QB#9468

FASTQ Processing Tools for Data Analysis

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TAS | 03/26/2020

Outline of the webinar

Overview of Illumina FASTQ generation

- Data analysis pipeline overview
- FASTQ format
- Demultiplexing and FASTQ generation

FASTQ tools

- Adapter trimming
- Quality trimming
- Read merging

Does my run look good vs does my FASTQ look good?

Illumina metrics vs third party QC tools

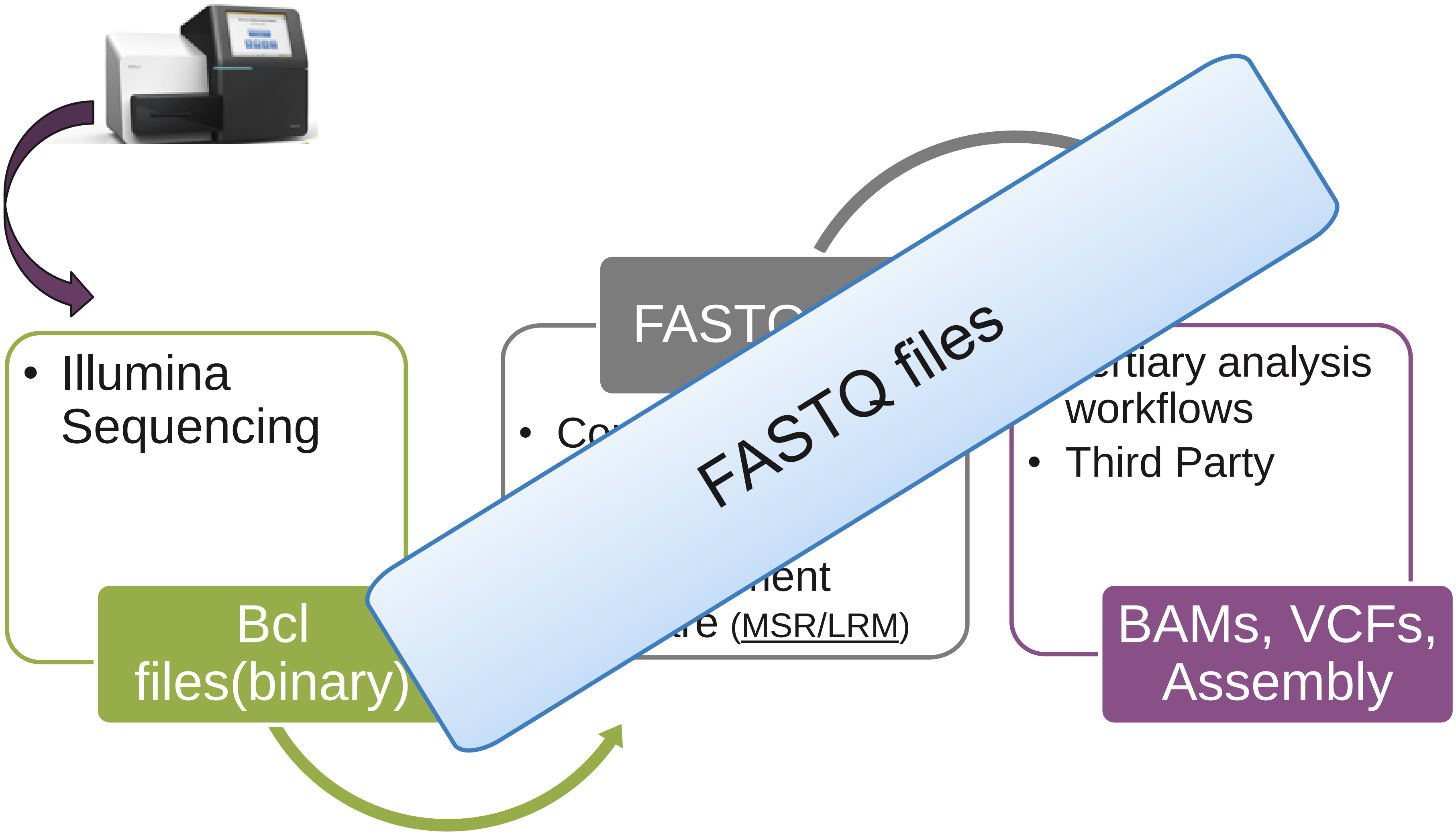
Intended audience

- **Users who are new to Illumina sequencing platforms**
  - End users who use a core facility to sequence their data and need some guidance on data optimization

- **Not intended for bioinformaticians**

Overview of Illumina FASTQ Generation

Pipeline for data analysis



- Illumina Sequencing

Bcl files(binary)

FASTC

- Co

ment

re (MSR/LRM)

rtiary analysis workflows
- Third Party

BAMs, VCFs, Assembly

FASTQ files

For Research Use Only. Not for use in diagnostic procedures.

BCL Files

- **BCL = Base CalL**
  - The “raw” data
  - Binary file containing base calls and quality scores for each tile for each cycle
  - Produced by on-instrument Real-Time Analysis Software (RTA)

FASTQ Files-format

- **Text file that contains the sequence data from clusters**

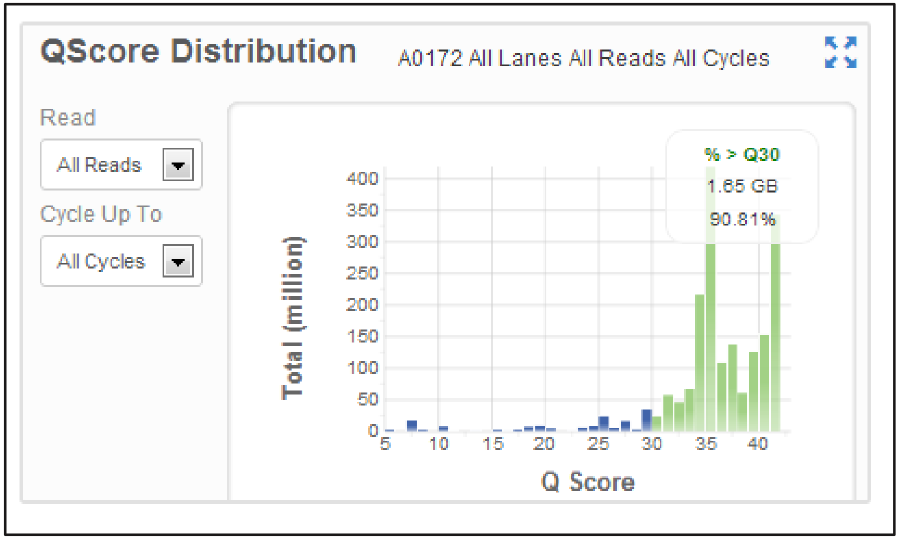
- **Each entry consists of 4 lines:**

1. Sequence identifier with information about the sequencing run and the cluster.
   i. Header format = @<instrument>:<run number>:<flowcell ID>:<lane>:<tile>:<xpos>:<y-pos>:<UMI> <read>:<is filtered>:<control number>:<index>
2. The sequence (the base calls; A, C, T, G and N).
3. A separator, which is simply a plus (+) sign.
4. The base call quality scores. Phred +33 encoded (ASCII)

```
@NB987655:1:ABCD12345:1:11401:6329:1045 1:N:0:ATCACG
TCGCACTCAACGCCCTGCATATGACAAGACAGAATC
+
<>;##=><9=AAAAAAAAAA9#:<#<;<<<????#=
```

Qscore – Quality Score

- The QScore is based on the Phred scale – Chance that a base call is incorrectly called
  - Q10: 10% chance of incorrect base call
  - Q20: 1% chance of incorrect base call
  - Q30: 0.1% chance of incorrect base call
  - Q40: 0.01% chance of incorrect base call
- Quality Scores for Next-Generation Sequencing



QScore Distribution A0172 All Lanes All Reads All Cycles

Read: All Reads

Cycle Up To: All Cycles

% > Q30
1.65 GB
90.81%

Symbol	ASCII Code	Q-Score
<	60	27
=	61	28
>	62	29
?	63	30
@	64	31
A	65	32
B	66	33
C	67	34
D	68	35
E	69	36
F	70	37

FASTQ Files- How to view

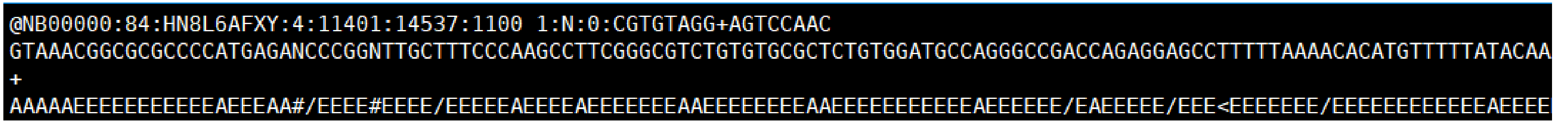
FASTQ files are large (file size) – best to not open these

How to view contents without opening?

- Use a terminal window on Linux or Mac
- zcat *.fastq.gz | less
- zcat *.fastq.gz | head

How to open a FASTQ file?

- gzipped
- 7-zip for windows to unzip
- Open using a notepad



```
@NB00000:84:HN8L6AFXY:4:11401:14537:1100 1:N:0:CGTGTAGG+AGTCCAAC
GTAAACGGCGCGCCCCATGAGANCCCGGNTTGCTTTCCCAAGCCTTCGGGCGTCTGTGTGCGCTCTGTGGATGCCAGGGCCGACCAGAGGAGCCTTTTTAAAACACATGTTTTTATACAA
+
AAAAAEEEEEEEEEEEAEEEAA#/EEEE#EEEE/EEEEEAEEEEAEEEEEEEAAEEEEEEEEAAEEEEEEEEEEEAEEEEEE/EAEEEEE/EEE<EEEEEEE/EEEEEEEEEEEEAEEEE
```

