ETH zürich

University of Zurich UZH

functional genomics center zurich

f. g. c. z.

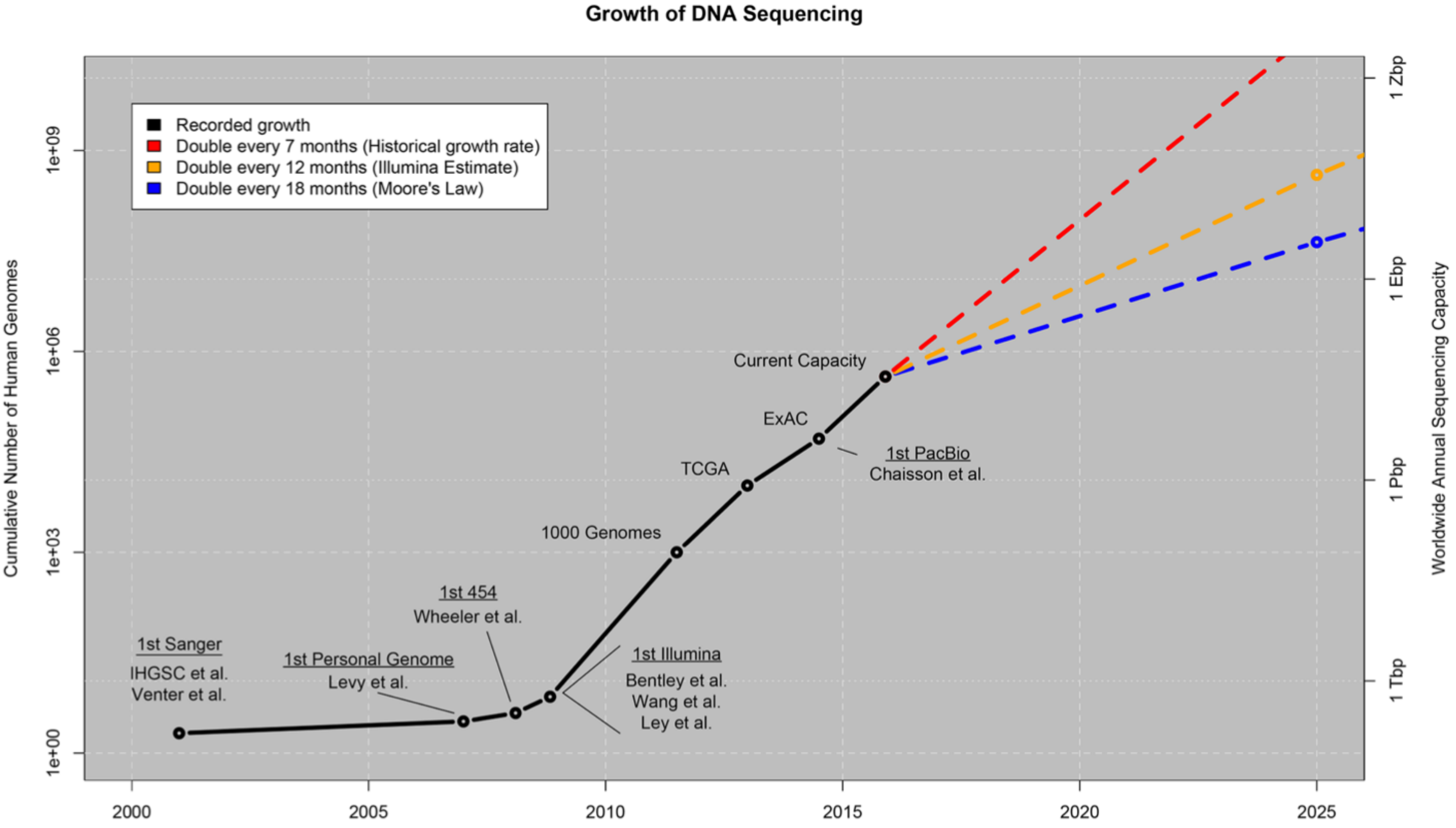
NGS Characteristics

Hubert Rehrauer

University of Zurich UZH

ETH
Eidgenössische Technische Hochschule Zürich
Swiss Federal Institute of Technology Zurich

NGS Data Increase



- NGS data increases faster than computer speed

Stephens ZD etal. (2015) Big Data: Astronomical or Genomical? PLoS Biol 13(7): e1002195.

