

Trimming and quality control (2015-06-03)

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PhD course: High throughput sequencing of non-model organisms

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After a general introduction to the UNIX command line, it is time for you to analyze your own fastq files. The first important step for any kind of sequencing data is to get rid of adapter contamination and bad quality reads. In this tutorial we will use the programs [FastQC](#) and [TrimGalore!](#) to check the quality of the sequenced libraries before and after trimming. We will also learn few more UNIX commands that extract important information from fastq files and that allow you to turn off your computer while the analysis continues to run on the remote server.

IMPORTANT NOTE Before you get started: to compare characteristics of your libraries, please keep record of the resulting numbers, like the number of raw reads, reads after quality control, number of mapped reads etc. This helps to identify peculiarities/outliers in your libraries which may either be due to biological peculiarities of your species or unknown technical issues.

Log on (with `ssh`) to the remote computer with the `-X` option to be able to use graphical interfaces.

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1 Overview of sequence lengths

Next Generation Sequencing data is generally stored in fastq files. Most of the time the data are compressed, either in .zip or in .gz format.

If your file is zip-compressed, you can use the following command to unzip it:

```
1 unzip FILE.fastq.zip
```

If your file is gz-compressed, use the following command instead:

```
1 gunzip FILE.fastq.gz
```

To get a quick impression of the minimum and maximum read lengths in your fastq file, you can use the following commands (replace `FILE.fastq` with your own filename):

```
1 awk '{if(NR%4==2) print length($0)}' FILE.fastq | sort -n | head -n1
2 awk '{if(NR%4==2) print length($0)}' FILE.fastq | sort -n | tail -n1
```

It reads like this: measure the length of every second line in every group of 4 lines (the sequence line in a fastq file), `sort` it (numerically with `-n`) and print out either the first (smallest) value with `head` or the last (biggest) value with `tail`. `NR` represents the current line number and the `%` sign is the modulus operator, which divides the line number by 4 (`NR%4`) and returns only the remainder. This extracts all the sequences, which are on line 2,6,10,14...

The following command allows you to count the sequence lengths:

```
1 awk '{if(NR%4==2) print length($0)}' FILE.fastq | sort -n | uniq -c > read_length.txt
```

The lines that follow makes use of the program R. If you copy and paste the code into the command line, you will get an overview graphic of the sequence length distribution

```
1 cat >> Rplot.r << 'EOF'
2 reads<-read.csv(file="read_length.txt", sep=" ", header=FALSE)
3
4 png(filename = "SequenceLengthDistribution.png",
5       width = 480, height = 480, units = "px", pointsize = 12,
6       bg = "white")
7 plot(reads$V2,reads$V1,type="l",xlab="read length",ylab="occurrences",col="blue")
8 dev.off()
9
10 EOF
11
12
13 R CMD BATCH Rplot.r
```

You can open the created figure with the GNOME image viewer using the following command:

```
1 eog SequenceLengthDistribution.png
```

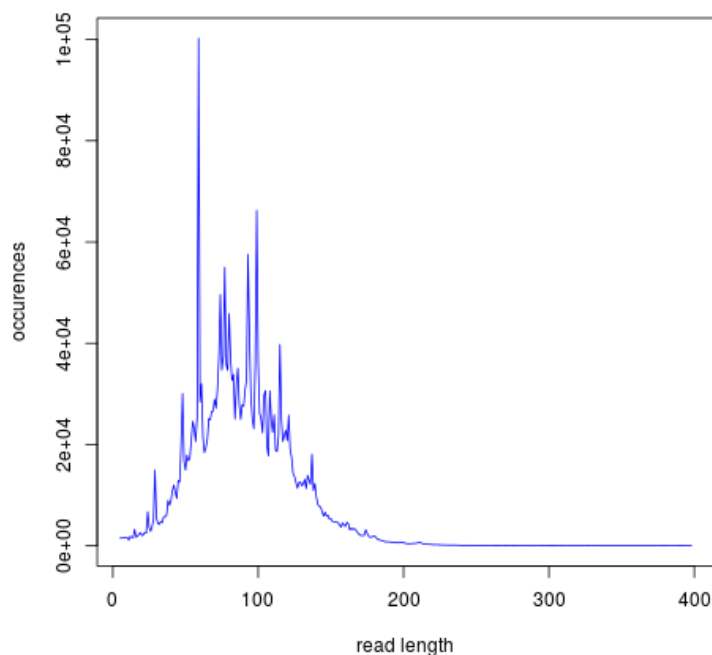


Figure 1: Example graphic of the length distribution in a fastq file

2 Quality control

To inspect the quality of the sequencing data, we use [FastQC](#). In the installation and setup instructions of the program ([link](#)), you will find that FastQC can run in an interactive mode or in a command line mode. This tutorial uses the command-line version but feel free to play around yourself with the interactive version of FastQC.

