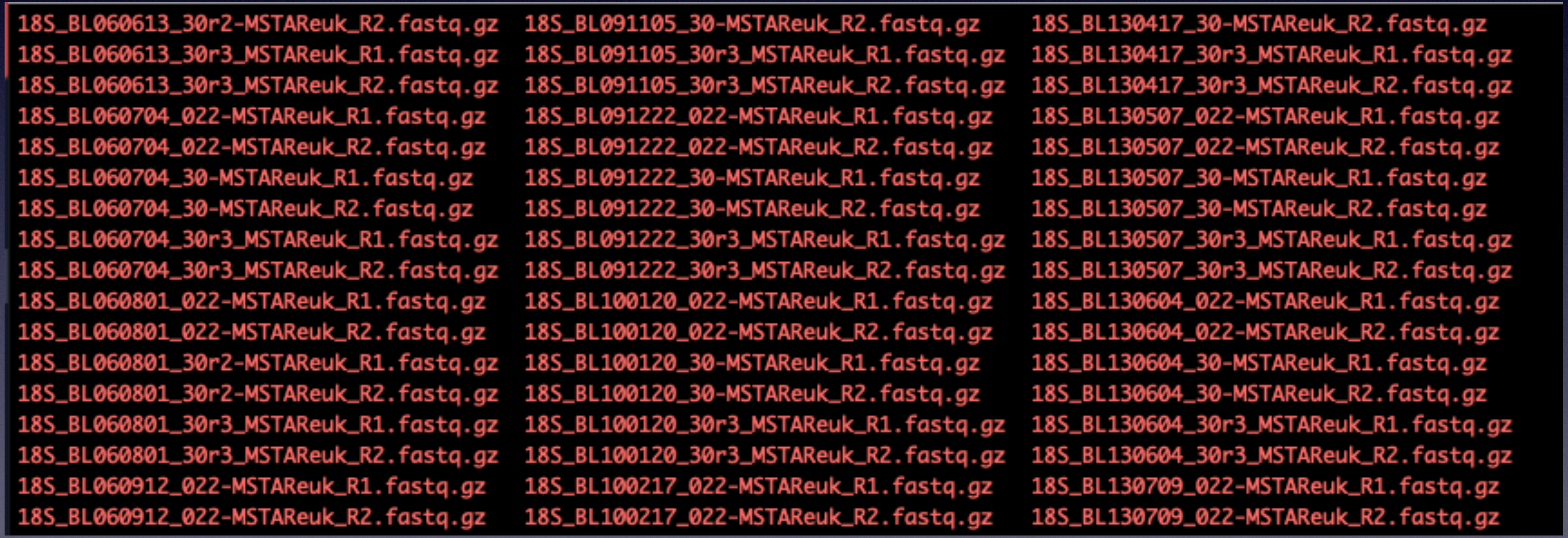
Sequence format and pre-processing

Ramiro Logares, ICM, Barcelona

Illumina short reads

- We receive files in fastq format from the sequencing center
- Two files per sample, 1 forward (R1) and 1 reverse (R2)
- Normally, reads overlap
- Depending on the library preparation, all reads are in the same direction (5’-3’) or their directions are mixed in both R1 and R2
- We usually work with gzipped fastq files to save space

How files look like when they are received



```
18S_BL060613_30r2-MSTAReuk_R2.fastq.gz  18S_BL091105_30-MSTAReuk_R2.fastq.gz    18S_BL130417_30-MSTAReuk_R2.fastq.gz
18S_BL060613_30r3_MSTAReuk_R1.fastq.gz  18S_BL091105_30r3_MSTAReuk_R1.fastq.gz  18S_BL130417_30r3_MSTAReuk_R1.fastq.gz
18S_BL060613_30r3_MSTAReuk_R2.fastq.gz  18S_BL091105_30r3_MSTAReuk_R2.fastq.gz  18S_BL130417_30r3_MSTAReuk_R2.fastq.gz
18S_BL060704_022-MSTAReuk_R1.fastq.gz   18S_BL091222_022-MSTAReuk_R1.fastq.gz   18S_BL130507_022-MSTAReuk_R1.fastq.gz
18S_BL060704_022-MSTAReuk_R2.fastq.gz   18S_BL091222_022-MSTAReuk_R2.fastq.gz   18S_BL130507_022-MSTAReuk_R2.fastq.gz
18S_BL060704_30-MSTAReuk_R1.fastq.gz    18S_BL091222_30-MSTAReuk_R1.fastq.gz    18S_BL130507_30-MSTAReuk_R1.fastq.gz
18S_BL060704_30-MSTAReuk_R2.fastq.gz    18S_BL091222_30-MSTAReuk_R2.fastq.gz    18S_BL130507_30-MSTAReuk_R2.fastq.gz
18S_BL060704_30r3_MSTAReuk_R1.fastq.gz  18S_BL091222_30r3_MSTAReuk_R1.fastq.gz  18S_BL130507_30r3_MSTAReuk_R1.fastq.gz
18S_BL060704_30r3_MSTAReuk_R2.fastq.gz  18S_BL091222_30r3_MSTAReuk_R2.fastq.gz  18S_BL130507_30r3_MSTAReuk_R2.fastq.gz
18S_BL060801_022-MSTAReuk_R1.fastq.gz   18S_BL100120_022-MSTAReuk_R1.fastq.gz   18S_BL130604_022-MSTAReuk_R1.fastq.gz
18S_BL060801_022-MSTAReuk_R2.fastq.gz   18S_BL100120_022-MSTAReuk_R2.fastq.gz   18S_BL130604_022-MSTAReuk_R2.fastq.gz
18S_BL060801_30r2-MSTAReuk_R1.fastq.gz  18S_BL100120_30-MSTAReuk_R1.fastq.gz    18S_BL130604_30-MSTAReuk_R1.fastq.gz
18S_BL060801_30r2-MSTAReuk_R2.fastq.gz  18S_BL100120_30-MSTAReuk_R2.fastq.gz    18S_BL130604_30-MSTAReuk_R2.fastq.gz
18S_BL060801_30r3_MSTAReuk_R1.fastq.gz  18S_BL100120_30r3_MSTAReuk_R1.fastq.gz  18S_BL130604_30r3_MSTAReuk_R1.fastq.gz
18S_BL060801_30r3_MSTAReuk_R2.fastq.gz  18S_BL100120_30r3_MSTAReuk_R2.fastq.gz  18S_BL130604_30r3_MSTAReuk_R2.fastq.gz
18S_BL060912_022-MSTAReuk_R1.fastq.gz   18S_BL100217_022-MSTAReuk_R1.fastq.gz   18S_BL130709_022-MSTAReuk_R1.fastq.gz
18S_BL060912_022-MSTAReuk_R2.fastq.gz   18S_BL100217_022-MSTAReuk_R2.fastq.gz   18S_BL130709_022-MSTAReuk_R2.fastq.gz
```

