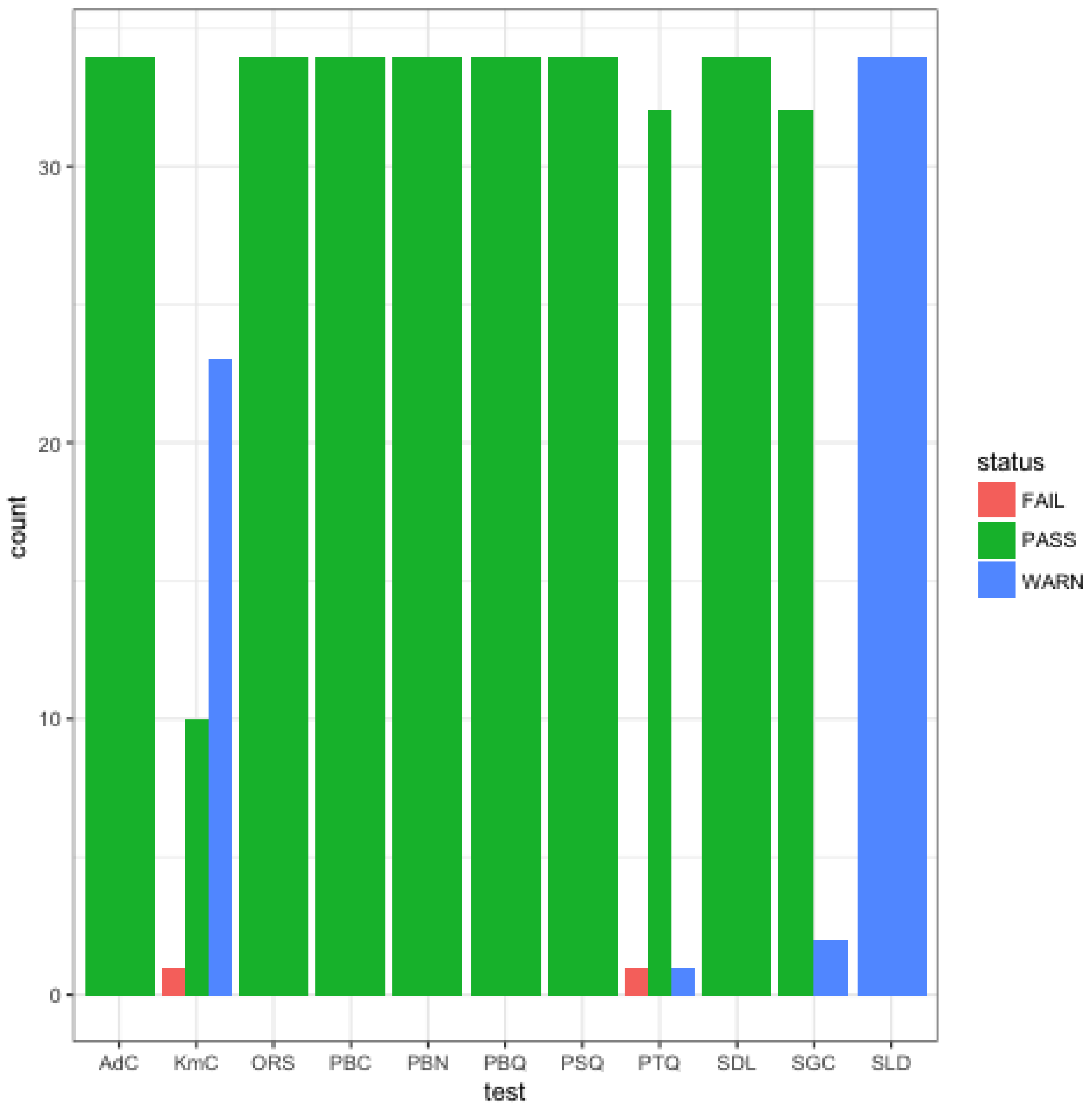
As we see above, running the script with default settings improved the file quality but there is a lot of room for improvement. After reviewing the intial quality reports and after trying a bunch of different flags and values, I ended up with this command for the example data. See the script documentation for more information about the different flags (`secapr clean_reads -h`).

