UBC Bioinformatics Class

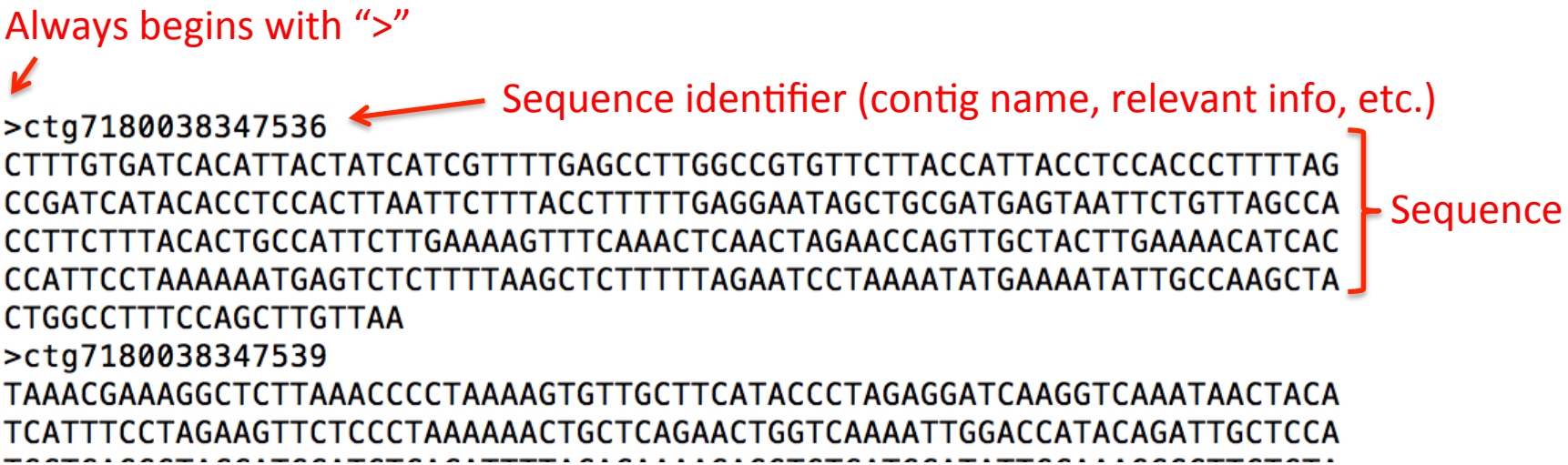
Topic 3: Fastq files and quality checking and trimming

Lecture outcomes

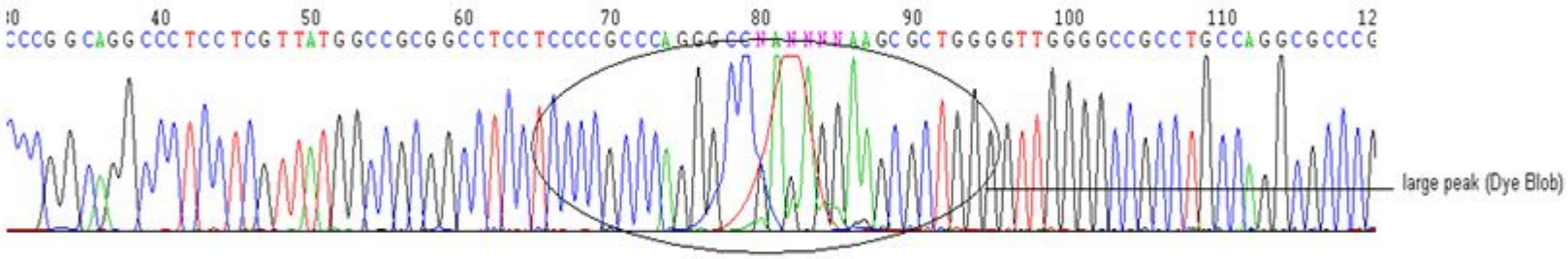
- Understand sequence file formats
- Identify the main steps for preparing NGS data for alignment/assembly