Anatomy of a FASTQ file

- **Naming**

  SampleName_SampleNumber_Lane_Read_FlowCellIndex.fastq.gz

  SampleName_S1_L001_R1_001.fastq.gz

  SampleName_S1_L001_R2_001.fastq.gz

- **e.g. NextSeq500/550, PE sequencing. 8 fastq files for each sample**

  SampleName_S1_L001_R1_001.fastq.gz

  SampleName_S1_L002_R1_001.fastq.gz

  SampleName_S1_L003_R1_001.fastq.gz

  SampleName_S1_L004_R1_001.fastq.gz

  SampleName_S1_L001_R2_001.fastq.gz

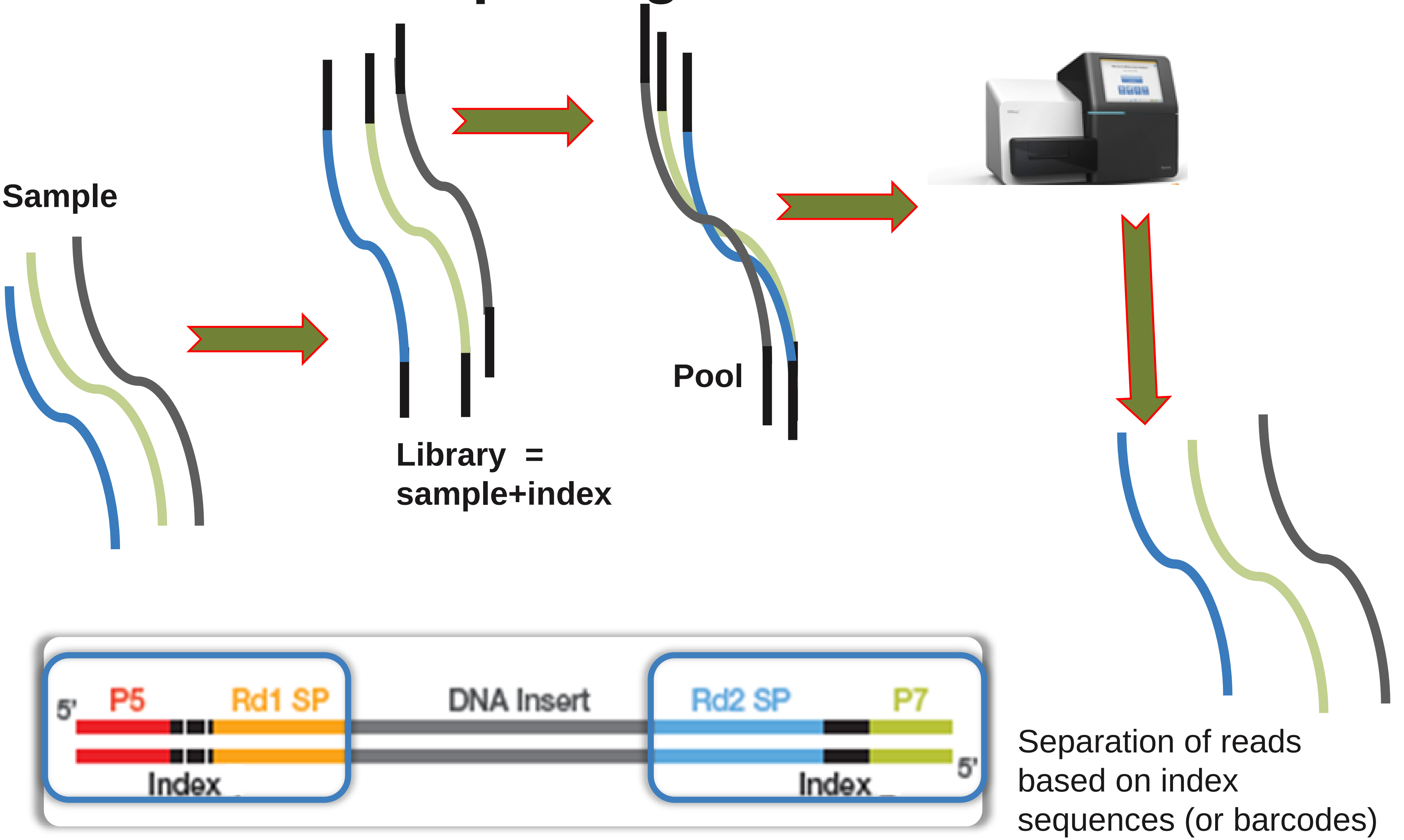
  SampleName_S1_L002_R2_001.fastq.gz

  SampleName_S1_L003_R2_001.fastq.gz

  SampleName_S1_L004_R2_001.fastq.gz

Demultiplexing and FASTQ generation

What is demultiplexing?



Sample

Library = sample+index

Pool

Separation of reads based on index sequences (or barcodes) using bioinformatics

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Illumina Tools for Demultiplexing and Fastq Generation

On-Board

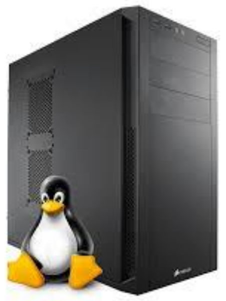
MiSeq™ Reporter

Local Run Manager

Cloud based

BaseSpace™ Sequence Hub (BSSH)

Linux based



Bcl2fastq

DRAGEN ™ bclconvert

Supports All Illumina Sequencing Systems

Bcl2fastq v2 is required for platforms using RTA 2 & 3.

Note: For sequencing systems running an earlier software version of Real-Time Analysis (RTA) than v1.18.54, use bcl2fastq v1.8.4.

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What is bcl2fastq v2?

- **Linux-based software to convert BCLs → FASTQs**
- **Optionally performs demultiplexing per sample sheet (created with IEM) instructions**
- **Command line example**

```
/usr/local/bin/bcl2fastq --runfolder-dir . --output-directory
./Data/Intensities/BaseCalls/  --input-dir
./Data/Intesities/Basecalls  --sample-sheet ./SampleSheet.csv
```

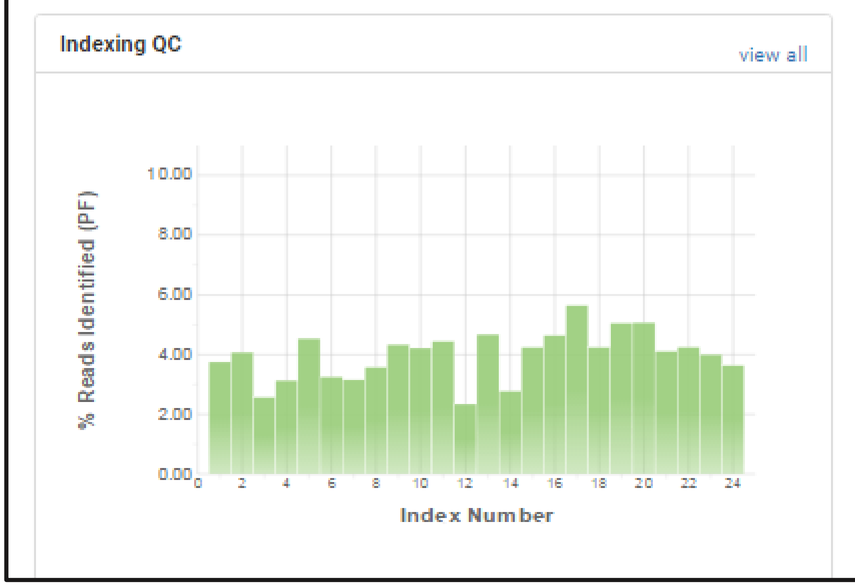
- **Command line configurable options**

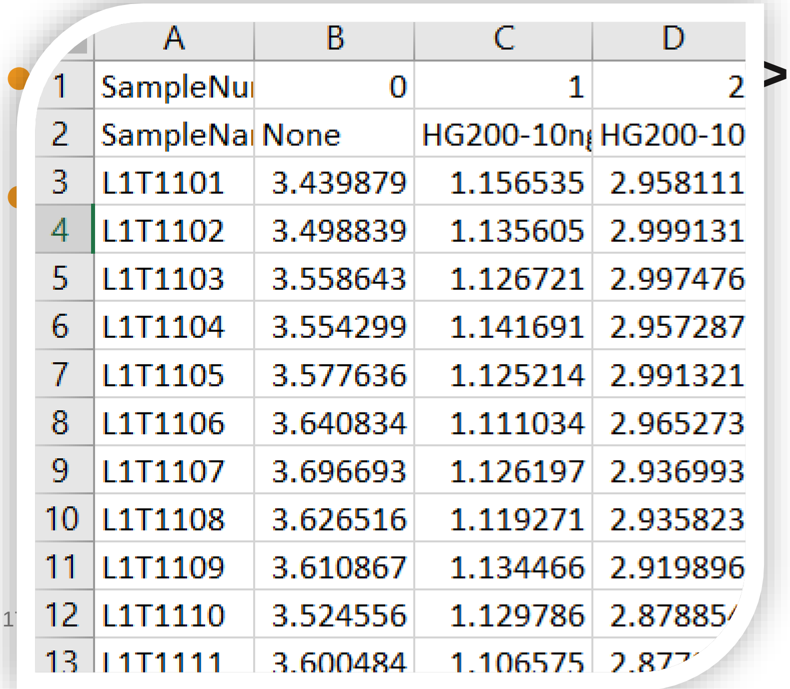
  E.g. `--no-lane-splitting`

  - SampleName_S1_R1_001.fastq.gz
  - SampleName_S1_R1_001.fastq.gz

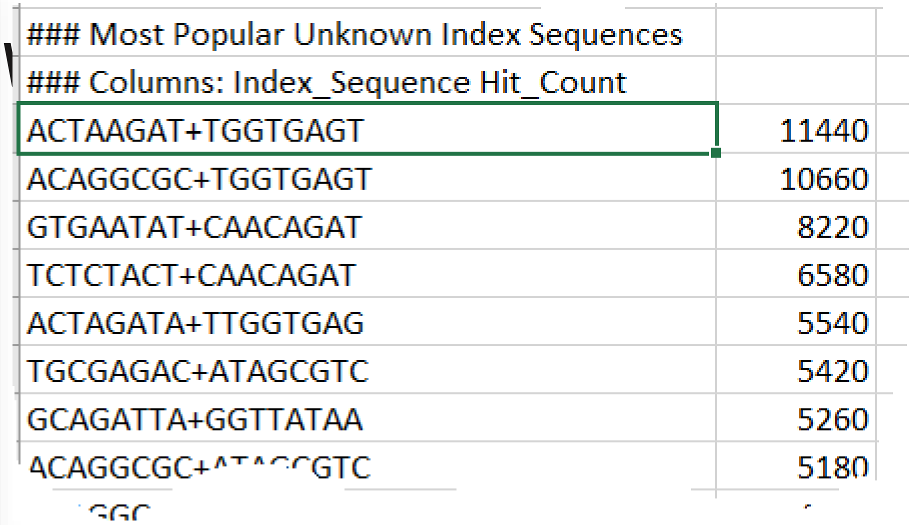
Demultiplexing reports

- **IndexQC on SAV**
- **DemultiplexSummaryF1L#.txt**
  - Helps troubleshoot sample dropouts
- **Depending on what software is used for demultiplexing, DemultiplexSummaryF1L1.txt location will vary**





	A	B	C	D
1	SampleNu	0	1	2
2	SampleNa	None	HG200-10n	HG200-10
3	L1T1101	3.439879	1.156535	2.958111
4	L1T1102	3.498839	1.135605	2.999131
5	L1T1103	3.558643	1.126721	2.997476
6	L1T1104	3.554299	1.141691	2.957287
7	L1T1105	3.577636	1.125214	2.991321
8	L1T1106	3.640834	1.111034	2.965273
9	L1T1107	3.696693	1.126197	2.936993
10	L1T1108	3.626516	1.119271	2.935823
11	L1T1109	3.610867	1.134466	2.919896
12	L1T1110	3.524556	1.129786	2.87885
13	L1T1111	3.600484	1.106575	2.87



### Most Popular Unknown Index Sequences	
### Columns: Index_Sequence Hit_Count	
ACTAAGAT+TGGTGAGT	11440
ACAGGCGC+TGGTGAGT	10660
GTGAATAT+CAACAGAT	8220
TCTCTACT+CAACAGAT	6580
ACTAGATA+TTGGTGAG	5540
TGCGAGAC+ATAGCGTC	5420
GCAGATTA+GGTTATAA	5260
ACAGGCGC+ATAGCGTC	5180