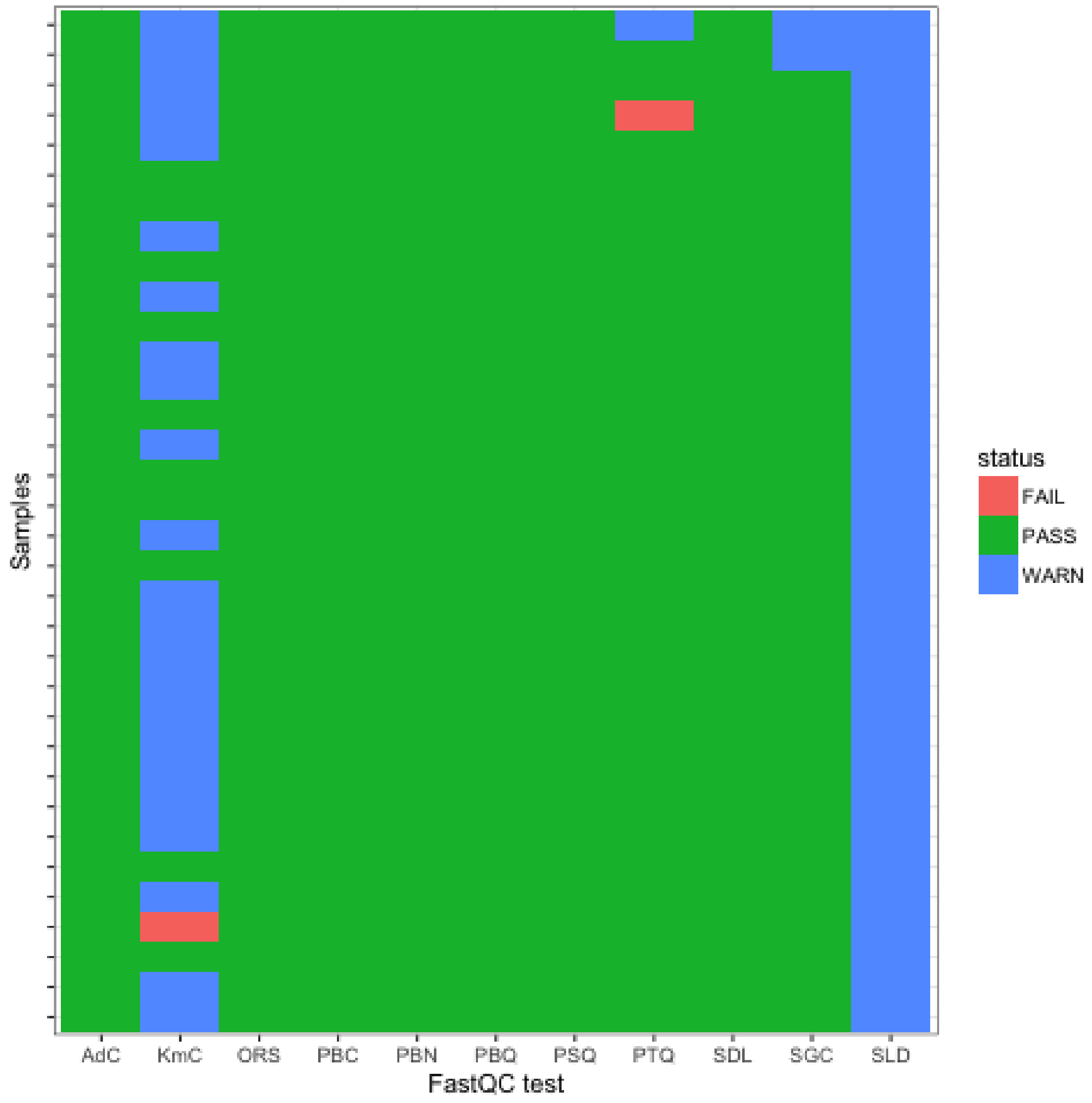
```
secapr clean_reads --input data/raw/fastq/ --config data/raw/adapter_info.txt --out
```

For producing the plots you repeat the commands from above (3. Clean reads with `secapr` (default settings)).

```
In [7]: from IPython.display import Image, display
        img1 = Image("../../data/processed/fastqc_results/summary_all_samples1.png"
        img2 = Image("../../data/processed/fastqc_results/summary_all_samples2.png"
        print("Fastqc results of properly cleaned fastq-files, using customized set
        display(img1,img2)
```

```
Fastqc results of properly cleaned fastq-files, using customized settings:
```





You can see how the overall quality of the data improves (compare R-plots to the ones resulting from the deafult settings). Once all samples are properly cleaned, move on to the next step, the contig assembly

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