NGS File Formats and Sequence Quality Check

Malay (malay@uab.edu)

February 24, 2016

Contents

1 NGS File formats

1.1 FASTQ

A file format for getting the raw reads and the `quality` values. This is what you get from the sequencer. An example file can be found in the `ShortRead` package of BC (Figure 1).

```
@ERR127302.8493430 HWI-EAS350_0441:1:34:16191:2123#0/1
GTCTGCTGTATCTGTGTCGGCTGTCTCGCGGGACATGAAGTCAATGAAGGCCTGGAATGTCACTACCCCCAG
+
HHHHHHHHHHHHHHHHHHHHEBDBB?B:BBGG<DDAA?AABFEFBDBD@DDECEE3>:?;@@@>?=BAB?##
@ERR127302.21406531 HWI-EAS350_0441:1:88:9330:2587#0/1
CTAGGGCAATCTTTGCAGCAATGAATGCCAATGGGTAGCCAGTGGCTTTTGAGGCCAGAGCAGACCTTCGGG
+
IIIIHIIIGIIIIIIIHIIIIEGBGHIIIIHGIIHIIIIIIIHIIIHIIIIIGIIIEGIIGBGE@DDGGGIG
```

Figure 1: Example FastQ file.

1.2 BAM or SAM format

The FASTQ files are aligned against a reference genome using a software like BWA (http://bio-bwa.sourceforge.net/). The resulting alignment format is a BAM or SAM files. BAM files are binary, SAM files are plain text. The software for interconversion and analysis of these files are mainly `samtools` (http://www.htslib.org/). Sam file format specification can be found here http://samtools.github.io/hts-specs/SAMv1.pdf. A small example BAM files comes along with `Rsamtools` package ("example_from_SAM_Spec.sam"'):

```
@HD VN:1.3  SO:coordinate
@SQ SN:ref  LN:45
r001    163 ref 7   30  8M2I4M1D3M  =   37  39  TTAGATAAAGGATACTG   *
r002    0   ref 9   30  3S6M1P1I4M  *   0   0   AAAAGATAAGGATA      *
r003    0   ref 9   30  5H6M        *   0   0   AGCTAA              *   NM:i:1
r004    0   ref 16  30  6M14N5M     *   0   0   ATAGCTTCAGC         *
r003    16  ref 29  30  6H5M        *   0   0   TAGGC               *   NM:i:0
r001    83  ref 37  30  9M          =   7   -39 CAGCGCCAT           *
```

1.3 VCF

Once the alignment BAM files have been generated, a variant caller like GATK (https://www.broadinstitute.org/gatk/) is used to find the variants in the file. The resulting file is called VCF. The specification can be found here (http://samtools.github.io/hts-specs/VCFv4.2.pdf). A sample VCF line is given below:

```
chr1    873762  .       T   G   [CLIPPED] GT:AD:DP:GQ:PL    0/1:173,141:282:99:255,0,255
chr1    877664  rs3828047   A   G   [CLIPPED] GT:AD:DP:GQ:PL    1/1:0,105:94:99:255,255,0
chr1    899282  rs28548431  C   T   [CLIPPED] GT:AD:DP:GQ:PL    0/1:1,3:4:25.92:103,0,26
```

Once the variant is called they are annotated using variant annotation tools like SnpEff (http://snpeff.sourceforge.net/) or Annovar (http://www.openbioinformatics.org/annovar/) or `VariantAnnotation` package.

2 Quality score

2.1 ASCII charater sets

Remember that to a computer everything is binary. The difference between a text file and a binary file is just to make the software reading the file to interpret the binary string representing "newline (\n)" differently than the other characters in the file.

Traditionally, each character in a computer is represented by 8 binary charater or 1 byte. Although, 1 byte could represent 256 different characters only 128 used to get used. This charater set is called ASCII. Then all the 256 possible code was included for representing english lanauge. This character table is called **extended ASCII** or **latin1**. You can see the table just looking at the output of the command `man ascii`. Nowadays, due the demand to support non-english language, 256 characters possible by 1 byte is no longer enough, and a new standard for **multibyte** character set emerged, called unicode or UTF.

