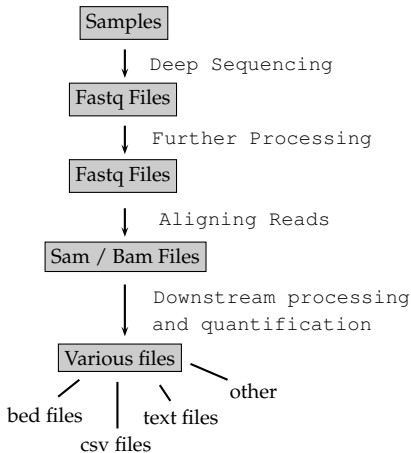


Genomic Files

University of Massachusetts Medical School

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A Typical Deep-Sequencing Workflow



Deep Sequencing Data pipelines involve a lot of text processing.

This is an oversimplified model and your workflow can look different from this!

Unix has very useful tools for text processing.
Some of them are:

- Viewing: `less`
- Searching: `grep`
- Table Processing: `awk`
- Editors: `nano`, `vi`, `sed`

Using Pipes

We don't want `grep` print everything all at once.

We want to see the output line-by-line.

Pipe the output to `less`.

```
$ grep AAA control.rep1.1.fq --color=always | less
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```

We have escape characters but `less` don't expect them by default. So

```
$ grep AAA control.rep1.1.fq --color=always | less -R
```

Unix pipes direct the (standard) output of the LHS of | to the RHS of | as standard input.

```
$ command_1 | command_2 | ... | command_n
```

The (standard) output of **command_i** goes to **command_{i+1}** as (standard) input.

Exercise

Submit two dummy jobs to the long queue and three short dummy to the short queue. Then get a list of your jobs that have been submitted to the **long** queue **only**.

Hint 1: Use `sleep 300` to create a dummy job.

Hint 2: Use **`bsub`** to submit a job. Remember that `-q` parameter is used to specify the queue.

Hint 3: Recall that **`bjobs`** can be used to list your jobs in the cluster.

Hint 4: Use what you have learned so far and put the pieces together.

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$ bsub -q short "sleep 300"
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```
$ bsub -q long "sleep 300"
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```
$ bjobs | grep long
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```

```
$ bjobs | grep long
```

Homework: Read the manual page of `bqueues` and find a way to do this without using a pipe.

What about saving the result?

We can make grep print all the reads we want on the screen.

But how can we save them? View them better?

For this we need to redirect the **standard output** to a textfile.

```
$ grep AAA control.rep1.1.fq > ~/AAA.txt
```

Standard Input, Output and Error

When a process is started, by default, several places are setup for the process to read from and write to.

- **Standard Input:** This is the place where process can read input from. It might be your keyboard or the output of another process.
- **Standard Output:** This is the place where the process writes its output.
- **Standard Error:** This is the place where the process writes its error messages.

By default, all these three places point to the terminal. Consequently, standard output and error are printed on the screen and the standard input is read from the keyboard.

Redirecting Standard Input, Output and Error

- We can redirect the standard output using ">".
Let's have the output of echo to a text file.

```
$ echo echo hi > out.txt
```

- We can redirect the standard input using "<".
Let's use the file we created as input to bash.

```
$ bash < out.txt
```

- We can redirect the standard error using "2>".
- We can redirect both the standard output and error using "&>".

As the ultimate product of sequencing, for each fragment of DNA, we get three attributes.

- Sequence Identifier
- Nucleotide Sequence
- Sequencing quality per nucleotide

The sequencing information is reported in **fastq** format. For each sequenced read, there are four lines in the corresponding fastq file.

Fastq Example

| | | |
|--------------------|---|-------------------------------------|
| @61DFRAAXX100204:2 | ← | Identifier |
| ACTGGCTGCTGTGG | ← | Nucleotide Sequence |
| + | ← | Optionally Identifier + description |
| 789::=<<==;9<==<;; | ← | Phred Quality |
| @61DFRAAXX100304:2 | ← | Identifier |
| ATAATGAGTATCTG | ← | Nucleotide Sequence |
| + | ← | Optionally Identifier + description |
| 4789;:=<=:<<=: | ← | Phred Quality |
| : | : | : |

Some aligners may not work if there are comments after the identifier (read name).

There are 4 rows for each entry. This is a simplified example and the actual sequences and the identifiers in a fastq file are longer.

Phred Quality Score

The sequencer machine is not error-free and it computes an error probability for each nucleotide sequenced.

Say, for a particular nucleotide position, the probability of reporting the wrong nucleotide base is P , then

$$Q_{Phred} = -10 \times \log_{10} P$$

is the *Phred Quality Score* of the nucleotide position.

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The above formula is for Sanger format which is widely used today. For Solexa format, a different formula is used.

Q_{Phred} is a number. But we see a character in the fastq file.
How do we make the conversion?

There are two conventions for this.

- 1 Phred 33
- 2 Phred 64

| ASCII TABLE | |
|-------------|-----------|
| Decimal | Character |
| 0 | NULL |
| ⋮ | ⋮ |
| 33 | ! |
| 34 | " |
| ⋮ | ⋮ |
| 64 | @ |
| 65 | A |
| ⋮ | ⋮ |
| 90 | Z |
| ⋮ | ⋮ |
| 97 | a |
| ⋮ | ⋮ |
| 122 | z |
| ⋮ | ⋮ |
| 127 | DEL |

ASCII printable characters start at the position 33. The capital letters start at position 65.

Phred 33: The character that corresponds to $Q_{Phred} + 33$ is reported.

Phred 64: The character that corresponds to $Q_{Phred} + 64$ is reported.

Phred Example

Suppose that the probability of reporting the base in a particular read position is $\frac{1}{1000}$. Then

$$Q_{Phred} = -10 \times \log_{10} \frac{1}{1000} = -10 \times \log_{10} 10^{-3} = 30$$

Using Phred 33: $30+33 = 63 \rightarrow ?$

Using Phred 64: $30+64 = 94 \rightarrow ^$

Exercise

From a big fastq, you randomly pick one million nucleotides with Phred 33 quality reported as **I**. In how many nucleotides, amongst a total of one million nucleotides, would you expect to be a sequencing error?

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In the ASCII table, the decimal number corresponding to **I** is 73. For Phred 33, we have

$$73 - 33 = 40 = -10 \times \log_{10} P \rightarrow P = 10^{-4}$$

We have 1 million nucleotides with a probability 10^{-4} of sequencing error. So, we expect to see $10^6 \times 10^{-4} = 100$ reads with a sequencing error.

Say we want to find reads that **don't** contain AAA in a fastq file, then we use the `-v` option to filter out reads with AAA.