fastq format

- Four sequences per line

1. @sequence.ID
2. ACTGACTGACTG # nucleotide sequence
3. + (separator)
4. Quality scores (Phred +33: normally 0-41)

Quite a bit of information here

```
@M02696:67:000000000-B44VG:1:1101:11781:1257 1:N:0:57
CCAGCAGCTGCGGTAATTCCGGCTCCTTCAGCCTGAGGTAGAATTGTTGTAGTTAAAACGCTCGTAGTTGGATTTGTTAGAGTTTTGTGTGTGTTGGTTGCGTATATATTCGTATATTCGTGATTCTTCATGCCACTTTTATACTGA
TTGTGGATAATTTTCGGATTATTTGCAATATTACTGTGAGAAAAAGAGTGCGCTTAAGGGCGGCTTTATGCTAAGATCATTTAGCATGGAATAAACATAACGG
+
CCCCCGGGGFG@CGG;FDEFFGEFGGGG9E@CFGCGGGEFG<EFGFEFGGGGFGGGEG<FC@@@6@F8@FCGAFFFFF,6C6EC@FCFGGGGGGGGGCFGGDF:CFFFAFF,BCE<CFFEFF7F8?,CF<EBCF,AFDGFAFF<
9@BEFEG?FC9,CE<FD?A7CGEG:FDFG,3A;,CDFGGGFF,=CF,6,6BFGF,6+4@EEGGGG7>EC?FGGF@FCGED8CFFGG79D9CCF<?C4713?FFFCDE
@M02696:67:000000000-B44VG:1:1101:8695:1347 1:N:0:57
CCAGCACCCGCGGTAATTCCGGCTCCTTCAGCCTGAGGTAGAATTGTTGTAGTTAAAACGCTCGTAGTTGGATTTGTAAGAGTTTTGTGTGTGTTGGTTGCGTATATATTCGTATATTCGTGATTCTTCATGCCACTTTTATACTGA
TTGTGGATAATTTTCGGATTATTTGCAATATTACTGTGAGAAAAAGAGTGCGCTTAAGGGCGGCTTTATGCTAAGATCATTTAGCATGGAATAAACATAACGG
+
CCCCCGGGGGGGGGGGGGGGGGGGGGGGGGGGGFGGGGGGGGGGGFGGGGGGGGGGGGGGGGGGFGGGFGEFGGGGGGFGGGGGGGGGGGGGGGGGGGGGGGFFGGFFGGGGGGFGGGFGGDGGGGGGDFGGGGGGGGGGGGC
FFGGGGGGGGG9FGGGGGGGGGGGFFGGGFGGGFFGGGGGGGGGGGFFFGGG6CEEFGDGDEGEEDFFFCFFE@CFF;ACD7:F8CFGGFCFFFFFFGCA*::
@M02696:67:000000000-B44VG:1:1101:22691:1423 1:N:0:57
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TTGTGGATAATTTTCGGATTATTTGCAATATTACTGTGAGAAAAAGAGTGCGCTTAAGGGCGGCTTTATGCTAAGATCATTTAGCATGGAATAAACATAACGG
+
CCCCCGGGGGGGGGGEGFGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGFFGGGGGGGGGGGGGGC@D8,CFGGCFGGGGEF<FF9FGF6EGGFG<FG<BFGFGGEGCDGGGGGGGGGGG?FGGGFFGCGGGGGGGGGGFG9@FA,EFF<
FFGGFFGGGGG,AFGGDGGDFFEACDFFDE@DDFAFC<;FD>=@FCFGGCDGG6ED;AEGGCEDG5FFFCF,9=CFFF)=,,=,BFFCGF4CD76:C>9ACE>
@M02696:67:000000000-B44VG:1:1101:19965:1620 1:N:0:57
CCAGCAGCTGCGGTAATTCCGGCTCCTTCAGCCTGAGGTAGAATTGTTGTAGTTAAAACGCTCGTAGTTGGATTTGTAAGAGTTTTGTGTGTGTTGGTTGCGTATATATTCGTATATTCGTGATTCTTCATGCCACTTTTATACTGA
TTGTGGATAATTTTCGGATTATTTGCAATATTACTGTGAGAAAAAGAGTGCGCTTAAGGGCGGCTTTATGCTAAGATCATTTAGCATGGAATAAACATAACGG
+
CCCCCGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGFFGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
FGGGGGGGGGGGGGGGGGGGGGGGGGFGGGGGGGGGGGFFFGFGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGFGGGGGGGGGGGFFDFFGGGFFFFF
@M02696:67:000000000-B44VG:1:1101:17122:1672 1:N:0:57
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+
CCCCCGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGEGGGGGGGGGGGGGGGEGGGGGGGGGGGGFGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGFCFFGFFEGGGGGGGGGGGGGGDGCGGGGGCFGGGGFFFGGFGGGDFFGGGGGGGGGFGGGGFGFF
@M02696:67:000000000-B44VG:1:1101:21438:1779 1:N:0:57
CCAGCACCTGCGGTAATTCCGGCTCCTTCAGCCTGAGGTAGAATTGTTGTAGTTAAAACGCTCGTAGTTGGATTTGTAAGAGTTTTGTGTGTGTTGGTTGCGTATATATTCGTATATTCGTGATTCTTCATGCCACTTTTATACTGA
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+
CCCCCGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGFFGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGFFGGGFGGGGGFDCEEGGGGFGGGGGGD?FGGFGGGGFGGFGCEGGGFFFDGGGGFFGGGGFGED
```
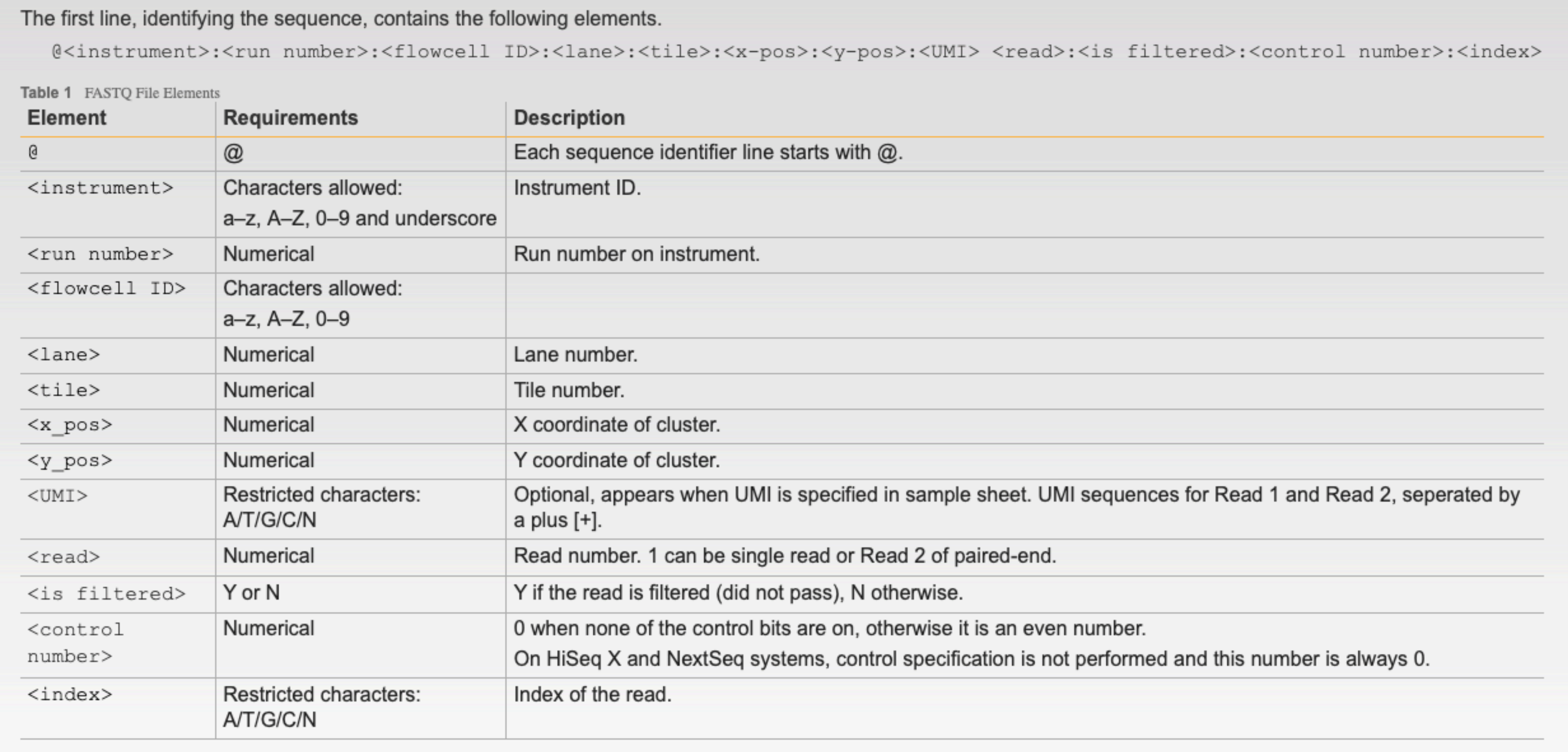

```
@M02696:67:000000000-B44VG:1:1101:11781:1257 1:N:0:57
```