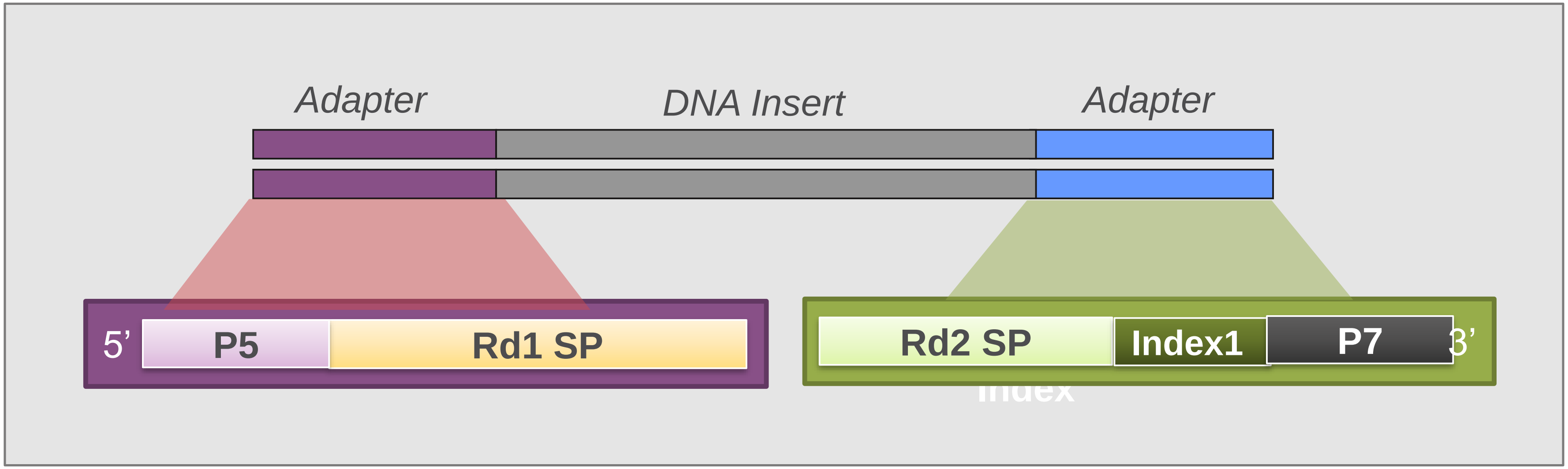
Tools: Adapter Trimming

Illumina library design

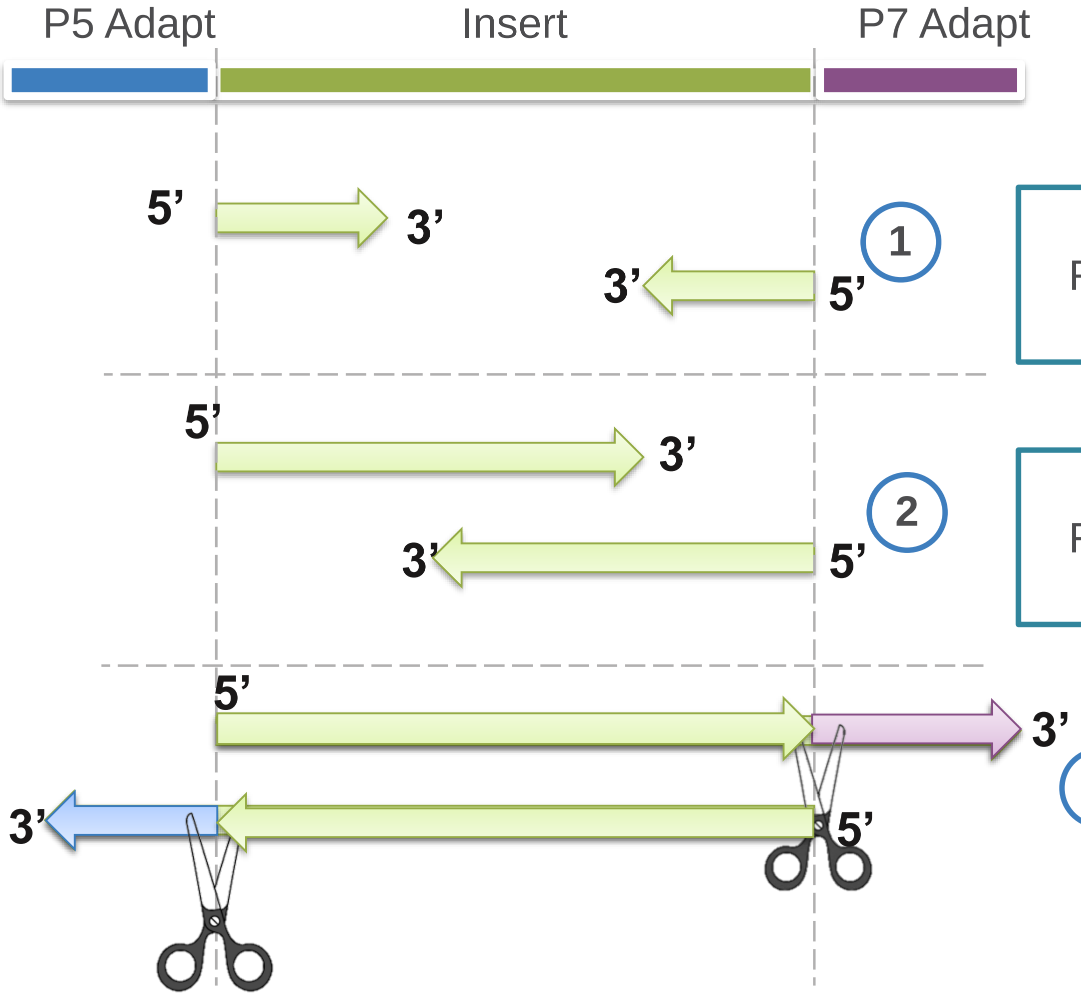
○ Library – DNA insert plus full adapter

○ Read 1 Sequencing Primer (Rd1 SP)

○ Read 2 Sequencing Primer (Rd2 SP)



Adapter trimming overview
PE 151



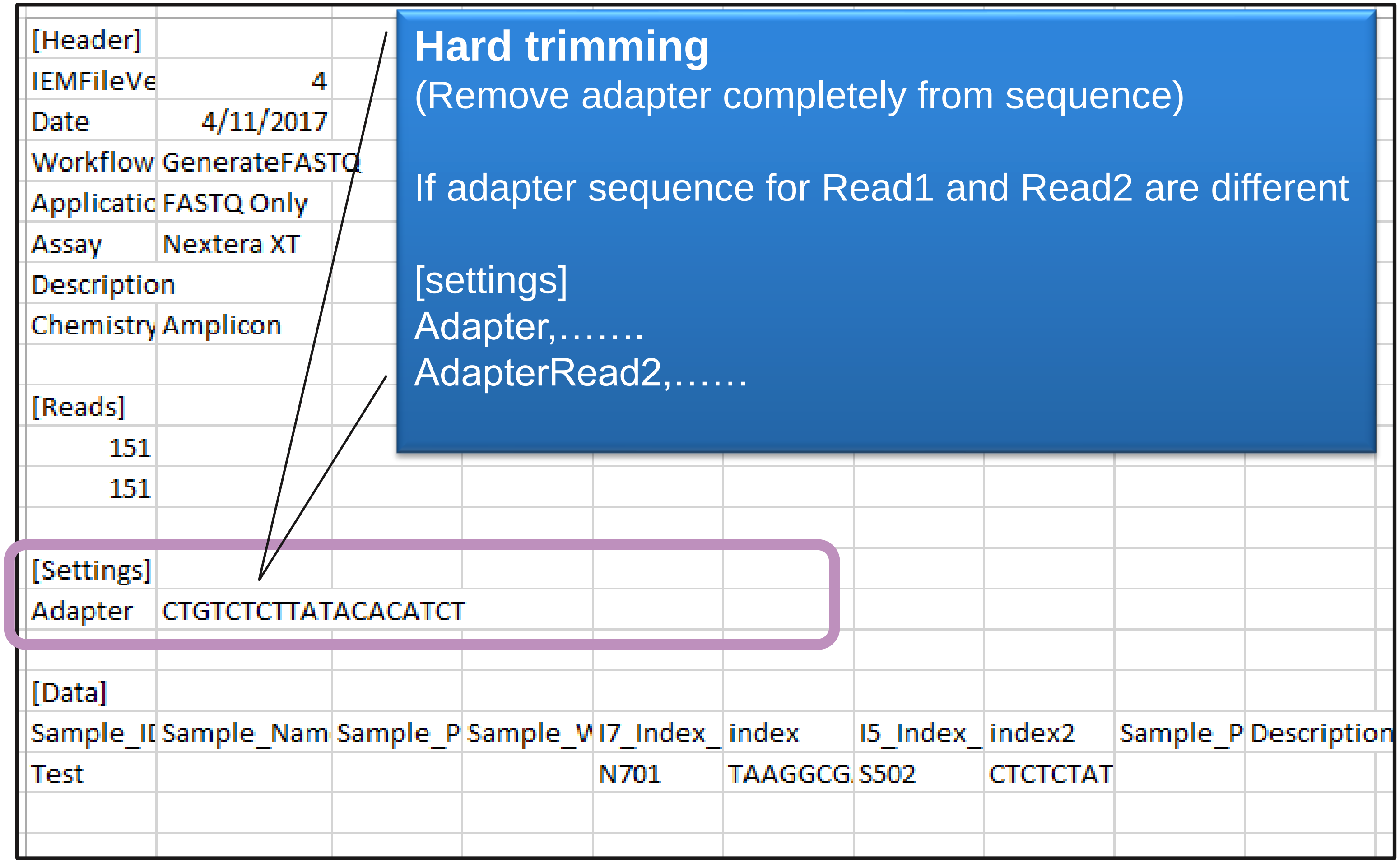
Insert size = 1000bp
Read length shorter than insert

Insert size =200bp
Read length shorter than insert (pairs overlap)

Insert size =100bp
Read length longer than insert

Adapter trimming options

MiSeq™ Reporter, Local Run Manager, BaseSpace™ Fastq Generation and Bcl2fastq



[Header]									
IEMFileVe	4								
Date	4/11/2017								
Workflow	GenerateFASTQ								
Applicatic	FASTQ Only								
Assay	Nextera XT								
Description									
Chemistry	Amplicon								
[Reads]									
151									
151									
[Settings]									
Adapter	CTGTCTCTTATACACATCT								
[Data]									
Sample_II	Sample_Nam	Sample_P	Sample_W	I7_Index_	index	I5_Index_	index2	Sample_P	Description
Test				N701	TAAGGCG.	S502	CTCTCTAT		

Hard trimming

(Remove adapter completely from sequence)

If adapter sequence for Read1 and Read2 are different

[settings]
Adapter,…….
AdapterRead2,……

If runs are setup using PrepTab for NextSeq / MiniSeq, adapter trimming is on by default.

https://support.Illumina.com/bulletins/2016/12/what-sequences-do-i-use-for-adapter-trimming.html

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Adapter trimming example (hard trimming)

Adapter match > 90% (default)

```
@M00000:71:000000000-D00LW:1:1101:16265:1658 1:N:0:1
ACTCTGCGTTGCGCTTCTGCTCGGCCTCCAGCTCACCCTCCCTGTCTCTTATACACATCTCCGAGCCCA
+
BCCCCFFCCBCCGGGGGGGGGGGGGGGHHHHHHHHHHHHHGGHHHHHHHHHHHHHHHHHHHHHGGGGGH
```

```
@M00000:71:000000000-D00LW:1:1101:16265:1658 1:N:0:1
ACTCTGCGTTGCGCTTCTGCTCGGCCTCCAGCTCACCCTCC
+
BCCCCFFCCBCCGGGGGGGGGGGGGGGHHHHHHHHHHHHHG
```

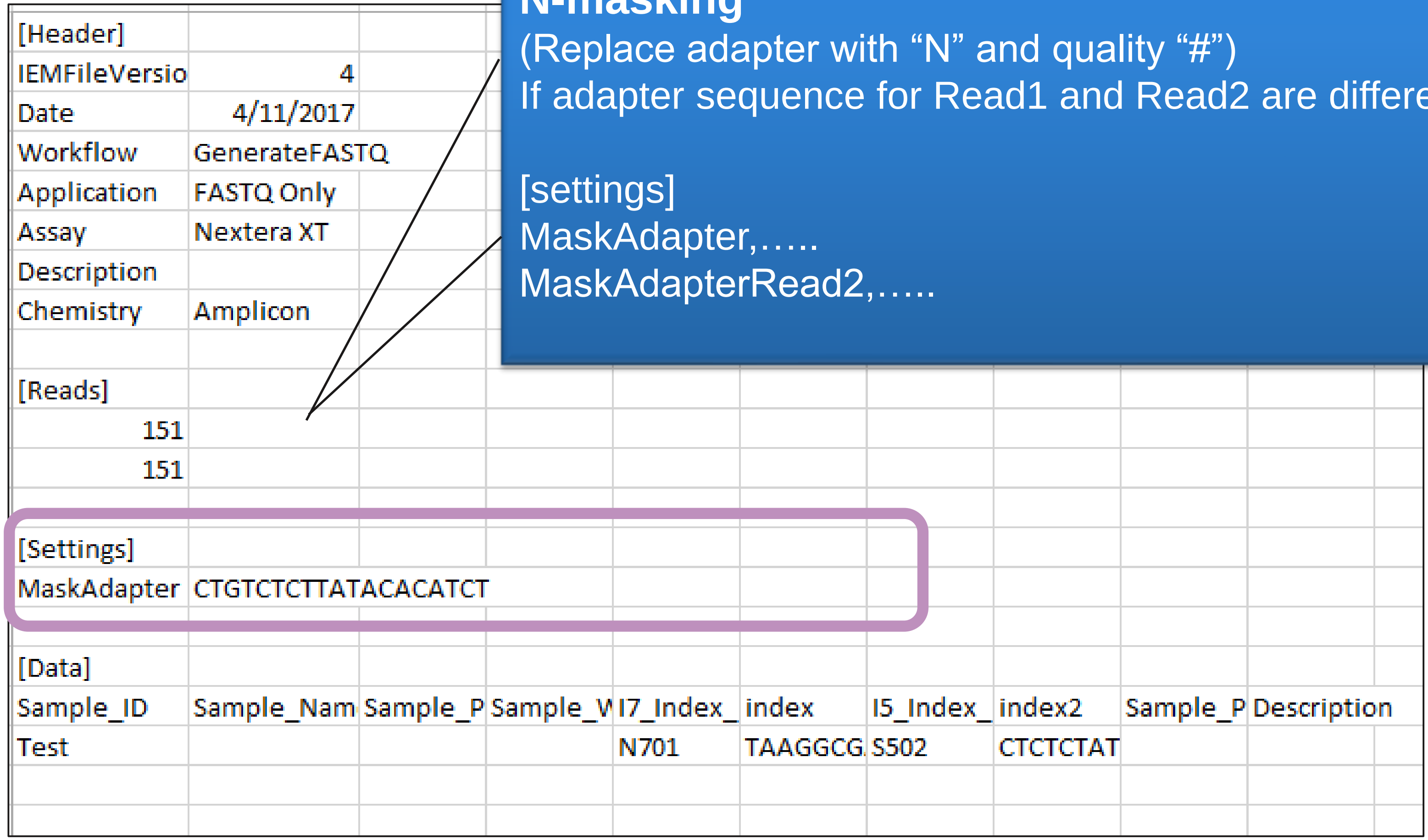
Bases trimmed (removed) from the start of the Adapter

Adapter masking options

Bcl2fastq

N-masking
(Replace adapter with “N” and quality “#”)
If adapter sequence for Read1 and Read2 are different

[settings]
MaskAdapter,…..
MaskAdapterRead2,…..