FastQC knows a number of standard adapter sequences used for HTS; however, it is not aware of the sequences used by the IonTorrent platform. To inform FastQC of the adapter sequences we have used we call FastQC with the `-a`, or `--adapters` option to specify a file containing the adapter sequences we have used.

So, to run FastQC on your file, simply type (where `adapters.txt` is a file containing the adapter sequences in a suitable format):

```
1 fastqc -a adapters.txt FILE.fastq
```

The output will be saved in a folder that has the name of your fastq file and ends with `fastqc`, like `FILE_fastqc`. Use the `cd` command to move into the folder and open the produced `fastqc_report.html` either with `firefox` or `chromium-browser` (one of the two should work).

```
1 firefox fastqc_report.html
2 chromium-browser fastqc_report.html
```

Get familiar with the output. Does the sequence length-distribution meet your expectations (400bp library)? You can find guidance on how to interpret the output of each module [here](#).

3 Trimming low quality reads and adapters

TrimGalore! is a wrapper script to automate quality and adapter trimming as well as quality control ([User Guide](#)).

When the program is installed, it can be used with

```
1 trim_galore [options] <filename(s)>
```

You can get an overview of the options with the `--help` option:

```
1 trim_galore --help
```

With the default settings, TrimGalore! trims low-quality ends with a Phred quality score threshold of 20 (can be changed with `-q`) and discards reads that become shorter than 20 bp (can be changed with `--length`).

The Ion-P1- and Ion-A-adapters are supposed to be automatically trimmed off on the Ion Server. So, the fastq files with the raw reads should not contain these adapters anymore. Nevertheless, try to trim them off anyway in order to check if there are still adapters left in your library - they can have negative effects on further analyses.

TrimGalore! uses the program [Cutadapt](#) to find and remove adapters from the 3' end of the reads (see Fig. 2). The program Cutadapt itself gives you more options for adapter trimming and allows you to remove adapters from the 5'-end of the sequence (see <http://cutadapt.readthedocs.org/en/latest/guide.html>)

The adapters used for Ion Torrent sequencing are shown in Fig. 3 and their orientation in the libraries is shown in Fig. 4.

.

To trim off the A-adapter, use TrimGalore! with the command:

```
1 trim_galore \  
2 -a CCATCTCATCCCTGCGTGTCTCCGACTCAG \  
3 --stringency 3 \  
4 FILE.fastq
```

The `\` sign just means that the command continues on the next line. You could type the entire command on a single line.

The option `--stringency 3` means that a `>3bp` overlap with the adapter sequence will be trimmed off the 3' end. The program writes a file that ends with `trimming_report.txt`, which



Figure 2: 3'- and 5'-adapter trimming ([source](#))

| |
|--|
| <p>Ion A Adapter (non-barcoded)</p> <p>5'- CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'</p> <p>3'-T*T*GGTAGAGTAGGGACGCACAGAGGCTGAGTC-5'</p> |
| <p>Ion P1 Adapter</p> <p>5'- CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGAT-3'</p> <p>3'-T*T*GGTGATCGCGAGGCGAAAGGAGAGATACCCGTCAGCCACTA-5'</p> |

Figure 3: Non-barcoded Ion-A and -P1 adapter sequences. In each sequence, a "*" indicates a phosphorothioate bond, for protection from nucleases and to preserve the directionality of adapter ligation

reports the number of reads that have been trimmed and/or removed.

The output file has the ending `trimmed.fq`. Use this file as input to TrimGalore! to trim off the P1-adapter:

```

1 trim_galore \
2 -a CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGAT \
3 --stringency 3 \
4 --fastqc FILE_trimmed.fq

```

The `--fastqc` option will automatically run FastQC in the default mode. Compare the FastQC outputs before and after trimming.