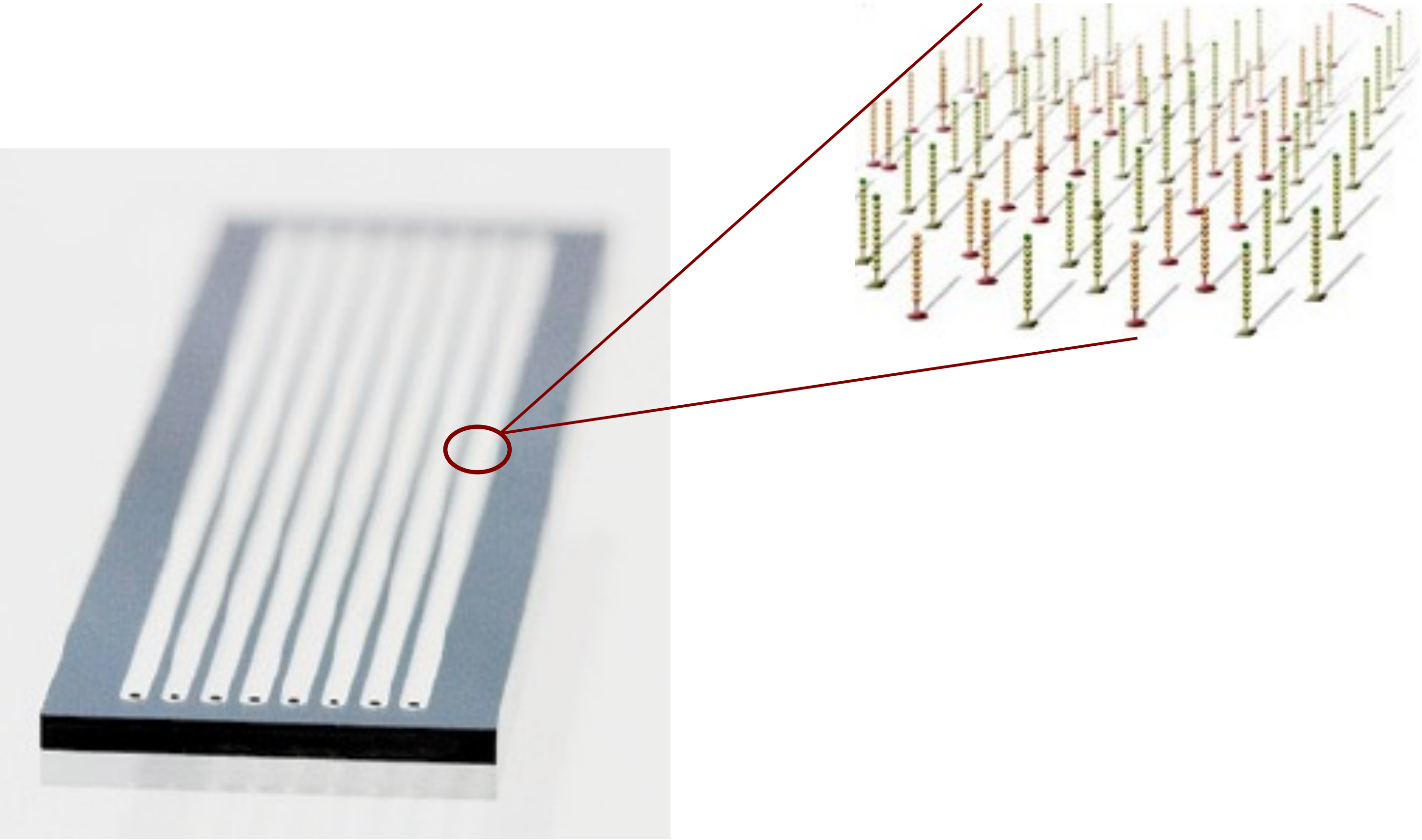
Ingredients for the success

- Evolution has yielded DNA and RNA molecules for information storage and transfer. They have good properties to be read (**measured**)
- NGS technologies rely on
  - processing of molecules is **massively parallel**
  - measurement process is done by individual molecules (**cheap and fast**)
  - actual readout is through fluorescent imaging (**massively parallel, cheap and fast**)