There are several versions of UTF. The simplest one is exactly like latin1 set called UTF+8. There are others, such as 2 byte UTF or UTF+16 or even 4 byte UTF called UTF+32. Which version a computer uses depends on `locale`. A sotware reading a file first looks at the locale and then interprets a text file based on the locale. If it is UTF+8, the software reads 1 byte at a time and then converts it into the character. For e.g., if the locale is UTF+8, and it finds the the following byte `01000001`, it knows that this is the decimal `65` or the english character `A`.

2.2 Phred score

It is obvious from the discussion that any number can also be represented using character code. Using `Latin1` character sets, it is possible represent a number between 0 and 256. `Phred` originally used in a software called `phred` is a standard for representing quality score in a sequence file. `Phred` is a part of software package called `Staden`.

There used to be two types of Phred scores: Phred+33 and Phred+64. This number indicates the starting value. Nowadays Illumina sequencers generally used Phred+33 score.

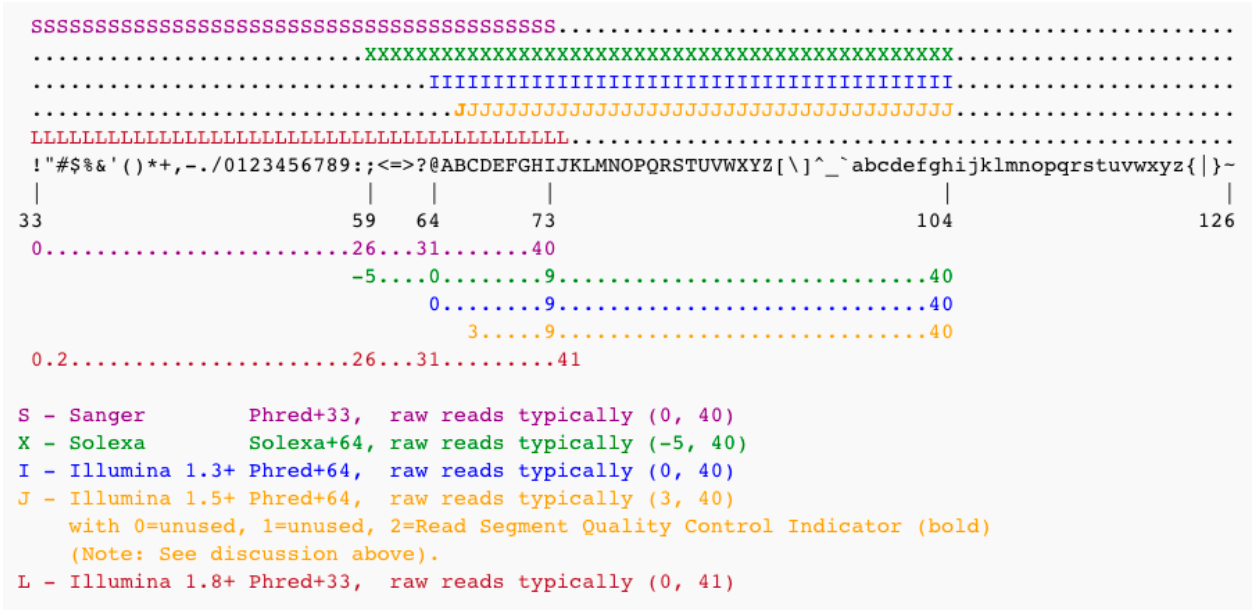


Figure 2: Phred score. (Source: wikipedia)

Phred quality scores are defined as scaled logarithmic probablity of an error in base-calling:

$Q = -10logP$

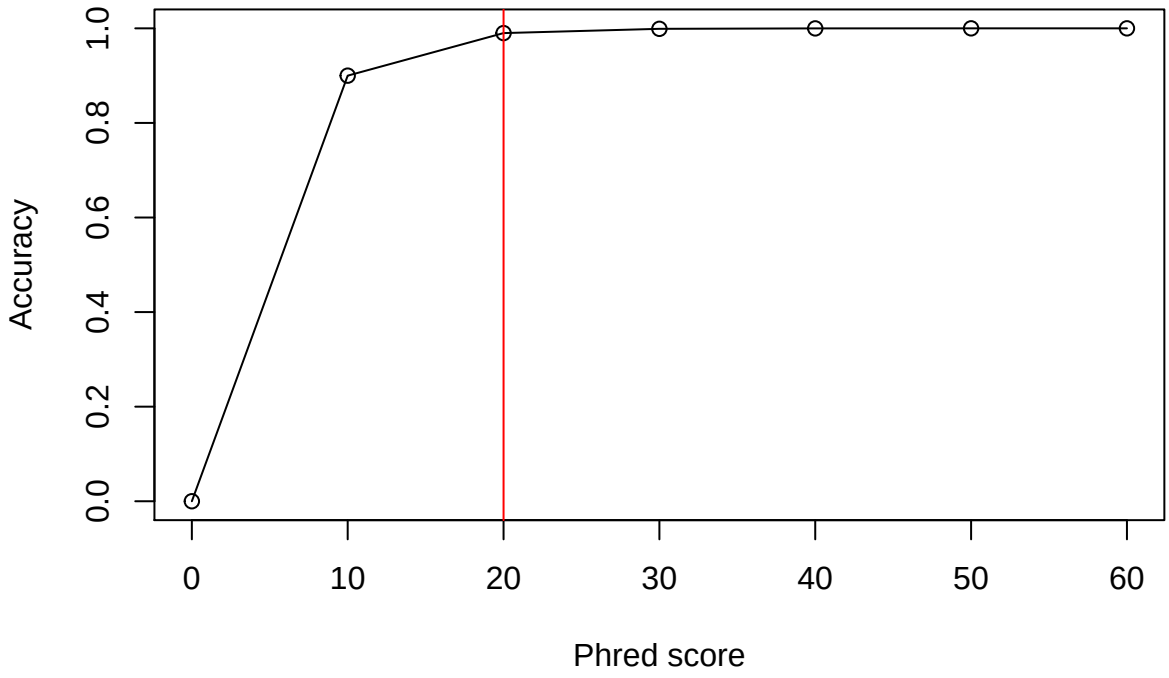
The number is then added to 33 to get the modern Phred+33 score. We can calculate the accuracy as follows:

$accuracy = 1 - 10^{-Q/10}$

2.3 Why Phred 20

A phred score of 20 is generally considered to be cutoff.

```
e <- seq(0,60,10)
a <- 1 - 10^(-(e/10))
plot(e,a,xlab="Phred score",ylab="Accuracy")
lines(e,a)
abline(v=20,col="red")
```



You can see there is sharp drop of quality below score 20. This is why Phred 20 is a good cutoff score. This actually $(20 + 33) = 53$ which `5` in ascii.

3 NGS Qaulity check

3.1 Fastqc

`Fastqc` is a program to check the quality of your file. Download Fastqc from here:

http://www.bioinformatics.babraham.ac.uk/projects/fastqc/fastqc_v0.11.2.zip

An example of good file is http://www.bioinformatics.babraham.ac.uk/projects/fastqc/good_sequence_short_fastqc.html. An example of a bad file is http://www.bioinformatics.babraham.ac.uk/projects/fastqc/bad_sequence_fastqc.html. I suggest that you run `fastqc` from commandline. See `fastqc --help` for details.

3.2 Read trimming

We will use `Trimmomatic` for read trimming and adapter removal. Download Trimmomatic from:

http://www.usadellab.org/cms/?page=trimmomatic

Generally, Illumina adapters are of two types: Nextera for WGS and exome sequencing and Truseq from RNAseq. We need to keep that in mind and provide the for trimming.

```
java -jar Trimmomatic-0.33/trimmomatic-0.33.jar PE \
D784G_R1.fq.gz D784G_R2.fq.gz  \
D784G_R1.trim_p.fq.gz D784G_R1.trim_u.fq.gz \
D784G_R2.trim_p.fq.gz D784G_R2.trim_ufq.gz
ILLUMINACLIP:Trimmomatic-0.33/adapters/TruSeq3-PE.fa:2:30:10 \
LEADING:2 \
TRAILING:2 \
SLIDINGWINDOW:4:15 \
MINLEN:30
```

We used `TruSeq3-PE` adapters for clipping. Some other options are:

1. Adapters will have max 2 mismatches and will be clipped if a score of 30 is reached.
2. Remove leading and trailing N bases if quality is below 2.
3. Move with a 4 bp window and cutting where the average quality falls below 15.
4. After trmming remove all sequences whose length is below 30.