[Header]									
IEMFileVersio	4								
Date	4/11/2017								
Workflow	GenerateFASTQ								
Application	FASTQ Only								
Assay	Nextera XT								
Description									
Chemistry	Amplicon								
[Reads]									
151									
151									
[Settings]									
MaskAdapter	CTGTCTCTTATACACATCT								
[Data]									
Sample_ID	Sample_Nam	Sample_P	Sample_W	I7_Index_	index	I5_Index_	index2	Sample_P	Description
Test				N701	TAAGGCG	S502	CTCTCTAT		

Adapter masking example

```
@M00000:71:000000000-D00LW:1:1101:16265:1658 1:N:0:1
ACTCTGCGTTGCGCTTCTGCTCGGCCTCCAGCTCACCCTCCCTGTCTCTTATACACATCTCCGAGCCCA
+
BCCCCFFCCBCCGGGGGGGGGGGGGGGHHHHHHHHHHHHHGGHHHHHHHHHHHHHHHHHHHHHGGGGGH
```

```
@M00000:72:000000000-D00LW:1:1101:16265:1658 1:N:0:1
ACTCTGCGTTGCGCTTCTGCTCGGCCTCCAGCTCACCCTCCNNNNNNNNNNNNNNNNNNNNNNNNNNNN
+
BCCCCFFCCBCCGGGGGGGGGGGGGGGHHHHHHHHHHHHHG############################
```

Bases masked to “N” and quality score to 2 “#” from the start of the adapter
(Useful if analysis program requires reads to be same read length)

illumina®

Masking short adapters – bcl2fastq

```
@M00000:71:000000000-D00LW:1:1101:16265:1658 1:N:0:1
ACCTGTCTCTTATACACATCTCCGAGTCTGCGTTGCGCTTCTGCTCGGCCTCCAGCTCACCCTCCCCCA
+
BCCCCFFCCBCCGGGGGGGGGGGGGGGHHHHHHHHHHHHHGGHHHHHHHHHHHHHHHHHHHHGGGGGH
```

```
@M00000:72:000000000-D00LW:1:1101:16265:1658 1:N:0:1
NNNNNNNNNNNNNNNNNNNNNNNNNNNN
+
############################
```

--minimum-trimmed-read-length 35
--mask-short-adapter-reads 22

Entire read is converted to 35bp Ns and quality score to "#" from the start of the sequence

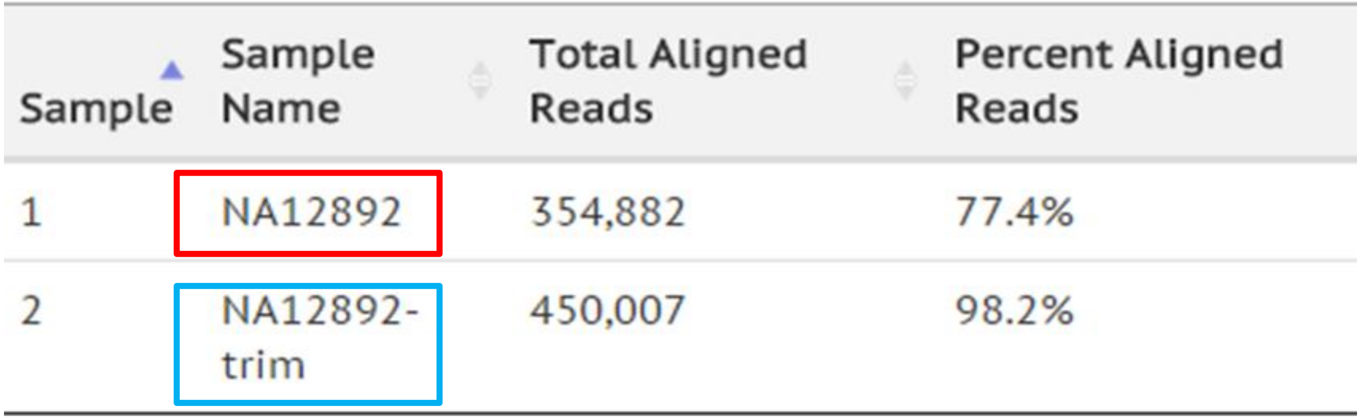
- Useful if adapter dimers in the sample
- Prevents generation of empty reads

Why adapter trim?

BWA
Enrichment
V2.1

1 Higher alignment %

BWA
(backtrace)



Sample	Sample Name	Total Aligned Reads	Percent Aligned Reads
1	NA12892	354,882	77.4%
2	NA12892-trim	450,007	98.2%

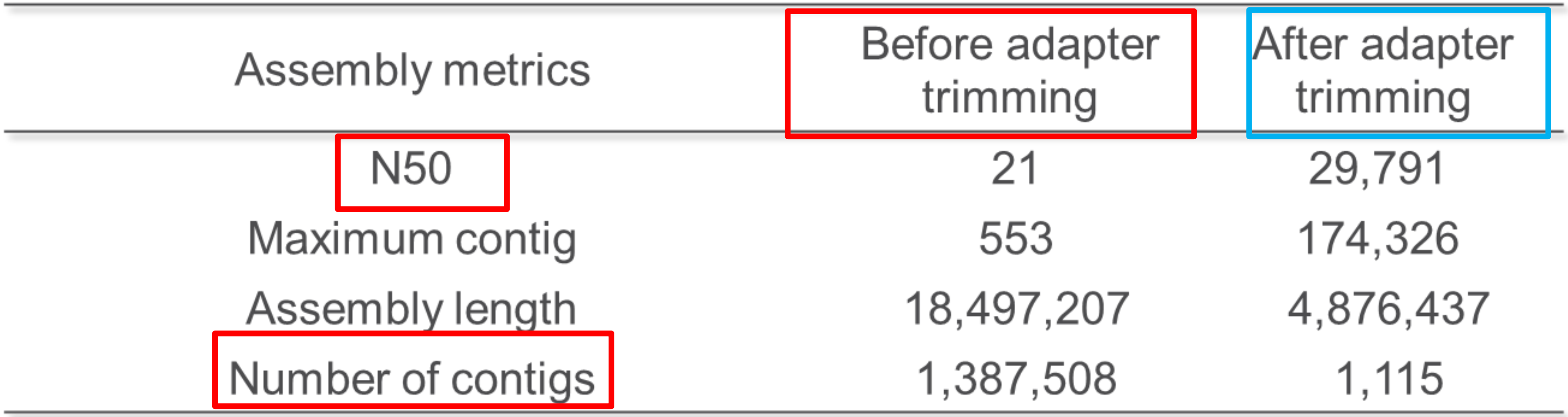
Why adapter trim?

Velvet de novo Assembly
BASESPACE LABS

2

Improved assemblies

Data: 2 x 250bp, *E.coli* (Nextera™ XT)



Assembly metrics	Before adapter trimming	After adapter trimming
N50	21	29,791
Maximum contig	553	174,326
Assembly length	18,497,207	4,876,437
Number of contigs	1,387,508	1,115

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Tools: Quality trimming

Quality trimming

- **Filter the end of reads based on read end average quality**
- **When to trim?**
  - Where algorithms sensitive to quality – De Novo assembly, merging reads, Metagenomics (using 16S rRNA gene for classification)
- **When not to trim?**
  - Resequencing. Most aligners take quality scores into account (i.e. BWA, Isaac) will soft clip the ends of reads if low quality
- **FASTQ toolkit app on BSSH (BaseSpace Sequence Hub) can be used to perform quality trimming**

Quality score trimming example

QualityScoreTrim,20

```
@M00000:72:000000000-D00LW:1:1101:22420:18334 1:N:0:1
CACCAAGGGCCTGGGGTGTCAATGGCGGGGCTTGTGACTGCACAAAAGGGGCCTCCCGCAGGGGCTCCCGCC
+
BBBBBBFBBBBBGGGGEEFGGGHHHHGGG00>A0B355@BB3@3BGB?E1///1/11//////////?////
```

```
@M00000:72:000000000-D00LW:1:1101:22420:18334 1:N:0:1
CACCAAGGGCCTGGGGTGTCAATGGCGGGGCTTGTGACTGCACAAAAGG
+
BBBBBBFBBBBBGGGGEEFGGGHHHHGGG00>A0B355@BB3@3BGB?E
```

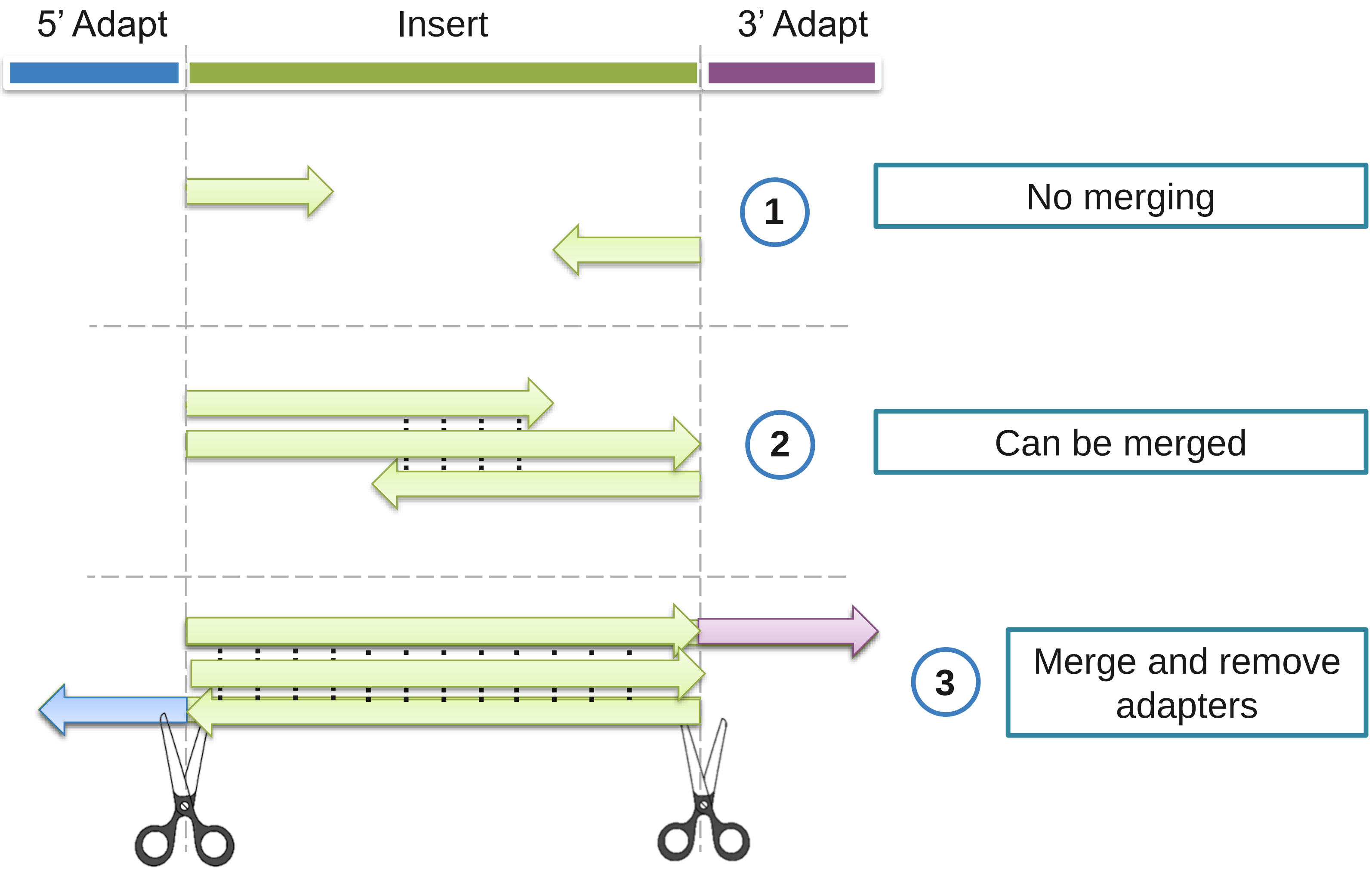
Q	ASC
13	.
14	/
15	0
16	1
18	3
20	5
22	7
25	9
30	?
31	@
32	A
33	B