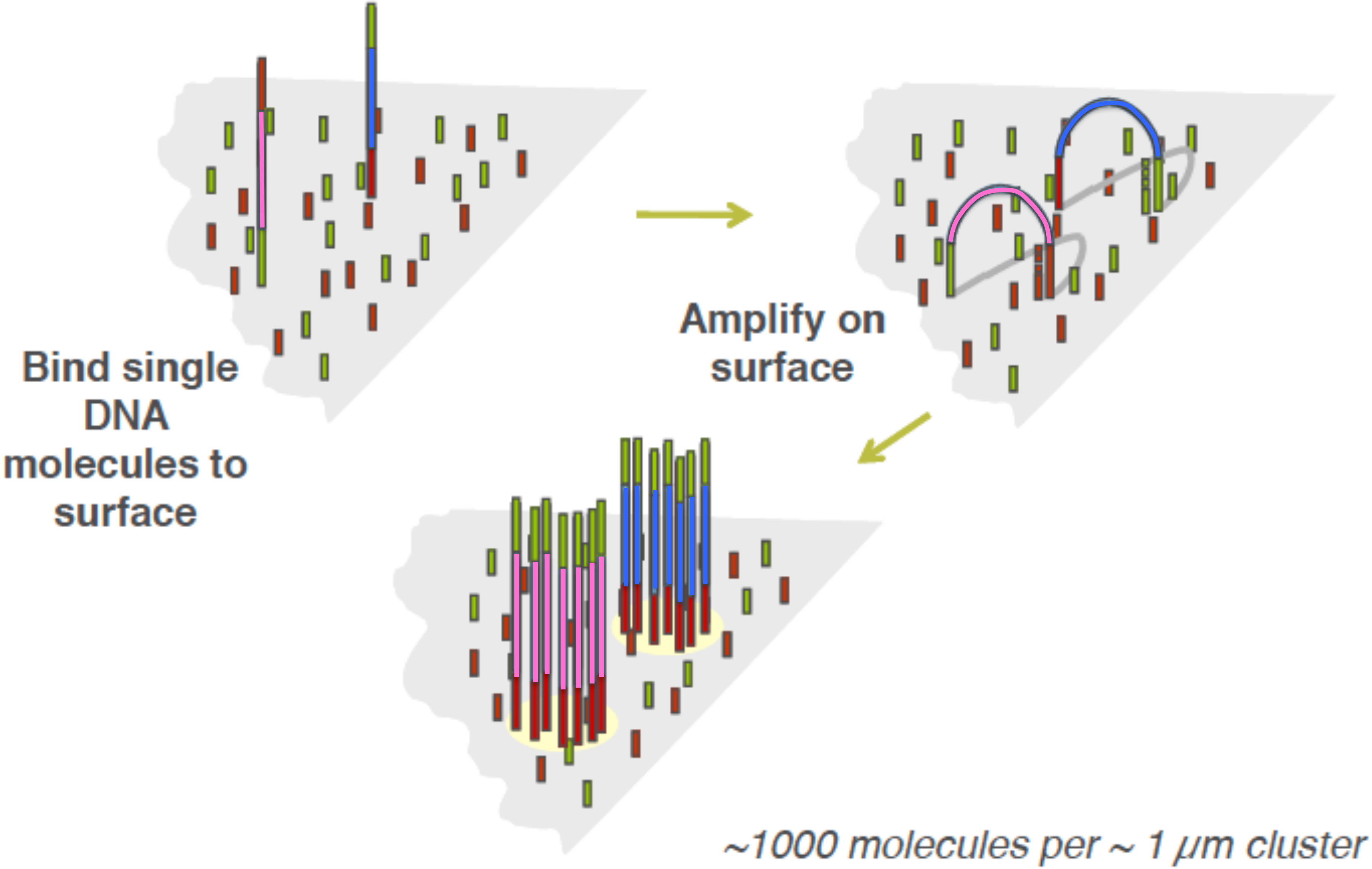
Sequencers

- short-read sequencers (up to ~ 600bp)
  - illumina (e.g. NovaSeq X)
  - Element Biosciences, Aviti
- long-read, single molecule technologies (500bp – megabases)
  - PacBio, Revio
  - Oxford NanoPore Technologies, PromethION
- example overview:
  - https://genohub.com/ngs-instrument-guide/