NGS file formats: Fasta

- Sequences with a header (.fasta, .fa, .fas)
- Now mainly used for storing reference sequences (no qual scores) as either nucleotides or peptides
- Can have quality scores are stored in separate files (usually .fasta or .fa & .qual)
- 2 lines/sequence read:



NGS file formats: Quality scores

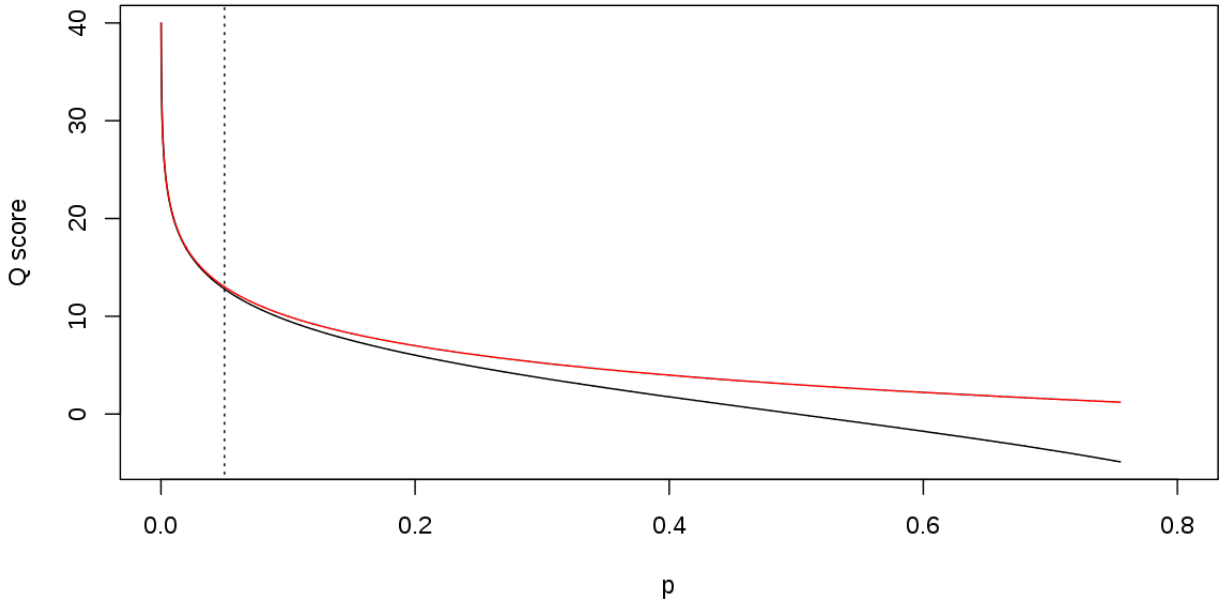


NGS file formats: Quality scores

Historically, two formats (now all are Sanger)

- $Q_{sanger} = -10 * \log_{10}(p)$
- $Q_{solexa} = -10 * \log_{10}(p / (1 - p))$

where p is the probability that a base call is incorrect



High quality scores are good

To calculate p from Q:

p = 10^(-Q / 10)

Q30 = 0.1% p[incorrect]
Q20 = 1% p[incorrect]
Q10 = 10% p[incorrect]

http://en.wikipedia.org/wiki/FASTQ_format

NGS file formats: Quality scores

```
 SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS.....................................................
 ..........................XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX.....................
 ..............................IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII.....................
 ..............................JJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJ.....................
 LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL.....................................................
 !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
 |                          |    |        |                                  |                     |
33                        59   64       73                                104                   126
 0........................26...31.......40
                          -5....0........9.............................40
                               0........9.............................40
                                  3.....9.............................40
 0.2......................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)
```

Fortunately, we seem to have settled on a standard in the community…for now!

http://en.wikipedia.org/wiki/FASTQ_format

Code break

Move into directory ~/data/Topic3

1) How many sequences so you have in the file Pine_reference_rnaseq_reduced.fa?

2) How many sequences do you have in the fastq file GBS12_brds_Pi_197A2_100k_R1.fastq?