Tools: Read merging

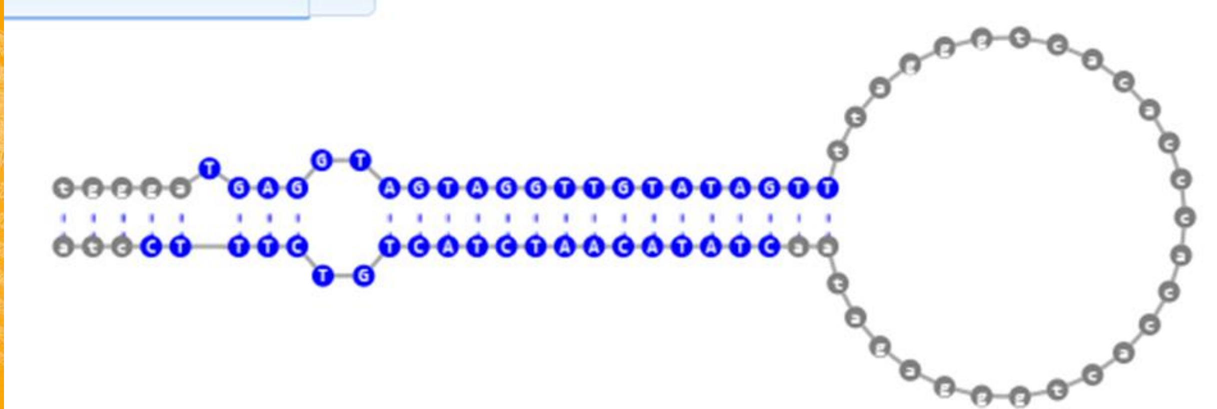
Read merging

- **Combines paired end read into a single read**
- **Generally requires substantially high overlap**
  - StitchReads,1 option in MiSeq™ Reporter
  - Workflows = Amplicon – DS, GenerateFASTQ workflow, TruSeq™ Amplicon
  - 10 bp overlap
  - Not supported in any other software
- **When to merge?**
  - Where read continuity is important
  - Improve indel detection in some cases (i.e. where Indel is in overlap region)
  - To use tools that only take single end reads (for example some metagenomics software)
  - Where majority of reads overlap
- **When not to merge?**
  - When some proportion of reads do not overlap
  - Where there are simple repeats in overlap region (i.e. sequencing amplicons with expansion repeats)
  - Most downstream aligners will handle unmerged reads

Read merging



Data optimization with an example:
Using FASTQ toolkit app for Small RNA workflow



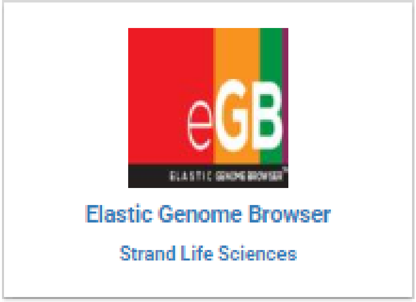
BaseSpace™

- BaseSpace™ is the Illumina cloud-based genomics computing environment for next-generation sequencing (NGS) data management and analysis.
- Is used to analyze or manipulate samples – results stored in the project. There are currently about 80+ apps

Core App

BaseSpace Lab App

Third party App



Please Read:
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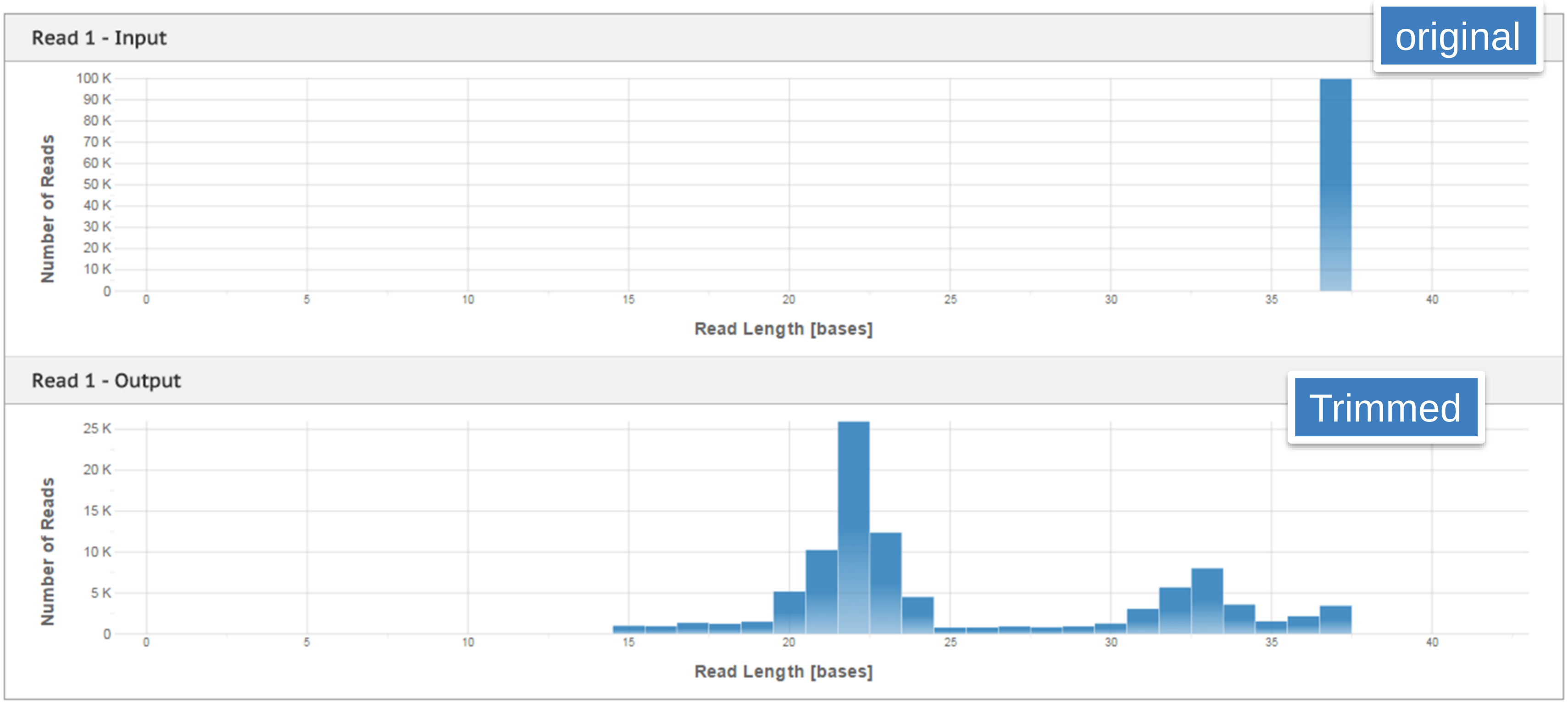
Small RNA sample prep kit

Small RNA

Illumina, Inc.

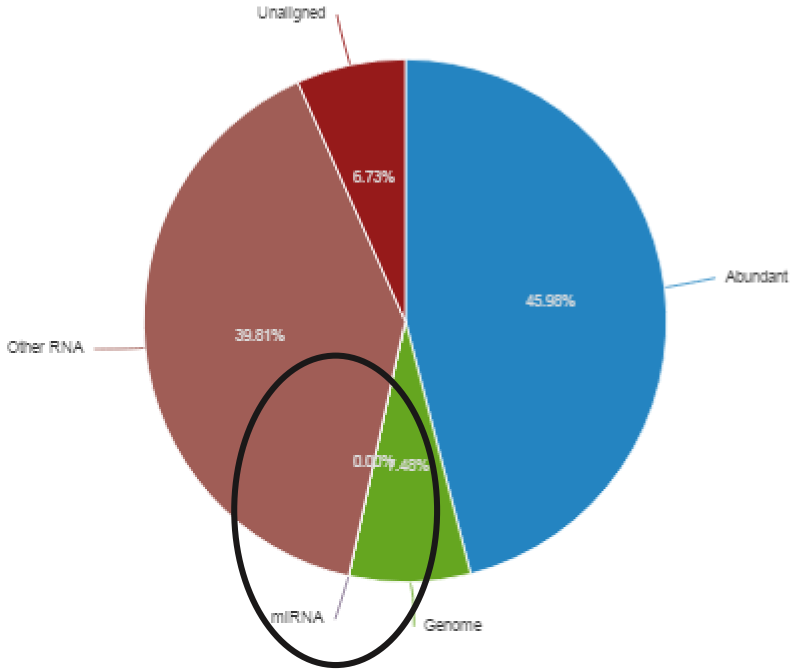
- **Human small RNA peaks at ~22bp**
- **Typical run setup is Single End = 36 to 50cycles. So reads have to be adapter trimmed**
- **Bcl2fastq**: --minimum-trimmed-read-length 20 and --mask-short-adapter-reads 20
- **Recommendation: For workflow GenerateFASTQ – do not turn on adapter trimming**
- **Generate FASTQs with 50bp and trim downstream using 3rd party tools or BSSH FASTQ toolkit**

FASTQ toolkit output- Results

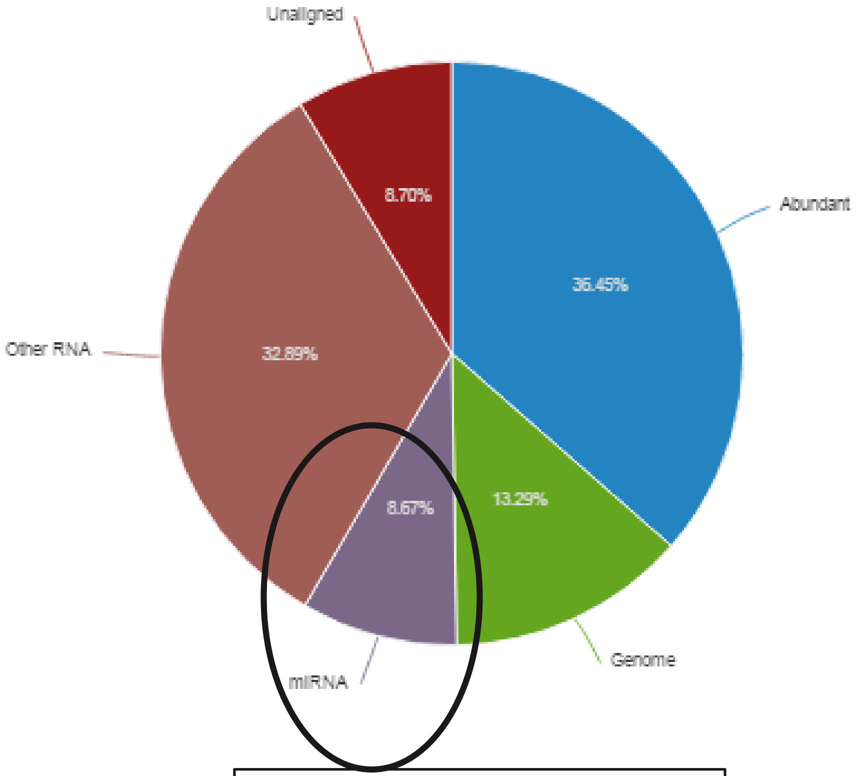


Small RNA app output

Small RNA
Illumina, Inc.