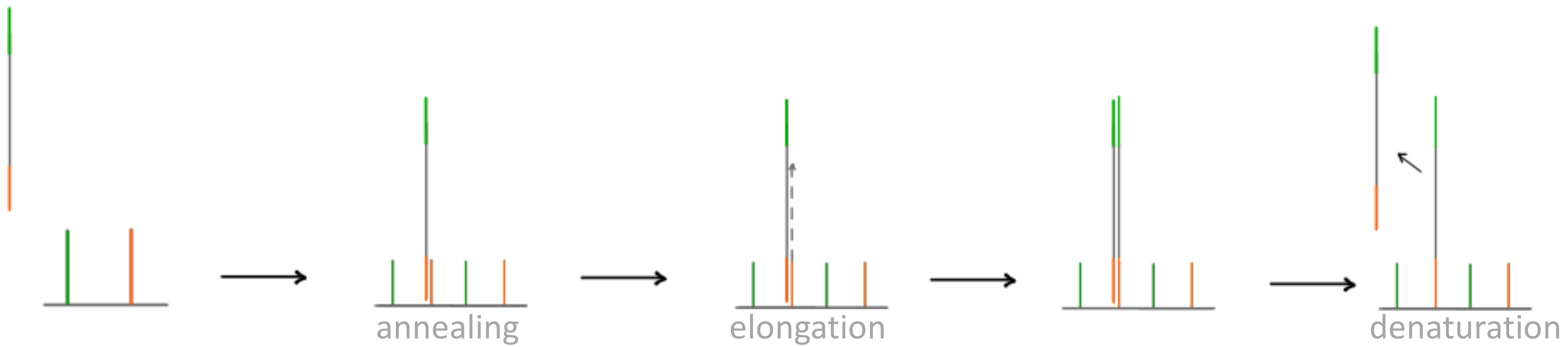
Illumina Flow cell



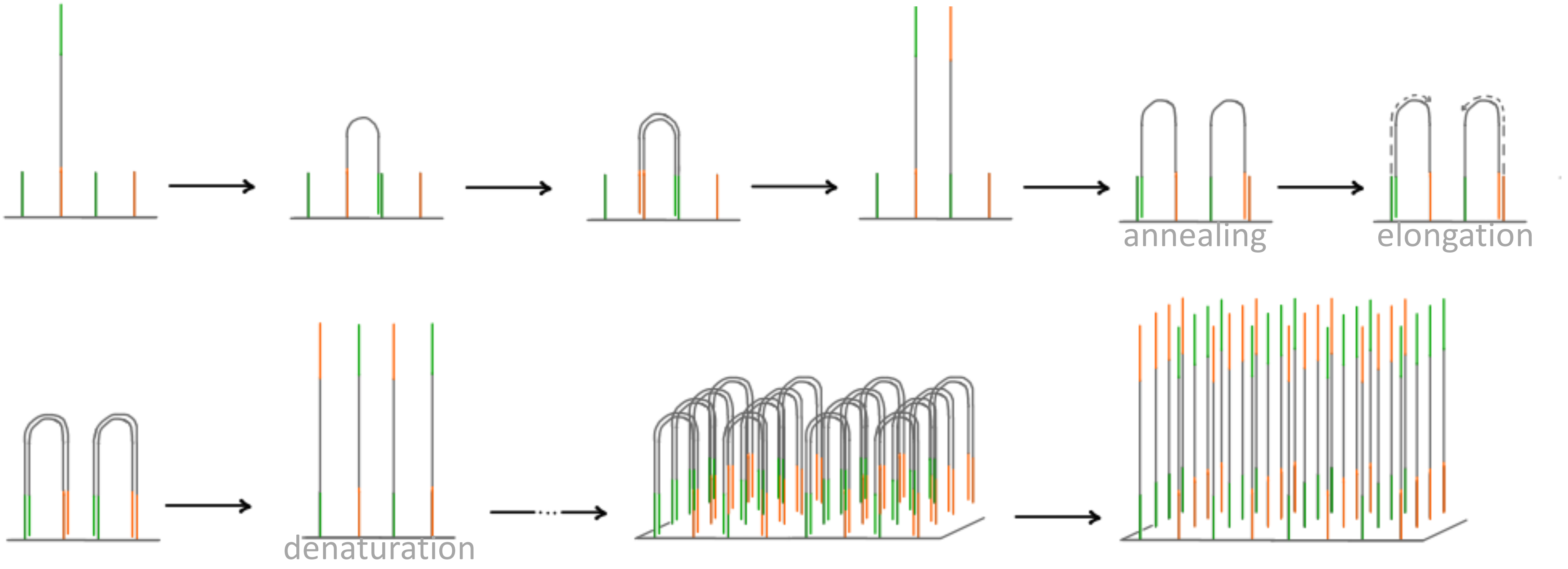
Cluster generation overview

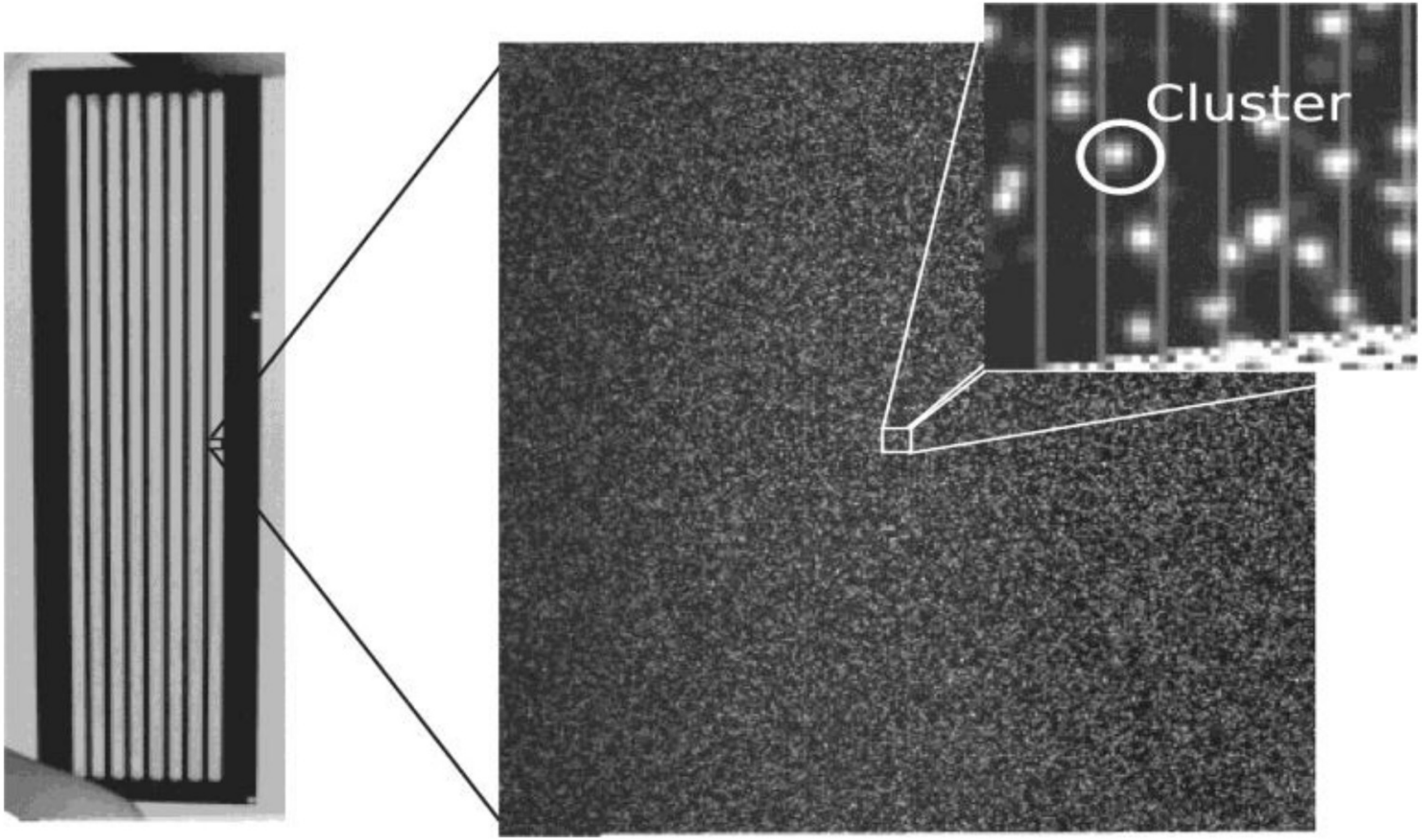


~1000 molecules per ~ 1 µm cluster

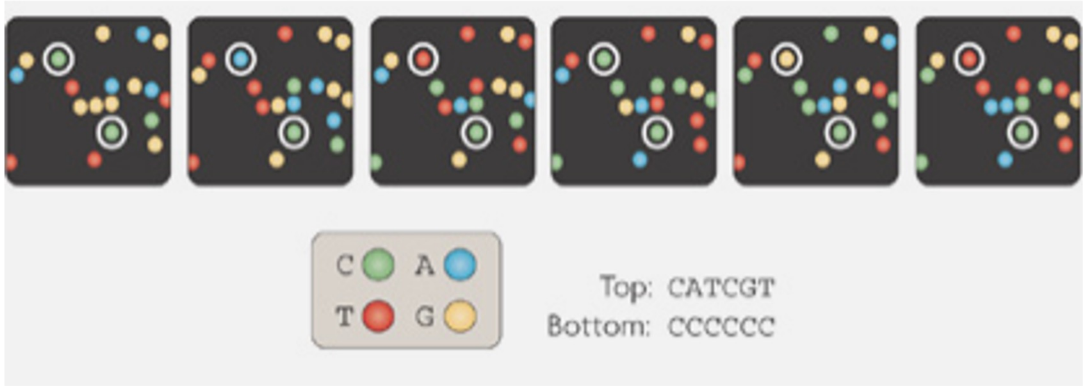


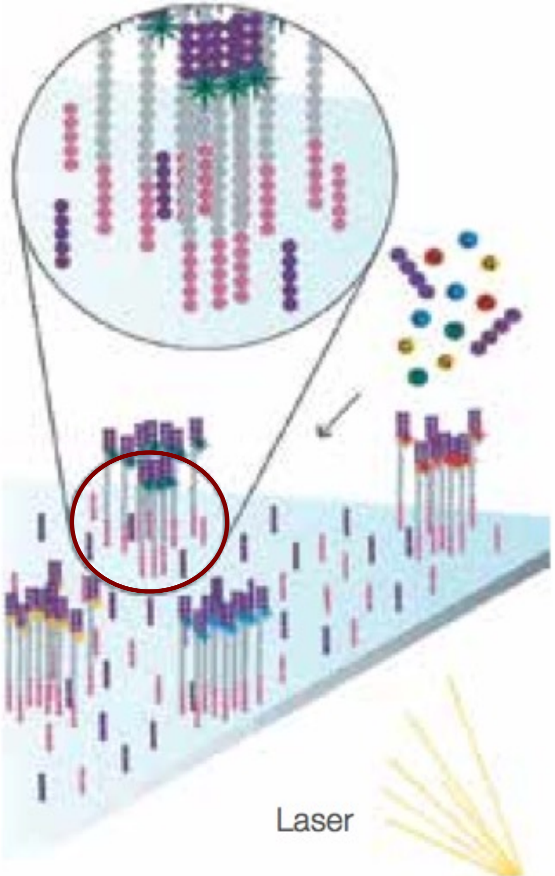