3) How many sequences contain a base with a Phred score of 2 GBS12_brds_Pi_197A2_100k_R1.fastq?

Note: there are more unix examples at the end of README_quality_trimming.txt file

Preparing Fastq for analysis

1) Check files for completeness, use md5 checksums if file corruption is suspected

2) Inspect quality statistics

3) Possible steps to clean files (choice of steps depends on the application)

- De-multiplex
- Trim adapters
- Filter low quality base calls
- Remove duplicate sequences
- Remove contaminant sequences
- Remove sequences that are mainly adapter

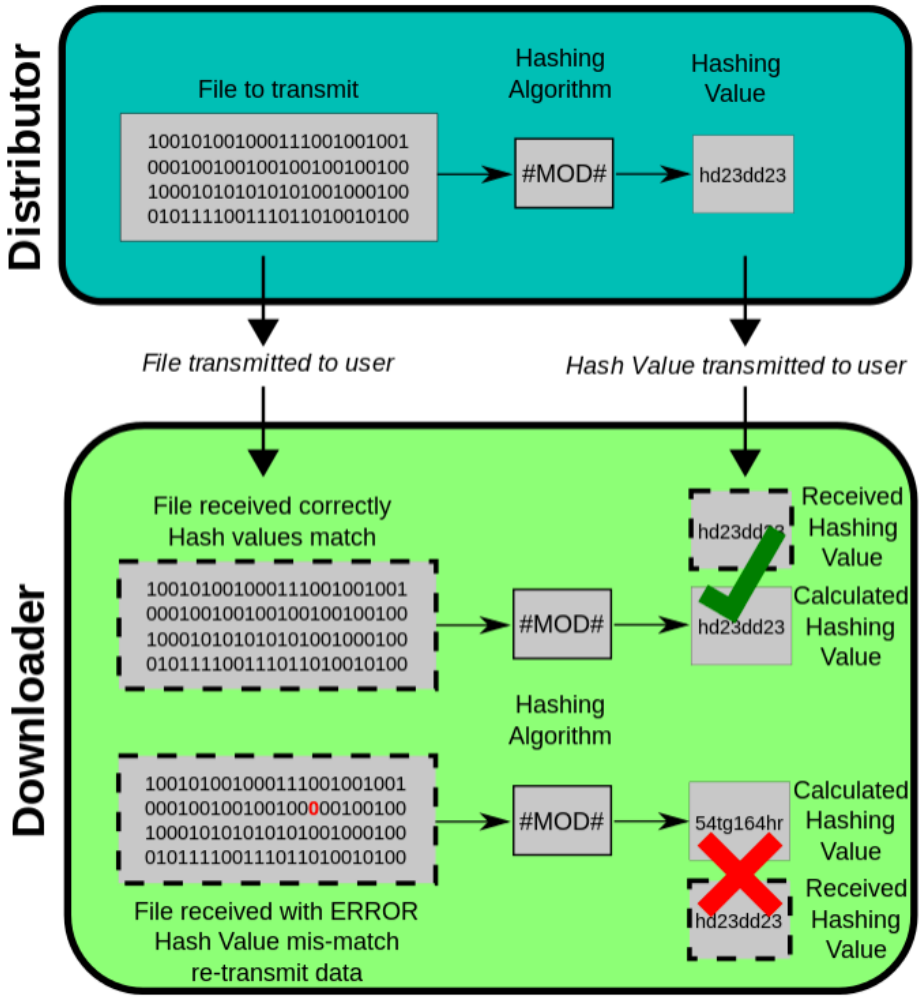
Usually done by sequencing center

Genotyping and RNAseq

Reference assembly

Many programs to implement these steps!