Untrimmed FASTQ

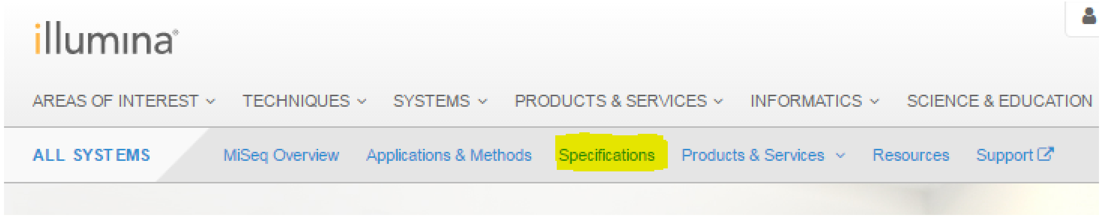


Trimmed FASTQ

Does my run look good?
vs
Does my FASTQ look good?

Previous Webinar -Sequence Analysis Viewer (SAV): A Beginner's Guide

Metrics



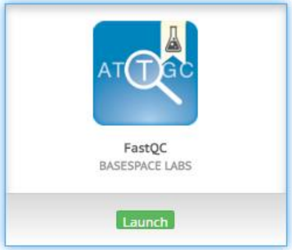
Illumina

- Based on Sequencing Analysis Viewer – taking a whole run into consideration
- Yield, Q30, Reads passing filter
- Used for assessing run quality – instrument performance
- Illumina.com > Systems > MiSeq™ > Specifications

3rd party tools *Eg.,* FASTQC

- Based on individual FASTQ file
- Sample dependent and hence library kit
- Used for assessing sample performance – primarily for troubleshooting individual sample

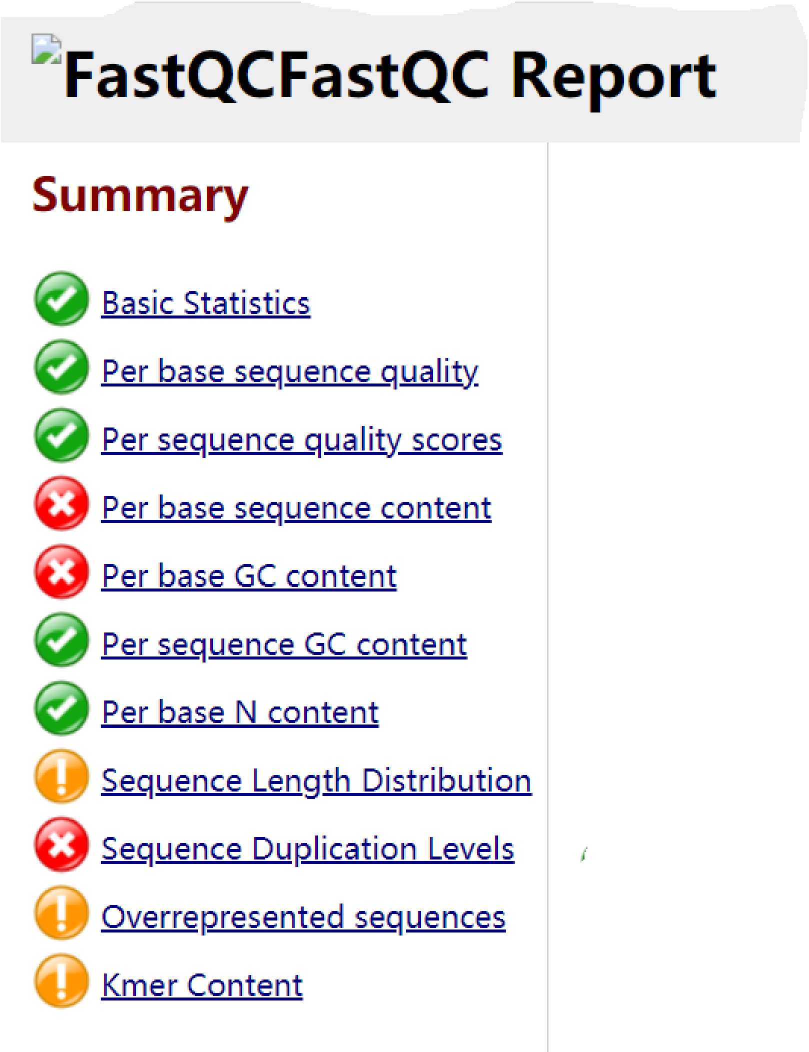
Does my FASTQ look good?



- FASTQC app quick QC step to assess sample quality - subsamples from read pool
- FASTQC tool helps understand the nature of your library
- Not all graphs are meaningful - Understand documentation
- Failure of QC plots in the app does not translate to poor data quality – understand your data
- Sometimes it needs more in-depth downstream data analysis

✓ ! ✗

What FastQC report looks like

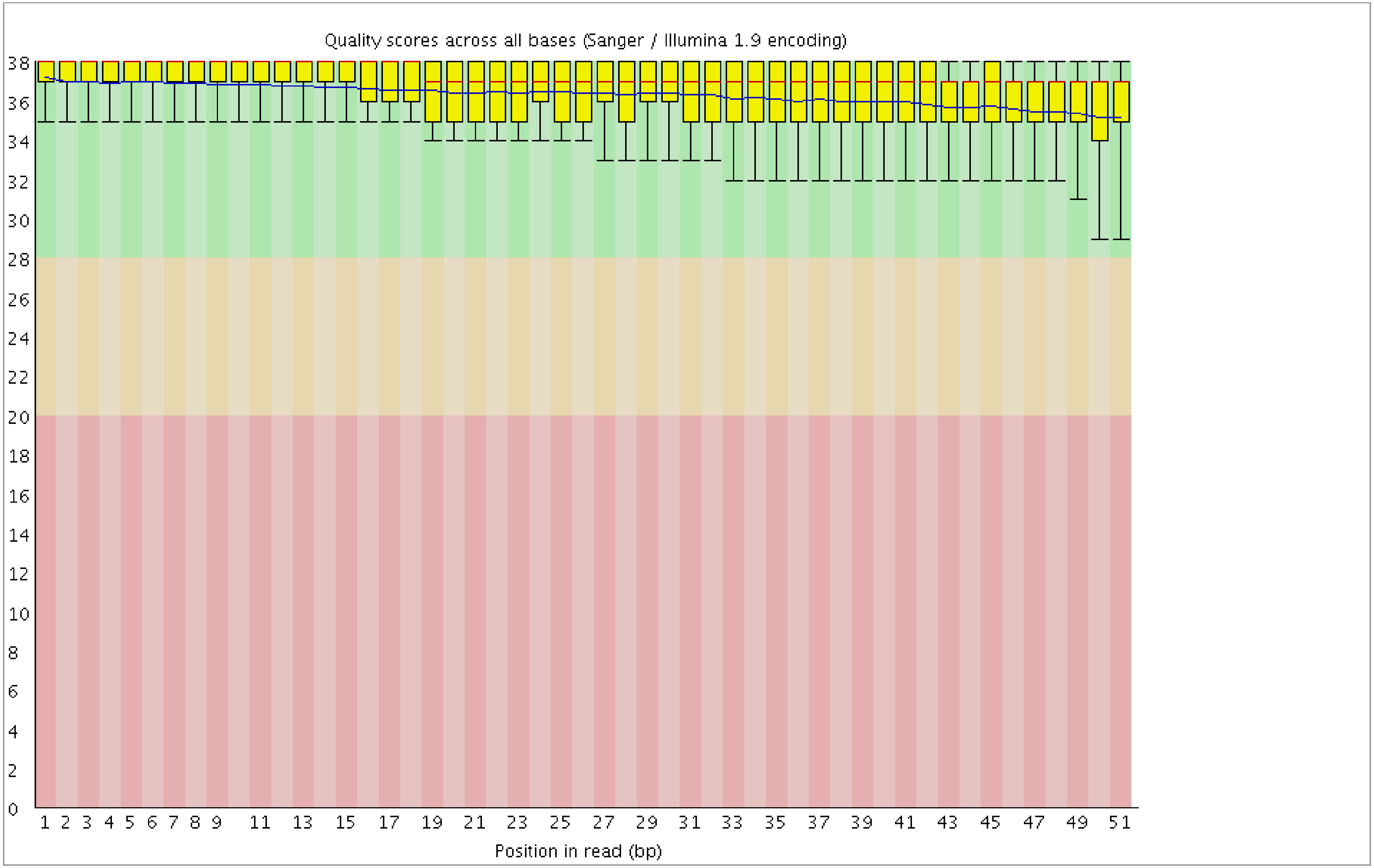


FastQCFastQC Report

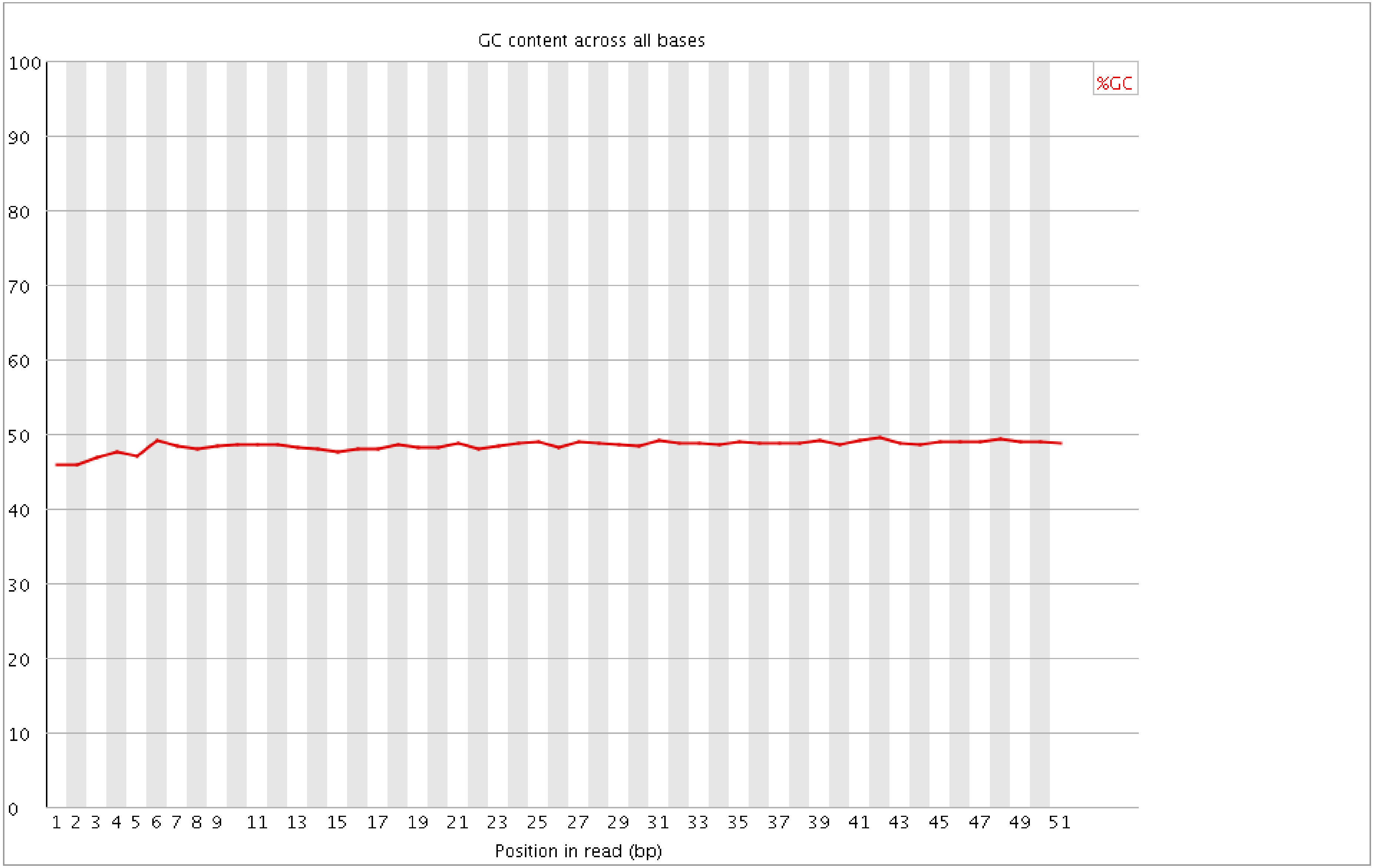
Summary

- ✓ Basic Statistics
- ✓ Per base sequence quality
- ✓ Per sequence quality scores
- ✗ Per base sequence content
- ✗ Per base GC content
- ✓ Per sequence GC content
- ✓ Per base N content
- ! Sequence Length Distribution
- ✗ Sequence Duplication Levels
- ! Overrepresented sequences
- ! Kmer Content