bridge amplification




Cluster

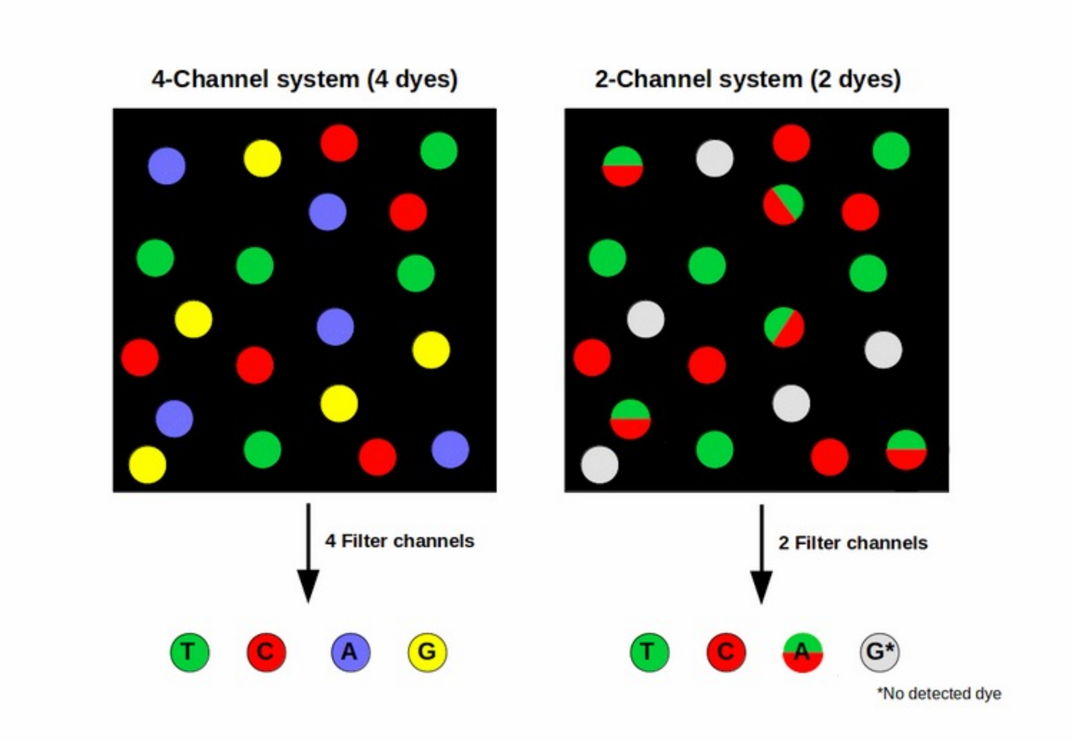
Illumina Sequencing

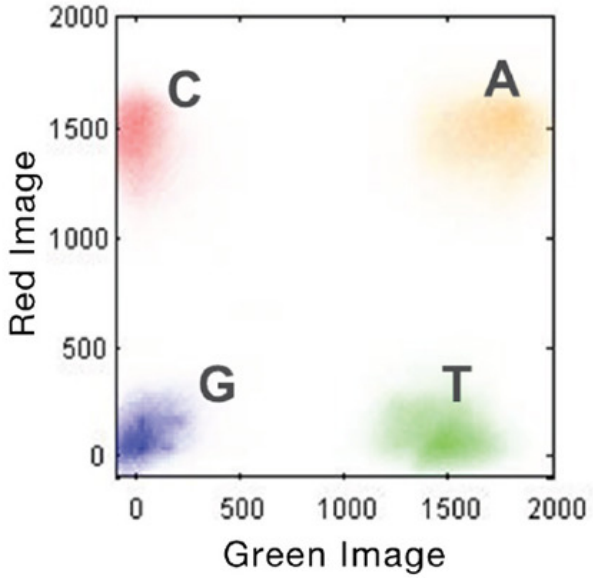




The first sequencing cycle begins by adding four labeled reversible terminators, primers, and DNA polymerase.

Color coding of bases