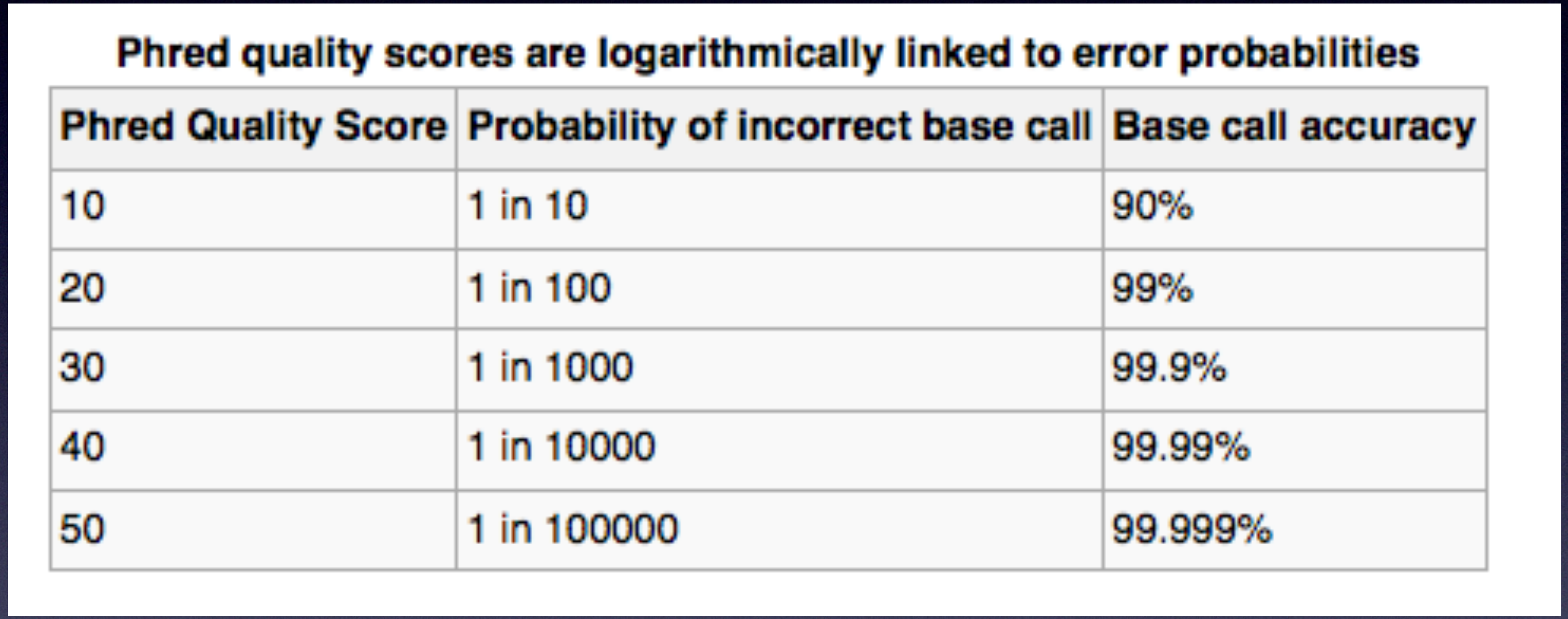
The first line, identifying the sequence, contains the following elements.

```
@<instrument>:<run number>:<flowcell ID>:<lane>:<tile>:<x-pos>:<y-pos>:<UMI> <read>:<is filtered>:<control number>:<index>
```