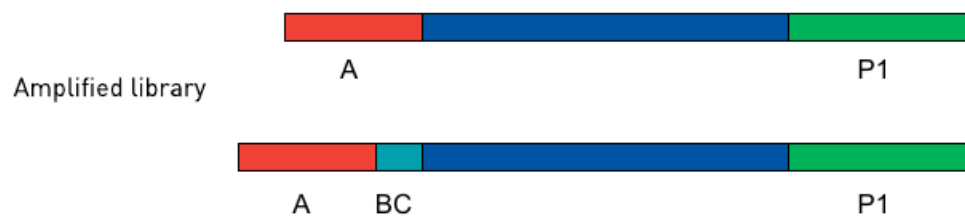


Figure 4: Ion adapters in the amplified library. BC is an optional barcode sequence.

4 Fraction of duplicate reads

Duplicate reads (identical reads present more than once in the library) can skew genotype estimates and thus should be identified and removed before SNP calling. Duplicates can result from primer or PCR bias towards these reads and poor libraries can have levels of duplicates >50%.

At this step, we will calculate the fraction of duplicates but we will remove them only after *de novo* genome assembly and read mapping. The approach is based on the [Simple fool's guide to population genomics via RNAseq](#) and makes use of `fastx_collapser` from the [FASTX-Toolkit](#) and a python script (`fastqduplicatecounter.py`).

First, use `fastx_collapser` to combine and count all identical reads.

```
1 fastx_collapser -Q 33 -v -i INPUTFILE.fq -o OUTPUTFILE.txt
```

The `INPUTFILE` is your trimmed fastq file. `-Q 33` specifies that quality scores are Phred33 encoded. The `OUTPUTFILE` is used in the next step with the python script '`fastqduplicatecounter.py`'.

```
1 fastqduplicatecounter.py OUTPUTFILE.txt OUTPUTFILE_header.txt > OUTPUTFILE_duplicatecount.txt
```

This script calculates the fractions of duplicate and singleton reads. Open the outputfile with `less OUTPUTFILE_duplicatecount.txt` and check the percentage of duplicate reads.

5 BONUS Running programs in the background with nohup

What if your data analysis on a remote server takes several hours, days, or even weeks, to finish? No worries, you don't need to be connected to the remote server while the data are being analysed. Here, you will learn the tools that allow you to start an analysis, disconnect from the server, and then look at the progress or the results at a later time point.

The `nohup` tool allows you to run a process in the background; which means that, while the analysis is running, you can do other tasks in parallel or log off from the remote server.

Imagine the `nohup` tool as a bracket which encloses the command that you want to run in the background:

```
1 nohup ... &
```

Always, `nohup` precedes and `&` follows the command that you want to run in the background (here shown as `...`). Let's say you want to run the command `ls -lhcr` (which lists all files and subdirectories in your current directory) in the background.

```
1 nohup ls -lhcr &
```

When you hit ENTER, the terminal prints out some information:

```
1 [1] 21118
2 nohup: ignoring input and appending output to 'nohup.out'
```

The number 21118 (which will differ in your case) in the first line is the process-ID of your background-process. The second line informs you that all 'results', that would be normally printed in the terminal window, are now redirected to the file `nohup.out`.

5.1 Using the process-ID

If you have started a process that takes several hours to finish, then you can use the process-ID to see if the process is still running. For this, you can use the `ps` command with the `-p` option, which reports the status of a process with a certain process ID. To see the status of the process I have started above, I would use:

```
1 ps -p 21118
```

The output is

```
1 PID TTY          TIME CMD
```

Since this is only the header line of the process specifications, the process must have finished. Here:

- PID indicates the process-ID
- TTY indicates the controlling terminal
- TIME shows the time that the process is running already
- CMD shows the command name

If the process would still run, you would get a line similar to:

```
1 PID TTY          TIME CMD
2 21118 ?                00:00:04 ls
```

The `top` tool provides an ongoing look at processor activity in real time, similar to Figure 5.

At the top of the screen, it lists processes ordered by their CPU usage system (with the most intensive on top). Besides other information, it shows which user is running which process, as well as the process-ID. You can quit the program by hitting `q`.