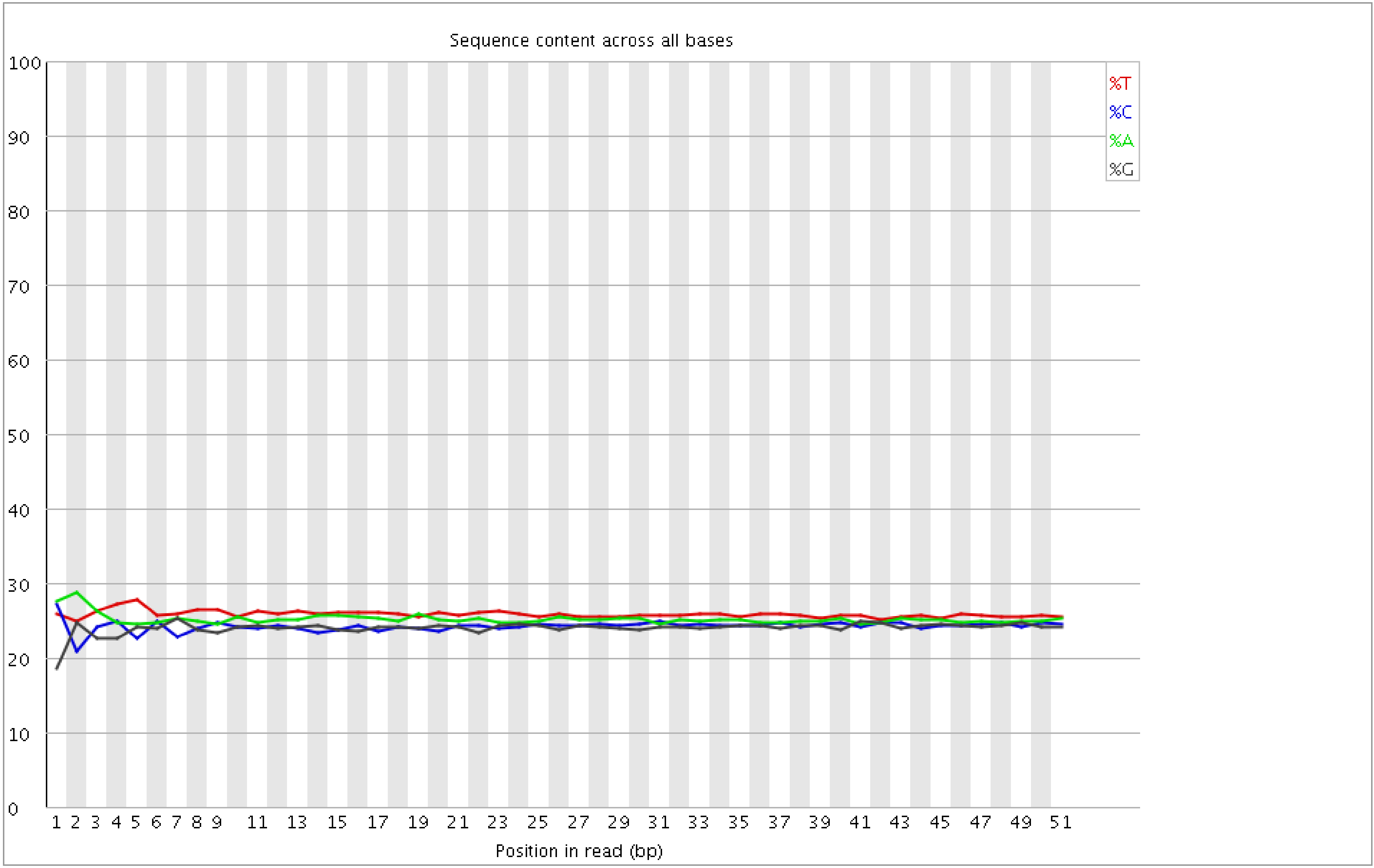
Per Base Sequence Quality



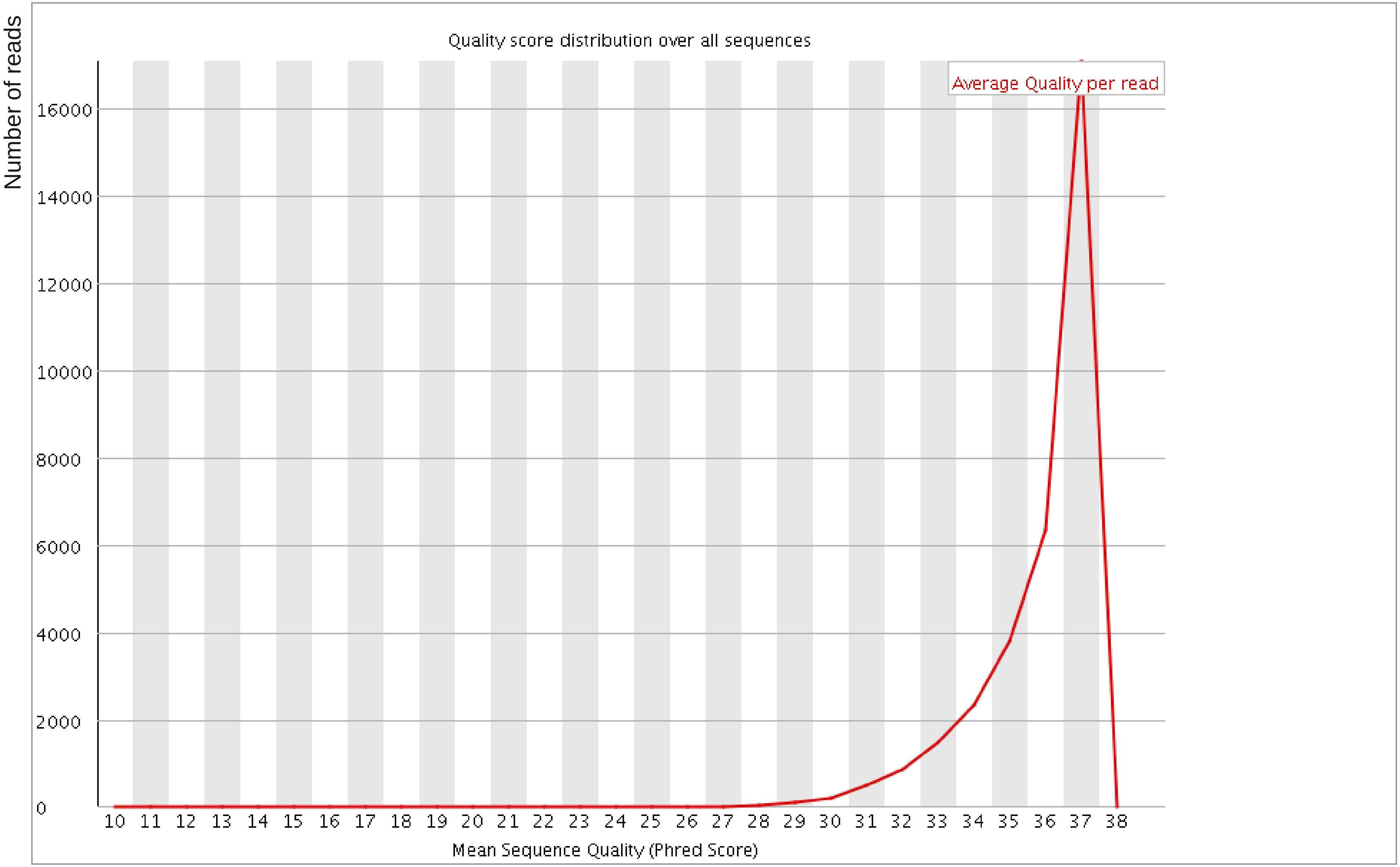
✓ Per Base GC Content



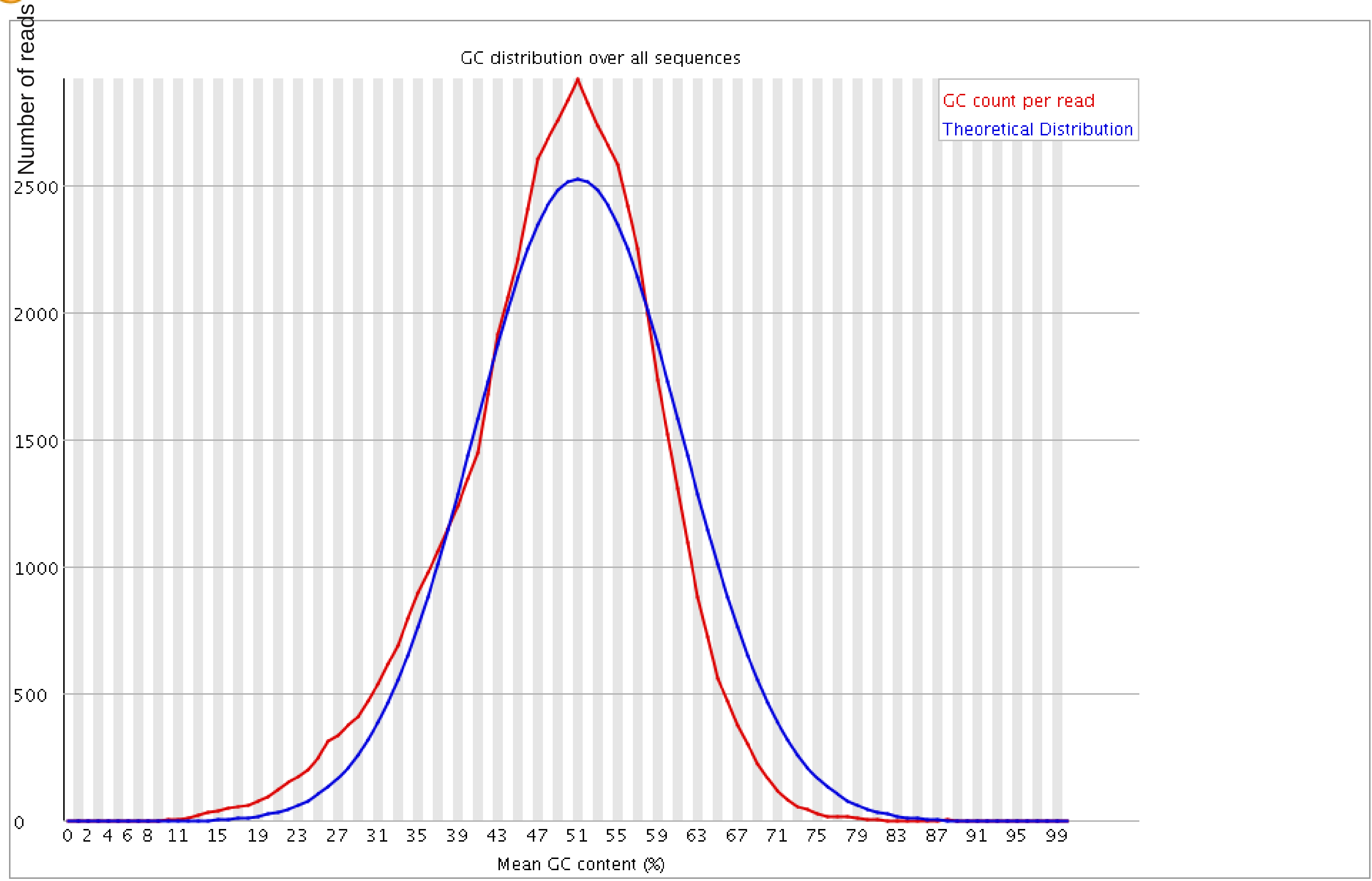
Per Base Sequence Content



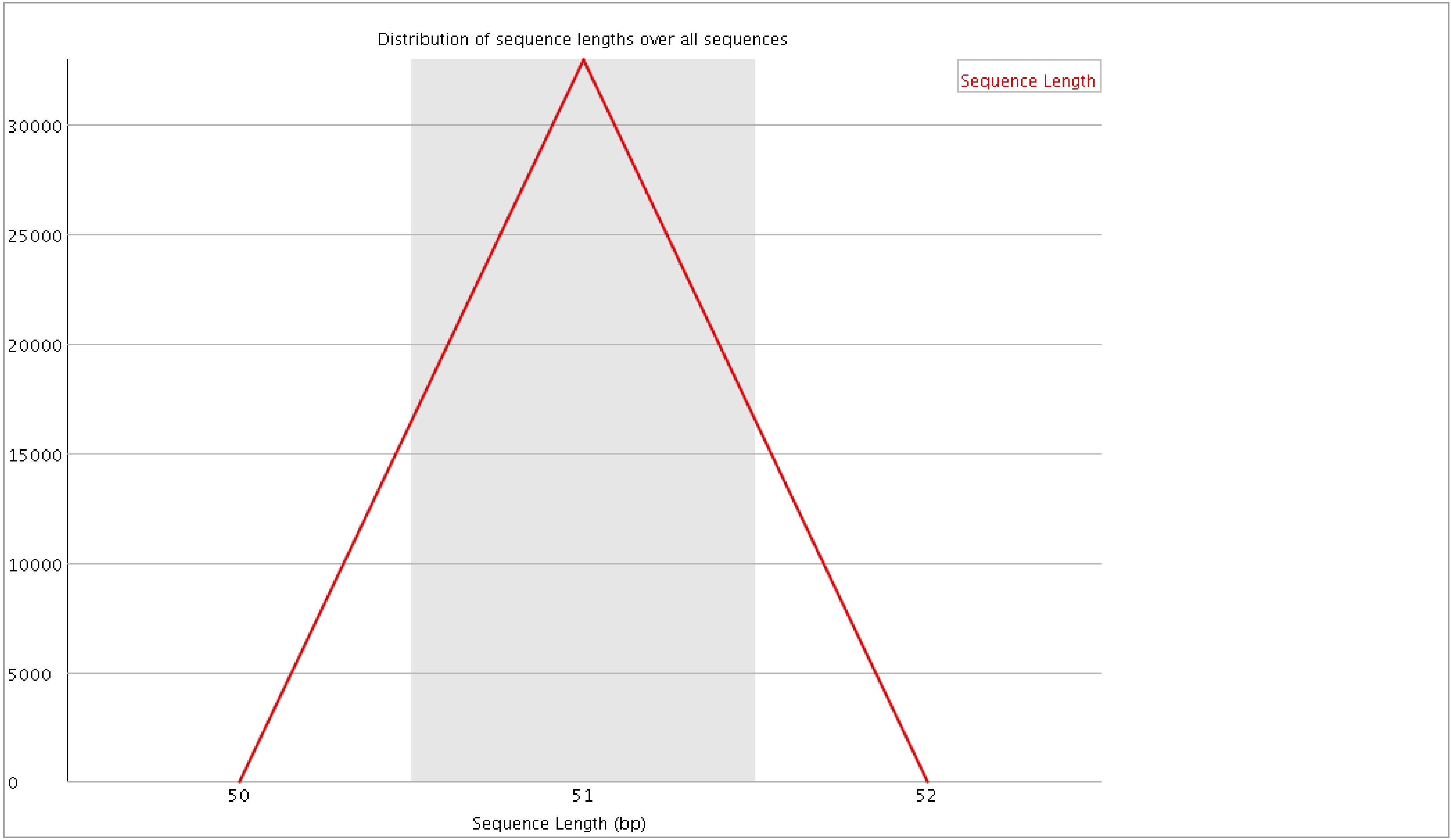
✓ Per Sequence Quality Scores



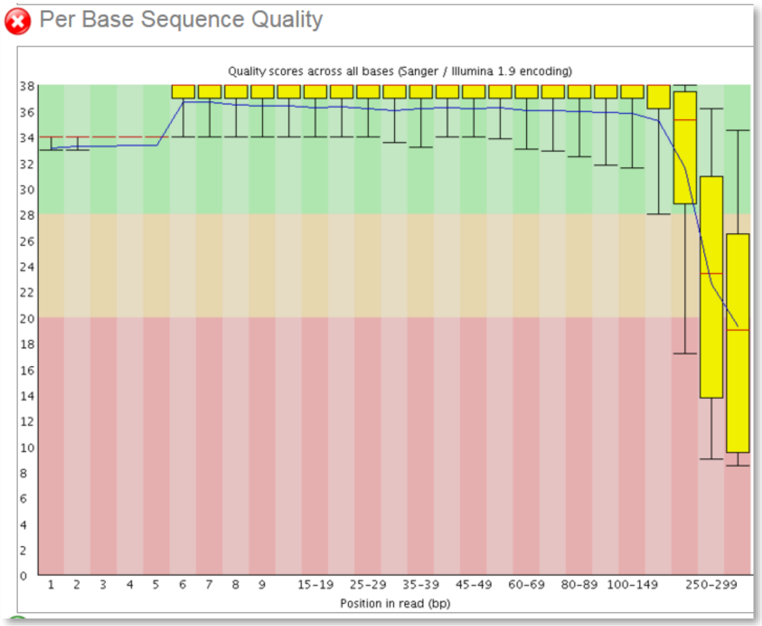
Per Sequence GC Content

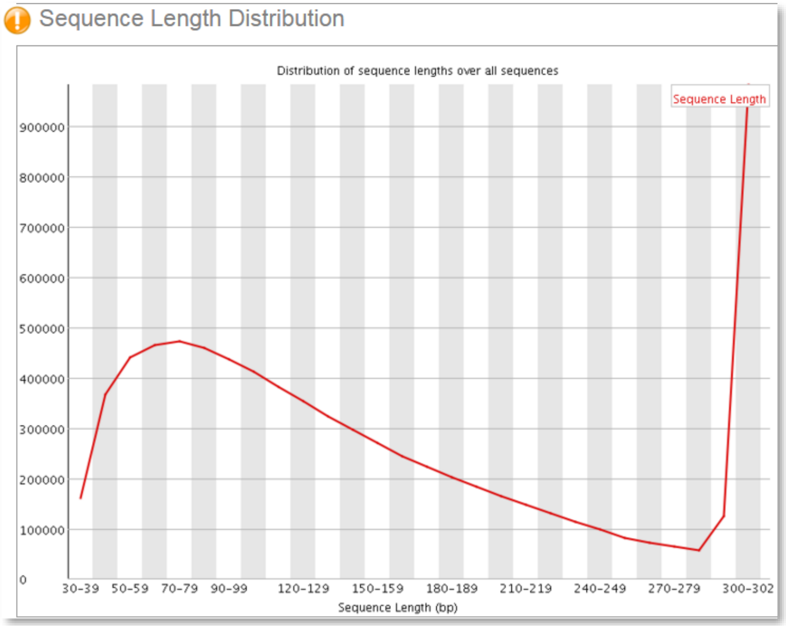


Sequence Length Distribution



Example of less than ideal data

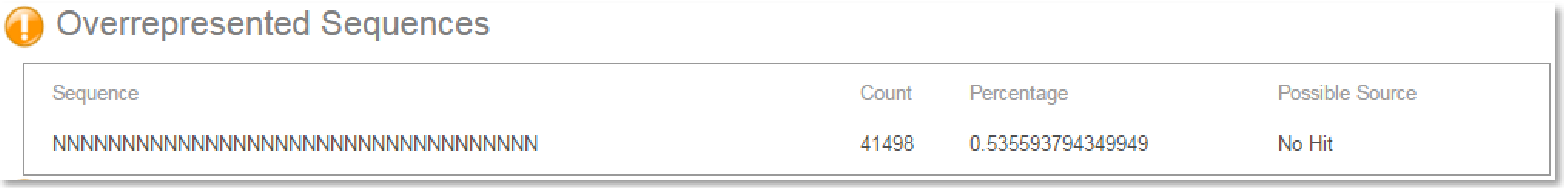




- Indication of potential run problem, check SAV data

- Indication of mixed library types or potential library problem?

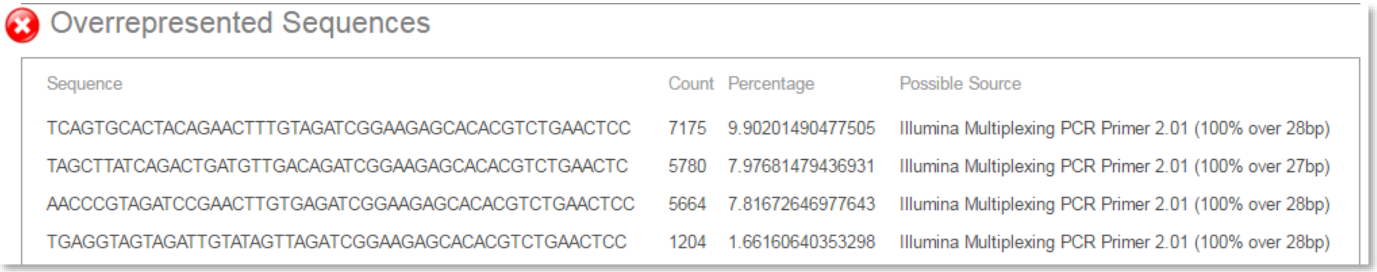
Example of less than ideal data



Overrepresented Sequences

Sequence	Count	Percentage	Possible Source
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN	41498	0.535593794349949	No Hit

- Indication of poor sequence quality, using non-PF data?
- Using adapter masks, short reads?



Overrepresented Sequences

Sequence	Count	Percentage	Possible Source
TCAGTGCACTACAGAACTTTGTAGATCGGAAGAGCACACGTCTGAACTCC	7175	9.90201490477505	Illumina Multiplexing PCR Primer 2.01 (100% over 28bp)