Preparing Fastq: md5 checksum



Preparing Fastq: Quality metrics

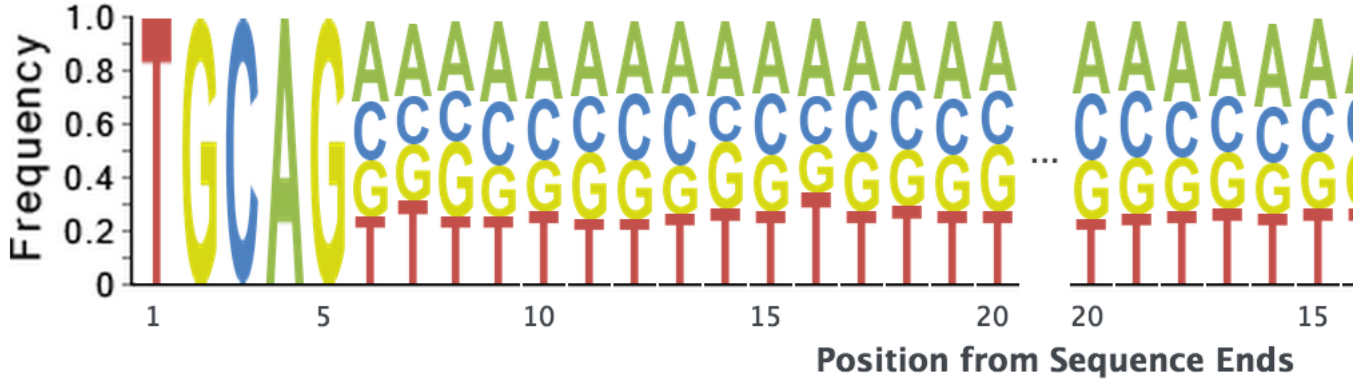
Many possible statistics to query:

- Number and length of sequences
- Base qualities
- Poly A/T tails
- Presence of tag sequences (stuff you added during preparation)
- Sequence complexity (e.g. ATATATATATATA…)

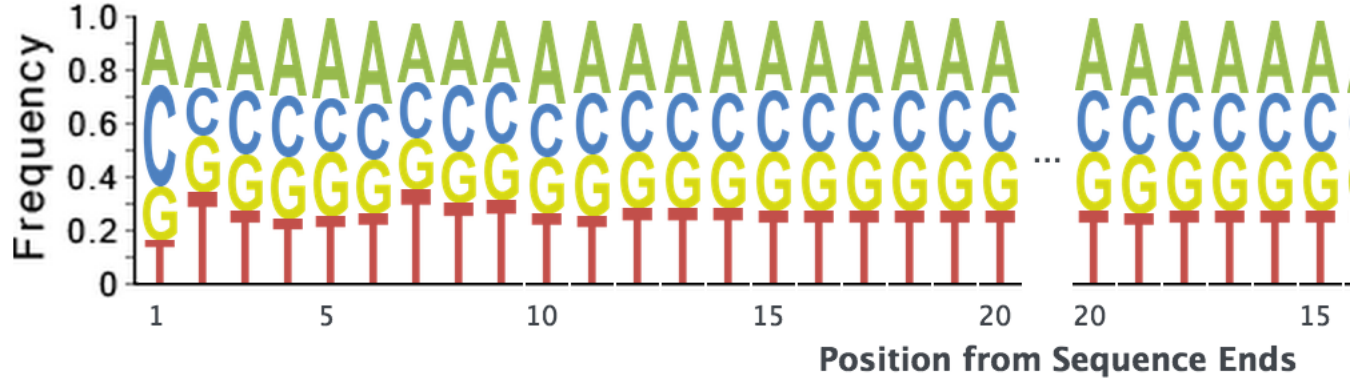
Recommended tools: prinseq, fastqc

Preparing Fastq: Quality metrics

Distribution of base frequencies in GBS reads with enzyme cut site:

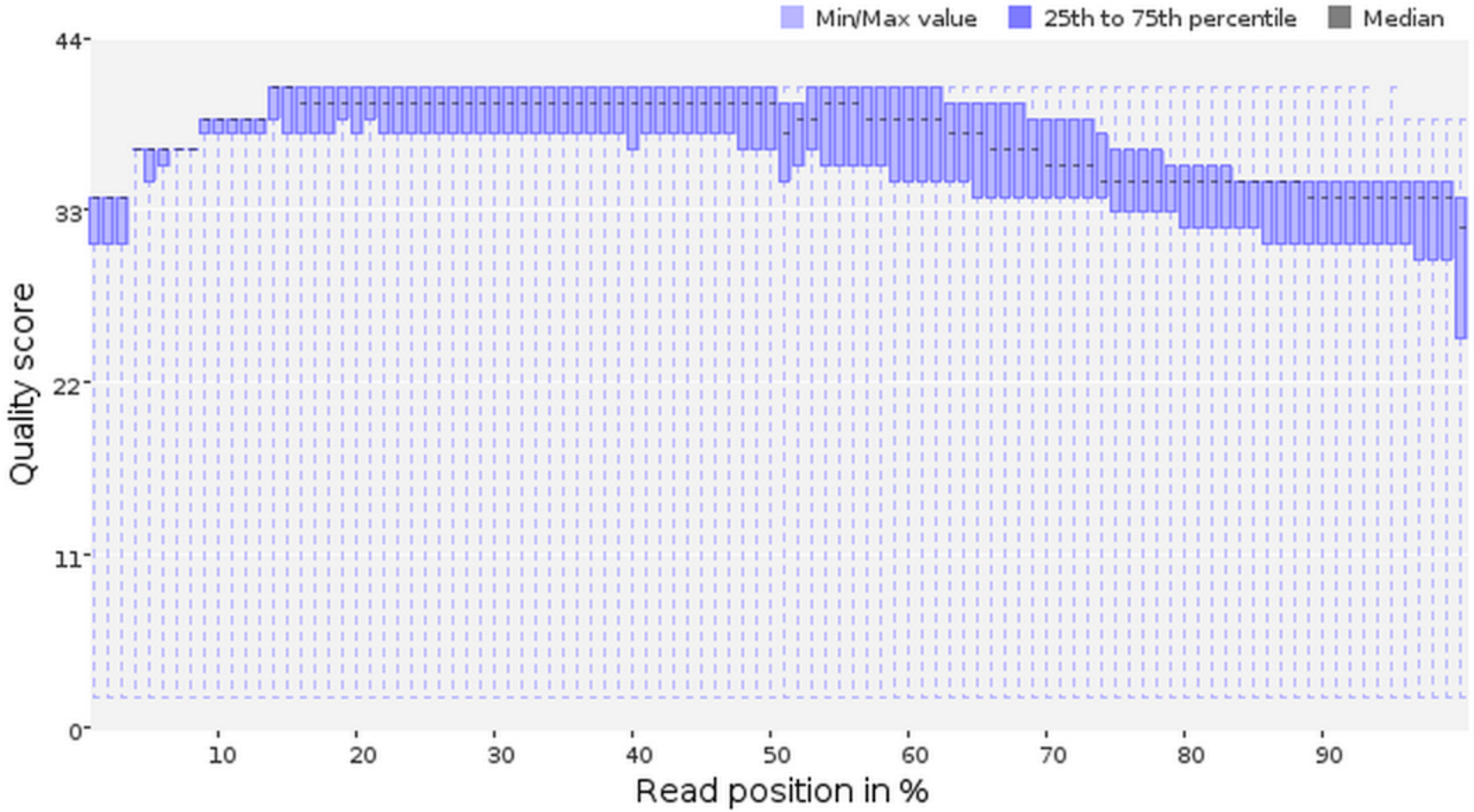


Distribution in RNAseq data, no adapters/tags:



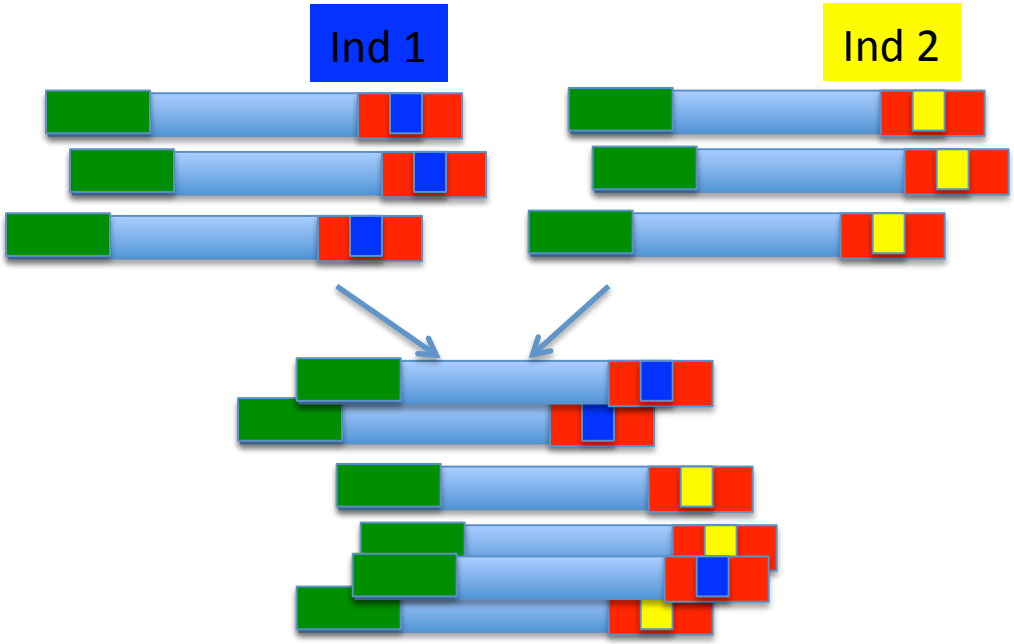
Preparing Fastq: Quality metrics

A normal quality score distribution for Illumina reads:



Preparing Fastq: De-multiplexing

Multiplexing is when several libraries are barcoded and sequenced on the same lane



- Most sequencing centers will de-multiplex the data
- Casava can be used for de-multiplexing and trimming barcodes from standard Illumina library preps

Preparing Fastq: Trimming

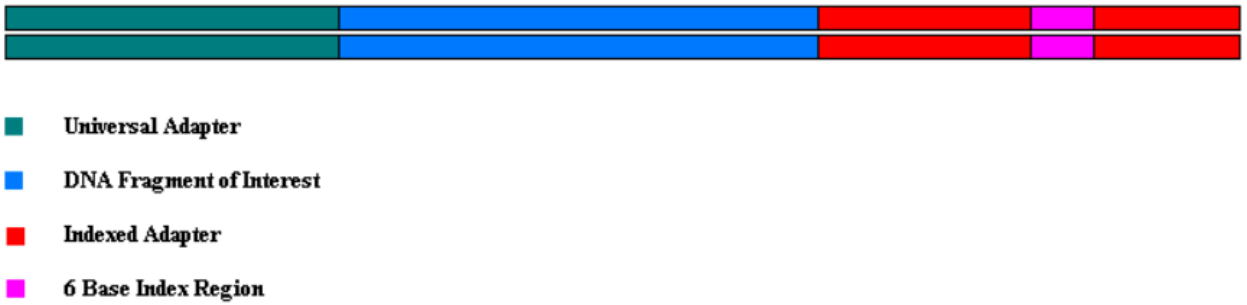
Table 1. Availability and characteristics of the trimming tools investigated in the current work.

Tool	Version	Link	Language	Algorithm family	Can work directly on gzip	Can work on paired end	PHRED format autodetection	Works on both read ends	Notes
Cutadapt	1.1	code.google.com/p/cutadapt/downloads/list	Python and C	Running sum	yes	no	no	no	Can also remove adapters, multi-threaded
ConDeTri	2.2	code.google.com/p/condetri/	Perl	Window based	yes (since v2.2)	yes	no	no	
ERNE-FILTER	1.2	sourceforge.net/projects/erne/files/	C++	Running sum	yes	yes	yes	yes	Can be combined with contaminant removal, multi-threaded
FASTX quality trimmer	0.0.13.2	hannonlab.cshl.edu/fastx_toolkit/download.html	C++	Window based	no	no	no	no	The default minimum read length parameter (-p) is set to zero
PRINSEQ	0:19:05	sourceforge.net/projects/prinseq/files/	Perl	Window based	no	no	no	yes	Also web interface for medium-size data
Trimmomatic	0.22	www.usadellab.org/cms/index.php?page=trimmomatic	Java	Window based	yes	yes	no	yes	Can also remove adapters
SolexaQA	1.13	sourceforge.net/projects/solexaqa/files/	Perl	Window based (Running sum with -bwa option)	no	no	yes	no	Cannot specify minimum read length to keep
Sickle	1.2	github.com/ucdavis-bioinformatics/sickle	C	Window based	yes	yes	no	yes	