The process-ID also allows you to cancel the process before it finishes. Cancelling processes comes in handy when you figure out that you started them with the wrong parameters or input


```

top - 12:50:11 up 53 days, 18:57, 4 users, load average: 0.70, 0.54, 0.46
Tasks: 372 total, 2 running, 369 sleeping, 0 stopped, 1 zombie
%Cpu(s): 4.0 us, 1.9 sy, 0.0 ni, 93.9 id, 0.1 wa, 0.0 hi, 0.0 si, 0.0 st
KiB Mem: 65957376 total, 65667884 used, 289492 free, 754908 buffers
KiB Swap: 21484337+total, 102644 used, 21474073+free, 57066652 cached Mem

  PID USER      PR  NI   VIRT   RES   SHR  S  %CPU  %MEM     TIME+  COMMAND
 2027 alj        20   0 1559984 90196 28140 S  10.6  0.1 165:51.91 mainframe
   539 root       20   0 2117932 1.540g 430008 S   9.3  2.4 32:22.12 Xorg
   910 alj        20   0 1616140 577452 62460 S   8.3  0.9 10:17.17 firefox
  1922 alj        20   0 1631036 309656 71040 S   5.0  0.5 31:10.48 compiz
11380 alj        20   0 1081100 165344 66576 S   3.6  0.3 35:14.26 spotify
  1614 alj         9 -11 659224   9880  6844 S   1.0  0.0  5:28.33 pulseaudio
13033 alj        20   0 629676 17464 12660 S   1.0  0.0  0:00.19 gnome-screensho
30045 alj        20   0 576248 106992 20264 S   1.0  0.2  3:13.00 emacs24
   527 alj        20   0 544088  53012 26820 S   0.7  0.1  0:34.94 plugin-containe
30931 alj        20   0 3187484 136468 29776 S   0.7  0.2  6:05.72 dropbox
    8 root       20   0 0 0 0 S   0.3  0.0 81:54.38 rcu_sched
    9 root       20   0 0 0 0 S   0.3  0.0 26:46.25 rcuos/0
   130 root       20   0 0 0 0 S   0.3  0.0 1:03.51 kswapd1
  1069 avahi      20   0 32488 1104 768 S   0.3  0.0  5:38.15 avahi-daemon
  1147 alj        -4   0 33600 9464 1156 S   0.3  0.0  1:29.39 wineserver
  1218 alj        20   0 2718888 167356 19840 S   0.3  0.3  1:49.00 Kindle.exe
  1416 alj        20   0 439076 15616 2892 S   0.3  0.0  1:03.34 ibus-daemon
  1520 root       20   0 4368 516 360 S   0.3  0.0 12:51.63 acpid
  7450 root       20   0 0 0 0 S   0.3  0.0  6:51.18 kworker/5:0

```

Figure 5: Screenshot of the top tool output

files and you want to re-start with different settings. The kill command allows you to cancel a specific process.

```
1 kill 21118
```

This would cancel the process that we started before in the background. If you can't remember the process-ID but want to cancel all ls processes, then you could use the pkill command in the following way:

```
1 pkill ls
```

Compared to the kill command, the pkill command allows you to specify the command-name instead of the process-ID of the running process that you want to cancel.

If you don't have a record of the PID you can find out the id of processes being run by a specific user by combining ps and grep:

```
1 ps aux | grep user_name
```

where user_name is your own user name. That will list all processes started by you (well using your id in any case). The aux option specifies all processes and the manner in which these are printed out. If you read the man file (man ps) you will see that there are ways in which you can get ps to only list processes started by the current user (ps -eu) in a similar manner and that ps aux is BSD syntax. So grep isn't really needed here, but I tend to like the way this formats the output.

5.2 Redirecting output

By default, the `nohup` command redirects all information from the terminal window to the `nohup.out` file. If the file exists already, it will not be overwritten. All new information will be appended to the end of the file. With the `>` operator, you can redirect the output to a different file. For example, to redirect the output of the `ls` command to the file `Directory-Listing.txt`, you can use the command

```
1 nohup ls -lhcr > Directory-Listing.txt &
```

So, the redirection-operator (`>`) is followed by the name of the target file and precedes the closing `&` operator of the `nohup` command. If you want to save the output to a file in a different directory, just specify the entire file-path that precedes your target file, like:

```
1 nohup ls -lhcr > /home/alj/Documents/DirectoryListing.txt &
```

Emacs 24.3.1 (Org mode 8.3beta)