Table 1 FASTQ File Elements

Element	Requirements	Description
`@`	@	Each sequence identifier line starts with @.
`<instrument>`	Characters allowed: a–z, A–Z, 0–9 and underscore	Instrument ID.
`<run number>`	Numerical	Run number on instrument.
`<flowcell ID>`	Characters allowed: a–z, A–Z, 0–9	
`<lane>`	Numerical	Lane number.
`<tile>`	Numerical	Tile number.
`<x_pos>`	Numerical	X coordinate of cluster.
`<y_pos>`	Numerical	Y coordinate of cluster.
`<UMI>`	Restricted characters: A/T/G/C/N	Optional, appears when UMI is specified in sample sheet. UMI sequences for Read 1 and Read 2, seperated by a plus [+].
`<read>`	Numerical	Read number. 1 can be single read or Read 2 of paired-end.
`<is filtered>`	Y or N	Y if the read is filtered (did not pass), N otherwise.
`<control number>`	Numerical	0 when none of the control bits are on, otherwise it is an even number. On HiSeq X and NextSeq systems, control specification is not performed and this number is always 0.
`<index>`	Restricted characters: A/T/G/C/N	Index of the read.

Sanger Phred quality scores



Phred quality scores are logarithmically linked to error probabilities

Phred Quality Score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10000	99.99%
50	1 in 100000	99.999%

$$Q = -10 \log_{10} P$$

Q = Phred quality scores
P = base calling error probability

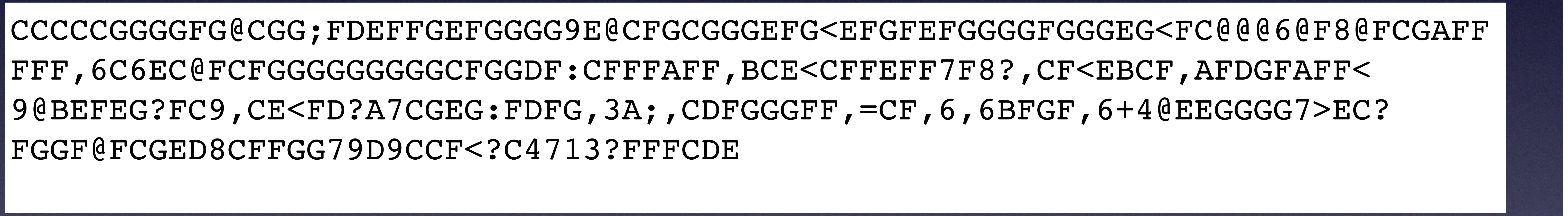
Calculating Phred scores

- To determine quality scores, Phred first calculates several parameters related to peak shape and peak resolution at each base.

- Phred then uses these parameters to look up a corresponding quality score in huge lookup tables.