```
$ grep -v AAA file.fastq
```


Problem

*How can we get **only** the nucleotide sequences in a fastq file?*

Problem

How can we get only particular columns of a file?

awk is an interpreted programming language designed to process text files. We can still use awk while staying away from the programming side.

awk {print (\$2)} sample.sam
awk statement columns sep. by a fixed character (def: space)

Some Awk Built-in Variables

| Content | Awk variable |
|----------------|---------------------|
| Entire Line | \$0 |
| Column 1 | \$1 |
| Column 2 | \$2 |
| : | : |
| Column i | \$i |
| Line Number | NR |

Example

Say, we only want to see the second column in a sam file,

```
$ awk '{print($2)}' sample.sam
```

Getting nucleotide sequences from fastq files

In fastq files, there are 4 lines for each read. The nucleotide sequence of the reads is on the second line respectively. We can get them using a very simple modular arithmetic operation,

```
$ awk '{if(NR % 4== 2)print($0)}' file.fq
```

NR = line number in the given file.

Using `awk`, get the sequencing quality from the fastq file.

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```
$ awk '{if(NR % 4== 0)print($0)}' file.fq
```

awk can be very useful when combined with other tools.

Problem

*How many reads are there in our fastq file that don't have the sequence **GC**?*

```
$ awk '{if(NR % 4== 2)print($0)}' file.fq | grep -v GC
```

gives us all such reads. How do we find the number of lines in the output?

Find sequences ending with AAA

Let's find all the sequences in our fastq file that ends with **AAA** using awk.

```
$ awk '{if(NR % 4== 2){if(substr( $0, length($0)-2, length($0) )=="AAA") print($0)}}'\  
file.fq
```

Using awk, find all sequences **starting** with AAA in a fastq file.

Using awk, find all sequences **starting** with **AAA** in a fastq file.

```
$ awk '{if(NR % 4== 2){if(substr( $0, 1, 3 )=="AAA") print($0)}}'\  
file.fq
```

wc: gives us the number of lines, words, and characters in a line.

with the `-l` option, we only get the number of lines.
Hence

```
$ awk '{if(NR % 4== 2)print($0)}' file.fq | grep -v GC | wc -l
```

gives us the number of reads that don't contain the sequence GC as a subsequence.

Example

Fasta File Format:

>Chromosome (or Region) Name

Sequence (possibly separated by new line)

>Chromosome (or Region) Name

Sequence (possibly separated by newline)

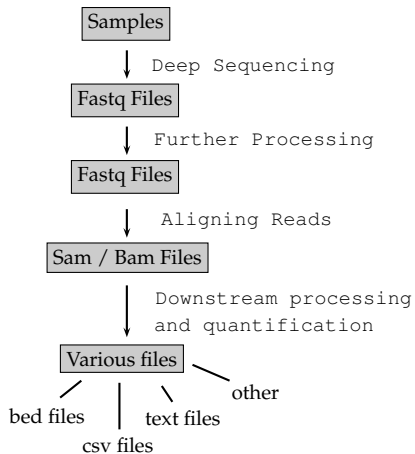
Let's find the number of chromosomes in the mm10.fa file. Each chromosome entry begins with ">", we get them by

```
$ grep ">" mm10.fa
```

Then we count the number of lines

```
$ grep ">" mm10.fa | wc -l
```

SAM / BAM Files



When a fastq file is aligned against a reference genome, a sam or a bam file is created as the ultimate output of the alignment. These files tell us where and how reads in the fastq file are mapped.