- 4-channel: Takes longer, readout is more "expensive"; can identify unknown bases (Ns) as lack of signal
- 2-channel: Faster, cheaper, but can not discriminate between failure to read (N) and a G

Color coding of illumina iSeq

- Single color system

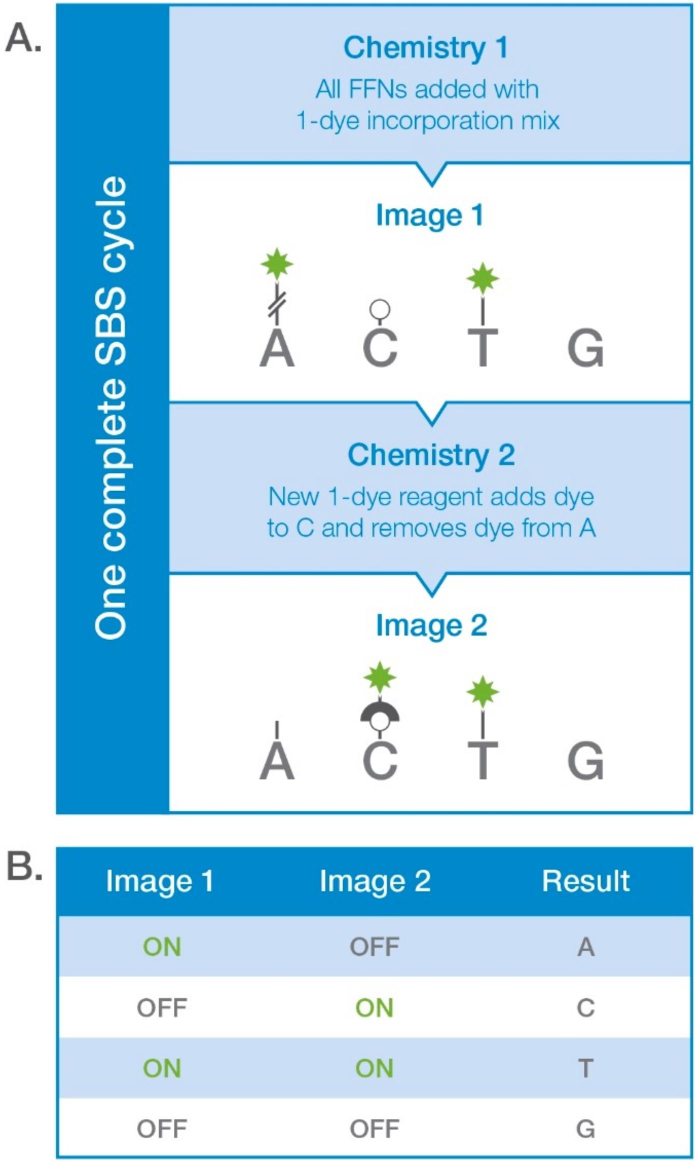
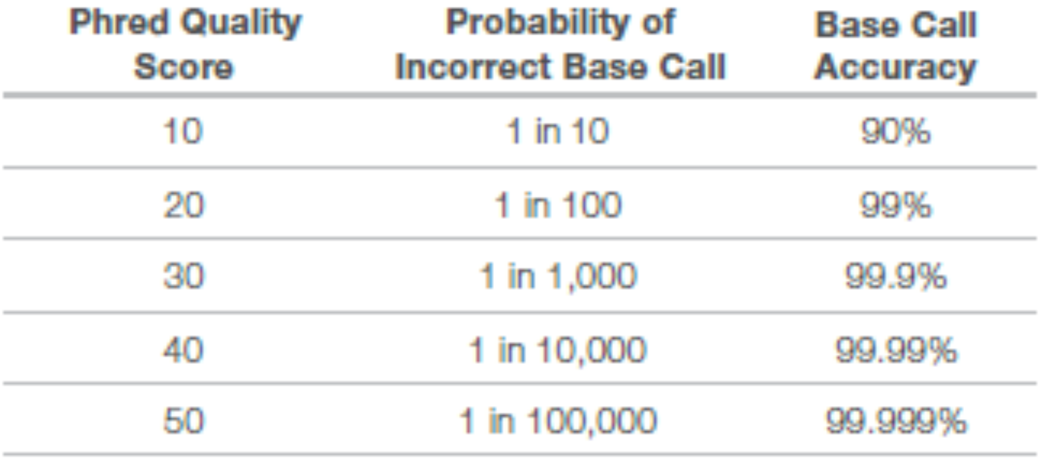