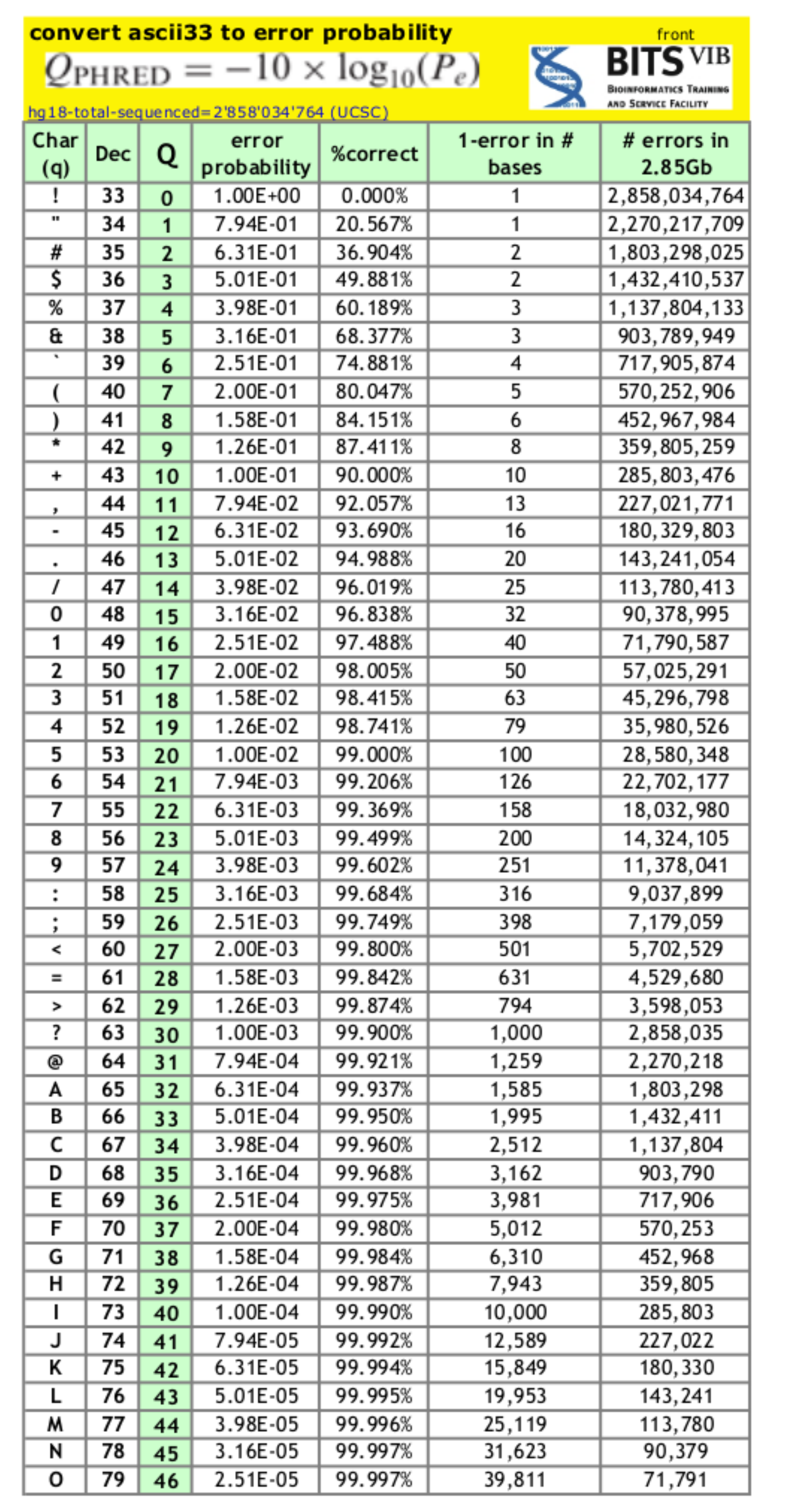
- These lookup tables were generated from sequence traces where the correct sequence was known, and are hard coded in Phred; different lookup tables are used for different sequencing chemistries and machines.

- Quality scores are encoded in ASCII (American Standard Code for Information Interchange)
- The start in character 33 (Phred+33)



```
CCCCCGGGGFG@CGG;FDEFFGEFGGGG9E@CFGCGGGEFG<EFGFEFGGGGFGGGEG<FC@@@6@F8@FCGAFF
FFF,6C6EC@FCFGGGGGGGGGCFGGDF:CFFFAFF,BCE<CFFEFF7F8?,CF<EBCF,AFDGFAFF<
9@BEFEG?FC9,CE<FD?A7CGEG:FDFG,3A;,CDFGGGFF,=CF,6,6BFGF,6+4@EEGGGG7>EC?
FGGF@FCGED8CFFGG79D9CCF<?C4713?FFFCDE
```

Q=ASCII code - 33



convert ascii33 to error probability

front

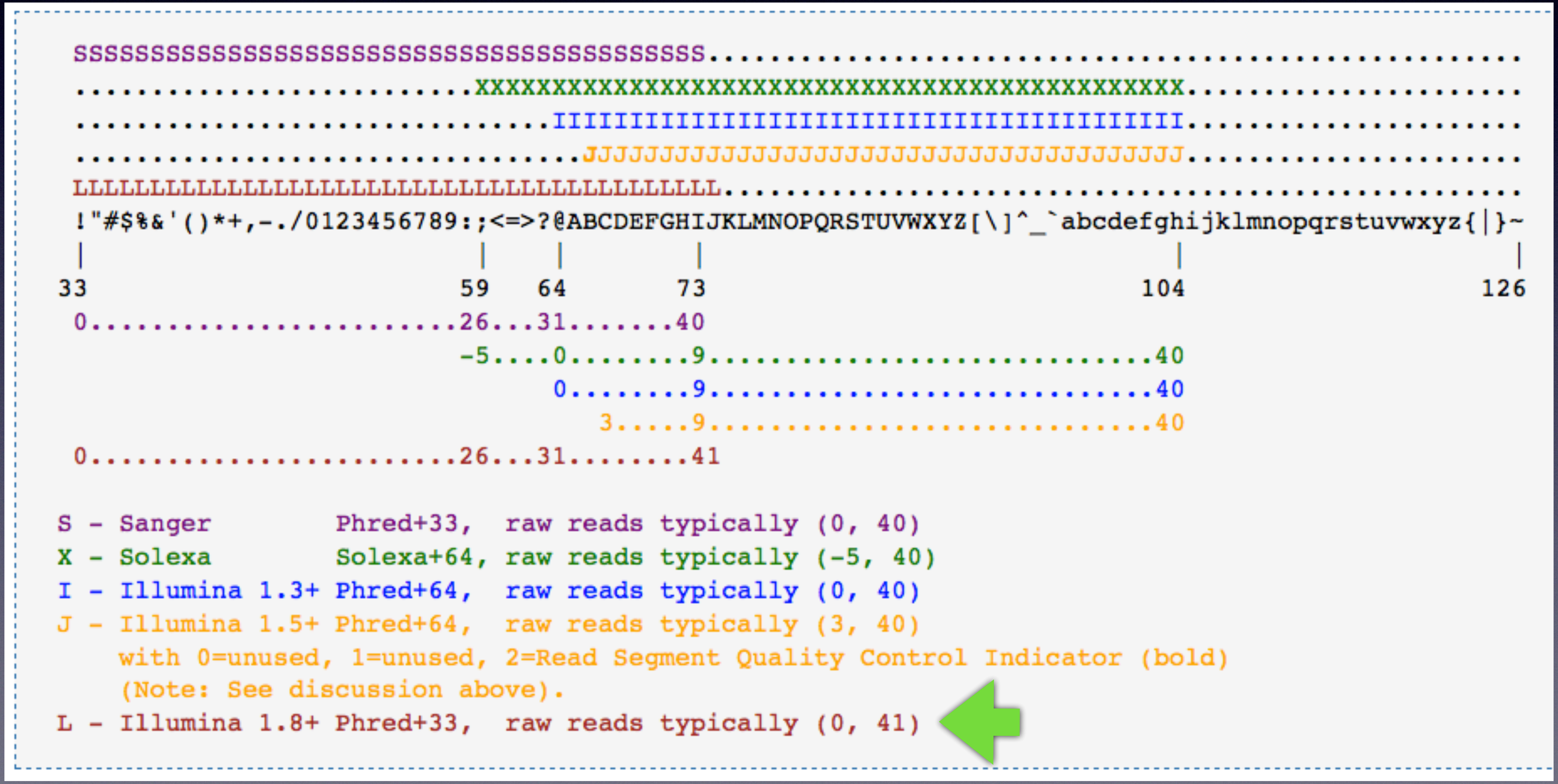
$Q_{\mathrm{PHRED}} = -10 \times \log_{10}(P_e)$