Sequence Aligners

Fastq File $\xrightarrow{\text{Aligner}}$ Sam / Bam File

| Short (Unspliced) Aligners | Spliced Aligners |
|----------------------------|------------------|
| Bowtie2 BWA | Tophat STAR |

miRNA Data:

Continuous reads so **Bowtie2** or **BWA** would be a good choice.

RNA-Seq Data:

Contains splice junctions, so **Tophat** or **STAR** would be a good choice.

Say a particular read is mapped somewhere in the genome by an aligner.

- Which chromosome?
- What position?
- Which strand?
- How good is the mapping?
- Are there insertions , deletions or gaps?

are some of the fundamental questions we ask on the alignment. A sam / bam file contains answers for these questions and possibly many more.

Sam is a text, Bam is a binary format

Recall:

A **text file** contains printable characters that are meaningful for us. It is big.

A **binary file** (possibly) contains nonprintable characters. Not meaningful to humans. It is small.

Sam File: Text file, tab delimited, big

Bam File: Binary file, relatively small

A bam file is a compressed version of the sam file and they contain the same information.

It is good practice to keep our alignment files in bam format to save space. A bam file can be read in text format using samtools.

Mandatory Fields of a Sam File

| Col | Field | Type | Regexp/Range | Brief Description |
|-----|-------|--------|--|-----------------------------------|
| 1 | QNAME | String | [!-?A-~] {1, 255} | Query template NAME |
| 2 | FLAG | Int | [0,2 ¹⁶ -1] | bitwise FLAG |
| 3 | RNAME | String | * [!-()+-<> -~][!-~]* | Reference sequence NAME |
| 4 | POS | Int | [0,2 ³¹ -1] | 1-based leftmost mapping Position |
| 5 | MAPQ | Int | [0,2 ⁸ - 1] | Mapping Quality |
| 6 | CIGAR | String | * ([0-9]+[MIDNSHPX=])+ | CIGAR string |
| 7 | RNEXT | String | * = [!-()+-<> -~][!-~]* | Ref. name of the mate/next read |
| 8 | PNEXT | Int | [0,2 ³¹ - 1] | Position of the mate/next read |
| 9 | TLEN | Int | [-2 ³¹ + 1,2 ³¹ - 1] | observed Template Length |
| 10 | SEQ | String | * [A-Za-z=.]+ | segment Sequence |
| 11 | QUAL | String | [!-\~]+ | Phred Qual. of the Seq. |

These are followed by optional fields some of which are standard and some others are aligner specific.

More detailed information on Sam format specification can be found at:
<http://samtools.github.io/hts-specs/SAMv1.pdf>

How do we convert sam files to bam files and bam files to sam files?

Use samtools.

Samtools is a software used to view and convert sam / bam files.

```
$ samtools command options
```

Don't have samtools?

What if we need a software that we don't have in the mghpc?

You can only install software **LOCALLY!**

What if we need a software that we don't have in the mghpc?

You can only install software **LOCALLY!**

There may be an easier way out!

the module system

The Module System in MGHPC

Many useful bioinformatics tools are already installed!
You need to *activate* the ones you need for your account.

To see the available modules:

```
$ module avail
```

To load a module, say samtools version 0.0.19:

```
$ module load samtools/0.0.19
```

If you can't find the software among the available modules, you can make a request to the admins via **ghpcc@list.umassmed.edu**

Converting Sam to Bam

```
$ samtools view -Sb sample.sam > sample.bam
```

By default, the input is in bam format. Using `-S`, we tell that the input is in sam format.

By default, the output is in sam format, by `-b`, we tell that the output is in bam format.

Converting Bam to Sam

```
$ samtools view -h sample.bam > output.sam
```

We need to provide the parameter `-h` to have the headers in the sam file.

Let's find all reads in a fastq file that **end** with **AAA**.
For this, we can use `grep -E` with *regular expressions*.

More on grep

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```
$ grep -E "AAA$" control.rep1.1.fq --color=always
```

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For this, we can use `grep -E` with *regular expressions*.

```
$ grep -E "AAA$" control.rep1.1.fq --color=always
```

Let's find all reads in a fastq file that **begin** with AAA.

```
$ grep -E "^AAA" control.rep1.1.fq --color=always
```

The character **\$** matches the end of a line and **^** matches the beginning of a line.

Exercise

Find all sequences in a fastq file that does **NOT** begin with a **CA** and that **does** end with an **A**.

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Find all sequences in a fastq file that does **NOT** begin with a **CA** and that **does** end with an **A**.

```
$ awk '{if(NR % 4 == 2){print($0)}}' file.fq | grep -v -E "^CA"\  
| grep -E "A$"
```

Exercise

Find all sequences in a fastq file that does **NOT** begin with a **CA** and that **does** end with an **A**.

```
$ awk '{if(NR % 4 == 2){print($0)}}' file.fq | grep -v -E "^CA"\  
| grep -E "A$"
```

Try doing this using awk only.