doi: 10.1371/journal.pone.0085024.t001

Preparing Fastq: Trimming

- Adapters are short sequences that are added to the beginning and end of DNA molecules to prepare them for sequencing



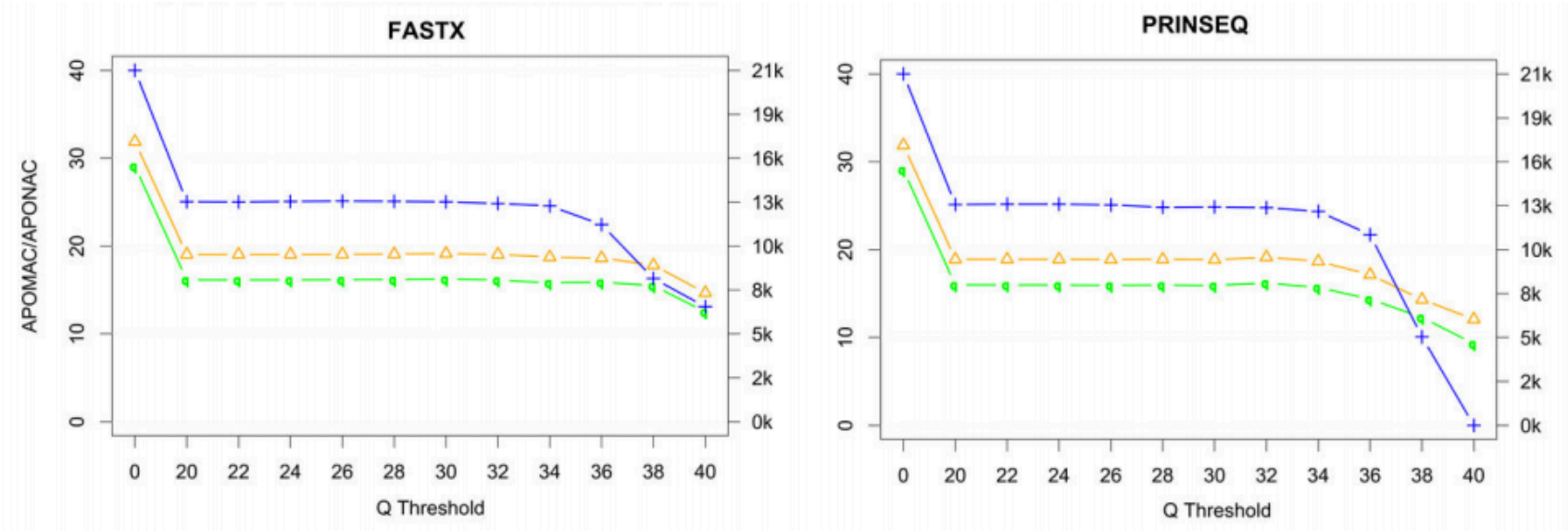
- Can compromise how well the reads align to a reference if not removed
- Detect during the quality control phase
- Removed by a range of tools (most sequencing centers will already have removed the adapters)

Preparing Fastq: Filtering

Choice of quality score to filter to depends upon the application:

- Too low a quality score cutoff:
  1) increase run times and RAM usage
  2) bad results (e.g. false SNP calls)
- Too high a quality score cutoff:
  1) faster run times
  2) lose useful data (e.g. more fragmented assemblies, missing SNPs)
- Usually Q20, but sometimes lower or higher