hg18-total-sequenced=2'858'034'764 (UCSC)

BITS VIB
Bioinformatics Training and Service Facility

Char (q)	Dec	Q	error probability	%correct	1-error in # bases	# errors in 2.85Gb
!	33	0	1.00E+00	0.000%	1	2,858,034,764
"	34	1	7.94E-01	20.567%	1	2,270,217,709
#	35	2	6.31E-01	36.904%	2	1,803,298,025
$	36	3	5.01E-01	49.881%	2	1,432,410,537
%	37	4	3.98E-01	60.189%	3	1,137,804,133
&	38	5	3.16E-01	68.377%	3	903,789,949
`	39	6	2.51E-01	74.881%	4	717,905,874
(	40	7	2.00E-01	80.047%	5	570,252,906
)	41	8	1.58E-01	84.151%	6	452,967,984
*	42	9	1.26E-01	87.411%	8	359,805,259
+	43	10	1.00E-01	90.000%	10	285,803,476
,	44	11	7.94E-02	92.057%	13	227,021,771
-	45	12	6.31E-02	93.690%	16	180,329,803
.	46	13	5.01E-02	94.988%	20	143,241,054
/	47	14	3.98E-02	96.019%	25	113,780,413
0	48	15	3.16E-02	96.838%	32	90,378,995
1	49	16	2.51E-02	97.488%	40	71,790,587
2	50	17	2.00E-02	98.005%	50	57,025,291
3	51	18	1.58E-02	98.415%	63	45,296,798
4	52	19	1.26E-02	98.741%	79	35,980,526
5	53	20	1.00E-02	99.000%	100	28,580,348
6	54	21	7.94E-03	99.206%	126	22,702,177
7	55	22	6.31E-03	99.369%	158	18,032,980
8	56	23	5.01E-03	99.499%	200	14,324,105
9	57	24	3.98E-03	99.602%	251	11,378,041
:	58	25	3.16E-03	99.684%	316	9,037,899
;	59	26	2.51E-03	99.749%	398	7,179,059
<	60	27	2.00E-03	99.800%	501	5,702,529
=	61	28	1.58E-03	99.842%	631	4,529,680
>	62	29	1.26E-03	99.874%	794	3,598,053
?	63	30	1.00E-03	99.900%	1,000	2,858,035
@	64	31	7.94E-04	99.921%	1,259	2,270,218
A	65	32	6.31E-04	99.937%	1,585	1,803,298
B	66	33	5.01E-04	99.950%	1,995	1,432,411
C	67	34	3.98E-04	99.960%	2,512	1,137,804
D	68	35	3.16E-04	99.968%	3,162	903,790
E	69	36	2.51E-04	99.975%	3,981	717,906
F	70	37	2.00E-04	99.980%	5,012	570,253
G	71	38	1.58E-04	99.984%	6,310	452,968
H	72	39	1.26E-04	99.987%	7,943	359,805
I	73	40	1.00E-04	99.990%	10,000	285,803
J	74	41	7.94E-05	99.992%	12,589	227,022
K	75	42	6.31E-05	99.994%	15,849	180,330
L	76	43	5.01E-05	99.995%	19,953	143,241
M	77	44	3.98E-05	99.996%	25,119	113,780
N	78	45	3.16E-05	99.997%	31,623	90,379
O	79	46	2.51E-05	99.997%	39,811	71,791

Phred encoding in different sequencers



```
  SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS.....................................................
  ..........................XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX......................
  ...............................IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII......................
  .................................JJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJ......................
  LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL....................................................
  !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
  |                         |    |        |                              |                     |
 33                        59   64       73                            104                   126
  0........................26...31.......40
                           -5....0........9.............................40
                                 0........9.............................40
                                    3.....9.............................40
  0........................26...31........41

 S - Sanger        Phred+33,  raw reads typically (0, 40)
 X - Solexa        Solexa+64, raw reads typically (-5, 40)
 I - Illumina 1.3+ Phred+64,  raw reads typically (0, 40)
 J - Illumina 1.5+ Phred+64,  raw reads typically (3, 40)
     with 0=unused, 1=unused, 2=Read Segment Quality Control Indicator (bold)
     (Note: See discussion above).
 L - Illumina 1.8+ Phred+33,  raw reads typically (0, 41)
```