TAGCTTATCAGACTGATGTTGACAGATCGGAAGAGCACACGTCTGAACTC	5780	7.97681479436931	Illumina Multiplexing PCR Primer 2.01 (100% over 27bp)
AACCCGTAGATCCGAACTTGTGAGATCGGAAGAGCACACGTCTGAACTCC	5664	7.81672646977643	Illumina Multiplexing PCR Primer 2.01 (100% over 28bp)
TGAGGTAGTAGATTGTATAGTTAGATCGGAAGAGCACACGTCTGAACTCC	1204	1.66160640353298	Illumina Multiplexing PCR Primer 2.01 (100% over 28bp)

- Indication of adapter presence
- adapter-dimer or adapter contamination

Summary

- **FASTQ format, demultiplexing and FASTQ generation using Illumina tools**
- **FASTQ processing tools for adapter trimming, quality trimming, read merging** *et.al.*
- **Illumina metrics are used for the overall run performance whereas third party tools such as FASTQC are used to look at sample performance**

Resources

- **FASTQC detailed documentation**
- What sequences do I use for adapter trimming?
- **Demultiplexing resource bulletin**
- FASTQ files explained
- Adapter trimming: Why are adapter sequences trimmed from only the 3' ends of reads?
- **Illumina在线技术培训研讨会 — Assessing Run Quality With SAV And FastQC**
- **Illumina在线技术培训研讨会 — Introduction To Bcl2fastq V2+**

QUESTIONS?