Preparing Fastq: Filtering



Preparing Fastq: Assembly

- Remove sequences consisting of adapter dimers (otherwise, they may be included as contigs). (e.g. tagdust)
- Clean out contaminants by blasting to known databases (can also be conducted post-assembly)
- Remove duplicate sequences: for *de novo* assembly, sequences that are exact copies will slow down the assembly without adding anything (e.g. fastx_collapser)

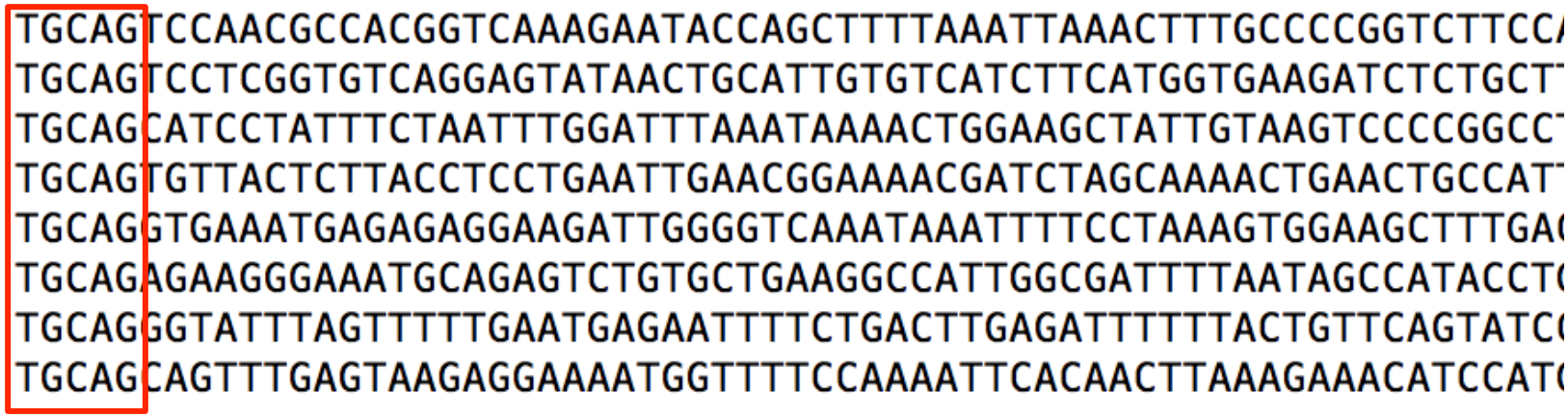
Preparing Fastq: Pairing

- With paired-end reads, if one read direction is removed but the other is not, then the _R1 and _R2 files are mismatched
- Need to run a script to eliminate unpaired reads from each _R1 and _R2 file

Some programs output reads in paired and unpaired files (e.g. prinseq, Trimmomatic). Others do not and custom scripts are required to re-pair data.

Preparing Fastq: GBS-specific filtering

- GBS / RAD use enzymes to cleave the DNA, so all reads will begin with the recognition sequence:



- Will need to de-multiplex using Stacks or custom script
- Clean GBS-specific adapters or other home-brew sequences that sequencing centers didn't remove

Further reading

- Del Fabbro et al. 2013. An Extensive Evaluation of Read Trimming Effects on Illumina NGS Data Analysis. PLoSOne. 8:e85024.

- http://prinseq.sourceforge.net/Data_preprocessing.pdf

- http://prinseq.sourceforge.net/manual.html#STANDALONE

Tutorial

- Navigate to the directory ~/data/Topic3
- Open the README file there, and follow the directions.

Questions:

1) Compare the two .html files for the initial filtering options. What kinds of differences do you see in the files? Why do you think these differences are found (think about the types of data you are analyzing)?

2) Try different filtering options for the GBS data (see http://prinseq.sourceforge.net/manual.html for options) and plot QC graphs. Discuss in a group of four which options you would choose to implement if this was your data. Be prepared to share your findings with the class.