Removing primers

- The sequences received from the sequencing center may contain primers used to amplify them

- Primers need to be removed as they normally contain ambiguous positions that can interfere with DADA2

- DADA2 assumes primers have been removed

Checking if sequences have primers in unix

- We know the primer sequence expected in the reads

- Fw :CCAGCA[ACGT]C[ACGT]GCGGTAATTCC
- Rv: ACTTTCGTTCTTGAT[AGCT][AGCT][AGCT]
  - Ambiguities are included to match sequences

- We use zgrep to match the primer against the gzipped sequences

```
[rlogares@marbits raw]$ ls
BL100525E-MSTAReuk_R1.fastq.gz  BL100706E-MSTAReuk_R1.fastq.gz  BL100914E-MSTAReuk_R1.fastq.gz  primers_R1_in_reads
BL100525E-MSTAReuk_R2.fastq.gz  BL100706E-MSTAReuk_R2.fastq.gz  BL100914E-MSTAReuk_R2.fastq.gz  primers_R2_in_reads
BL100622E-MSTAReuk_R1.fastq.gz  BL100803E-MSTAReuk_R1.fastq.gz  clipping_primers.sh
BL100622E-MSTAReuk_R2.fastq.gz  BL100803E-MSTAReuk_R2.fastq.gz  cutadapt.o40252

[rlogares@marbits raw]$ zgrep -c --color  CCAGCA[ACGT]C[ACGT]GCGGTAATTCC BL100525E-MSTAReuk_R1.fastq.gz
23843
```

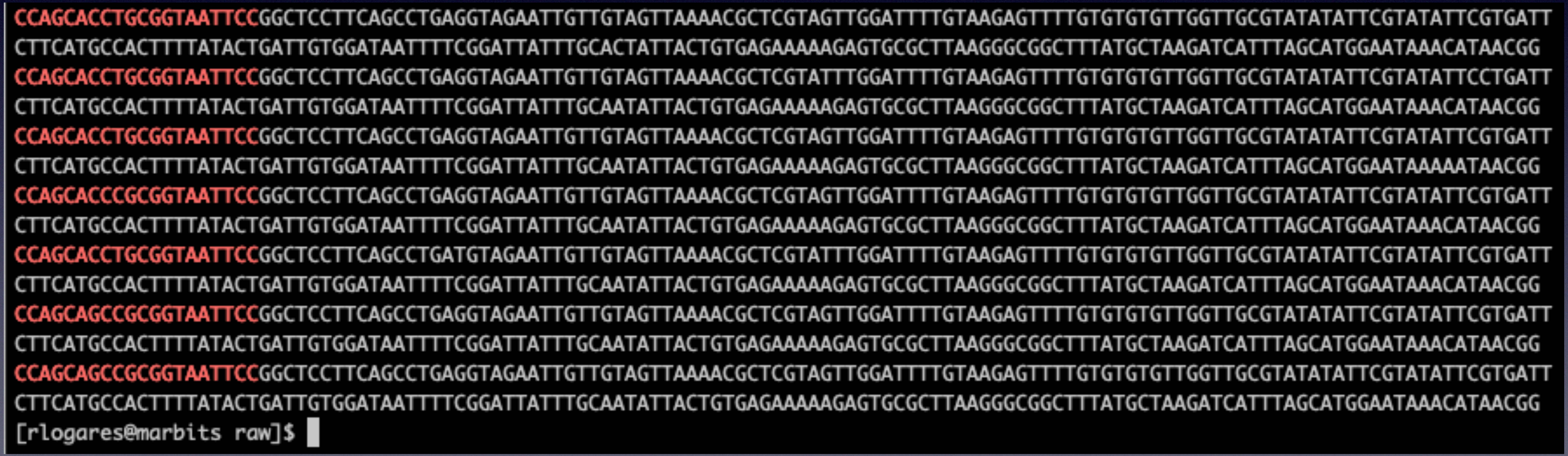
Forward primers

```
[rlogares@marbits raw]$ zgrep -c --color ACTTTCGTTCTTGAT[AGCT][AGCT][AGCT]  BL100525E-MSTAReuk_R2.fastq.gz
23856
```