A.
One complete SBS cycle
Chemistry 1
All FFNs added with 1-dye incorporation mix
Image 1
A C T G
Chemistry 2
New 1-dye reagent adds dye to C and removes dye from A
Image 2
A C T G
B.

Image 1	Image 2	Result
ON	OFF	A
OFF	ON	C
ON	ON	T
OFF	OFF	G

Phred scores measure base call accuracy

- P
    - error probability of a given base call
- *Q*
    - $-10\log_{10}P$
- Assign to each base
- Range from 0-41



Phred Quality Score	Probability of Incorrect Base Call	Base Call Accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1,000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%

Ewing B, Green P. 1998. Genome Res. 8(3):186-194.

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

Phred scores are stored with sequences

- FASTQ
  - 4 lines:
    1. Header line for Read (starts with “@” and the sequence ID)
    2. Sequence
    3. Header line for Qualities (starts with “+”)
    4. Quality score (represented in ASCII format)

```
@HWI-ST1034:40:C08PJACXX:2:1101:20681:1994 1:N:0:ATCACG

CTCGNAGACTGGCAACTTGTTCTGGTTTACTGCACCTTCTTTTAAAGGCAGAAAGGC

+

CCCF#2ADHHHGHJJJIJJHIIIJJHIJJJJJJJJJBGIIJJJJJJJJJJJJJJJIJ
```

Phred scores can be **ASCII encoded**

- Add an offset and convert the sum to ASCII

- Current format
  - **Illumina 1.9 (i.e. Sanger format)**
  - Phred scoring: 0-41;
  - Offset: 33
  - 41+33=74 (J)
  - All current sequencers
- This encoding would mean that there are 42 different values
- Illumina software bins the quality values to save space and better compress the quality scores

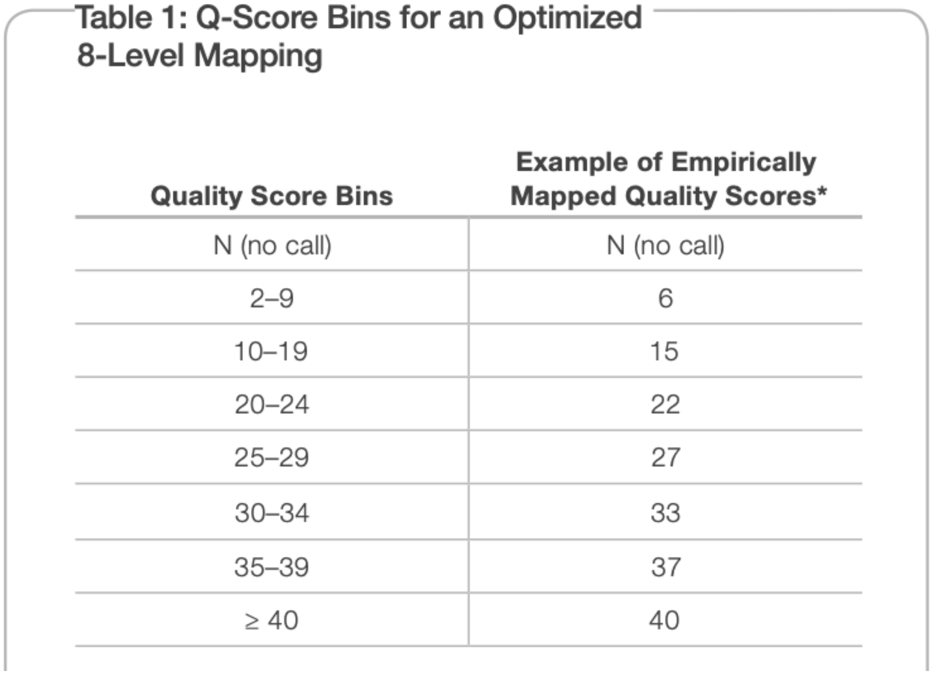
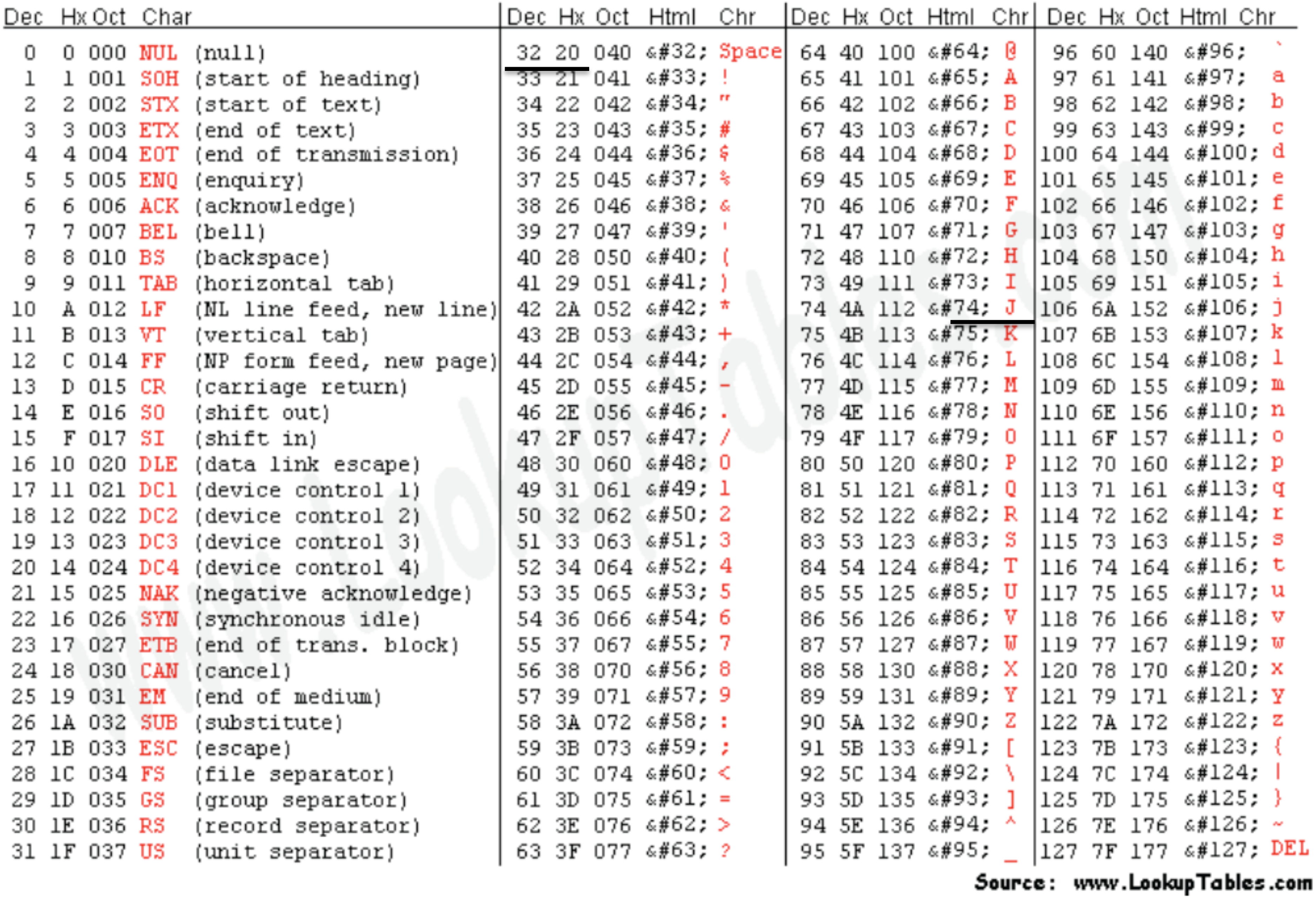