Reverse primers

- As several counts are given, we inspect the sequences visually

```
[rlogares@marbits raw]$ zgrep --color  CCAGCA[ACGT]C[ACGT]GCGGTAATTCC BL100525E-MSTAReuk_R1.fastq.gz
```



```
CCAGCACCTGCGGTAATTCCGGCTCCTTCAGCCTGAGGTAGAATTGTTGTAGTTAAAACGCTCGTAGTTGGATTTTGTAAGAGTTTTGTGTGTGTTGGTTGCGTATATATTCGTATATTCGTGATT
CTTCATGCCACTTTTATACTGATTGTGGATAATTTTCGGATTATTTGCACTATTACTGTGAGAAAAAGAGTGCGCTTAAGGGCGGCTTTATGCTAAGATCATTTAGCATGGAATAAACATAACGG
CCAGCACCTGCGGTAATTCCGGCTCCTTCAGCCTGAGGTAGAATTGTTGTAGTTAAAACGCTCGTATTTGGATTTTGTAAGAGTTTTGTGTGTGTTGGTTGCGTATATATTCGTATATTCCTGATT
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CCAGCACCTGCGGTAATTCCGGCTCCTTCAGCCTGATGTAGAATTGTTGTAGTTAAAACGCTCGTATTTGGATTTTGTAAGAGTTTTGTGTGTGTTGGTTGCGTATATATTCGTATATTCGTGATT
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CCAGCAGCCGCGGTAATTCCGGCTCCTTCAGCCTGAGGTAGAATTGTTGTAGTTAAAACGCTCGTAGTTGGATTTTGTAAGAGTTTTGTGTGTGTTGGTTGCGTATATATTCGTATATTCGTGATT
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CCAGCAGCCGCGGTAATTCCGGCTCCTTCAGCCTGAGGTAGAATTGTTGTAGTTAAAACGCTCGTAGTTGGATTTTGTAAGAGTTTTGTGTGTGTTGGTTGCGTATATATTCGTATATTCGTGATT
CTTCATGCCACTTTTATACTGATTGTGGATAATTTTCGGATTATTTGCAATATTACTGTGAGAAAAAGAGTGCGCTTAAGGGCGGCTTTATGCTAAGATCATTTAGCATGGAATAAACATAACGG
[rlogares@marbits raw]$ 
```
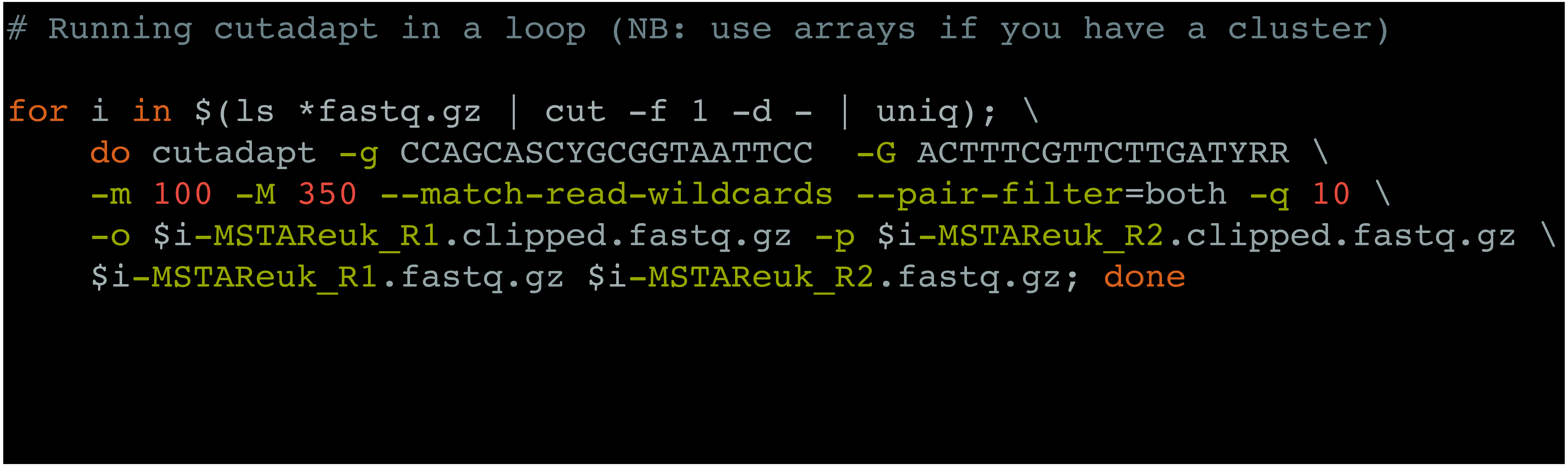

We use cutadapt to remove primers

- Program: https://cutadapt.readthedocs.io/en/stable/
- Runs in unix (we will run this in Google Colab)
- Cutadapt will search for primers in R1 and R2 sequences and remove them
- It can also remove all sequences where primers have not been found



```
# Running cutadapt in a loop (NB: use arrays if you have a cluster)

for i in $(ls *fastq.gz | cut -f 1 -d - | uniq); \
    do cutadapt -g CCAGCASCYGCGGTAATTCC  -G ACTTTCGTTCTTGATYRR \
    -m 100 -M 350 --match-read-wildcards --pair-filter=both -q 10 \
    -o $i-MSTAReuk_R1.clipped.fastq.gz -p $i-MSTAReuk_R2.clipped.fastq.gz \
    $i-MSTAReuk_R1.fastq.gz $i-MSTAReuk_R2.fastq.gz; done
```

Ambiguities in primers are interpreted
]

Cutadapt options

```
#     -g ADAPTER, --front=ADAPTER
#                        Sequence of an adapter ligated to the 5' end (paired
#                        data: of the first read). The adapter and any
#                        preceding bases are trimmed. Partial matches at the 5'
#                        end are allowed. If a '^' character is prepended
#                        ('anchoring'), the adapter is only found if it is a
#                        prefix of the read.
#       Paired-end options:
#       The -A/-G/-B/-U options work like their -a/-b/-g/-u counterparts, but
#       are applied to the second read in each pair.
#
#        -G ADAPTER          5' adapter to be removed from second read in a pair.
#        -m LENGTH, --minimum-length=LENGTH
#                        Discard reads shorter than LENGTH. Default: 0
#        -M LENGTH, --maximum-length=LENGTH
#                        Discard reads longer than LENGTH. Default: no limit
#        --match-read-wildcards
#                        Interpret IUPAC wildcards in reads. Default: False
#        --pair-filter=(any|both)
#                        Which of the reads in a paired-end read have to match
#                        the filtering criterion in order for the pair to be
#                        filtered. Default: any
#        -q [5'CUTOFF,]3'CUTOFF, --quality-cutoff=[5'CUTOFF,]3'CUTOFF
#                        Trim low-quality bases from 5' and/or 3' ends of each
#                        read before adapter removal. Applied to both reads if
#                        data is paired. If one value is given, only the 3' end
#                        is trimmed. If two comma-separated cutoffs are given,
#                        the 5' end is trimmed with the first cutoff, the 3'
#                        end with the second.
#        -o output file R1
#        -p FILE, --paired-output=FILE
#                        Write second read in a pair to FILE.
```

- After cutadap, sequences are ready to be used in dada2

- It is good to double check primers are gone using the same zgrep command used before

- We don’t analyse the overall quality of the sequences, as this will be done later with dada2

- We only remove entire sequences that look very wrong with cutadapt

- It is important to consider whether sequences come from sequencers with 4- or 2- color chemistries, as this will change cutadapt parameters

- There are alternative tools, such as Trimmomatic