Table 1: Q-Score Bins for an Optimized 8-Level Mapping

Quality Score Bins	Example of Empirically Mapped Quality Scores*
N (no call)	N (no call)
2–9	6
10–19	15
20–24	22
25–29	27
30–34	33
35–39	37
≥ 40	40



Dec	Hx	Oct	Char	Dec	Hx	Oct	Html	Chr	Dec	Hx	Oct	Html	Chr	Dec	Hx	Oct	Html	Chr
0	0	000	NUL (null)	32	20	040	&#32;	Space	64	40	100	&#64;	@	96	60	140	&#96;	`
1	1	001	SOH (start of heading)	33	21	041	&#33;	!	65	41	101	&#65;	A	97	61	141	&#97;	a
2	2	002	STX (start of text)	34	22	042	&#34;	"	66	42	102	&#66;	B	98	62	142	&#98;	b
3	3	003	ETX (end of text)	35	23	043	&#35;	#	67	43	103	&#67;	C	99	63	143	&#99;	c
4	4	004	EOT (end of transmission)	36	24	044	&#36;	$	68	44	104	&#68;	D	100	64	144	&#100;	d
5	5	005	ENQ (enquiry)	37	25	045	&#37;	%	69	45	105	&#69;	E	101	65	145	&#101;	e
6	6	006	ACK (acknowledge)	38	26	046	&#38;	&	70	46	106	&#70;	F	102	66	146	&#102;	f
7	7	007	BEL (bell)	39	27	047	&#39;	'	71	47	107	&#71;	G	103	67	147	&#103;	g
8	8	010	BS (backspace)	40	28	050	&#40;	(	72	48	110	&#72;	H	104	68	150	&#104;	h
9	9	011	TAB (horizontal tab)	41	29	051	&#41;	)	73	49	111	&#73;	I	105	69	151	&#105;	i
10	A	012	LF (NL line feed, new line)	42	2A	052	&#42;	*	74	4A	112	&#74;	J	106	6A	152	&#106;	j
11	B	013	VT (vertical tab)	43	2B	053	&#43;	+	75	4B	113	&#75;	K	107	6B	153	&#107;	k
12	C	014	FF (NP form feed, new page)	44	2C	054	&#44;	,	76	4C	114	&#76;	L	108	6C	154	&#108;	l
13	D	015	CR (carriage return)	45	2D	055	&#45;	-	77	4D	115	&#77;	M	109	6D	155	&#109;	m
14	E	016	SO (shift out)	46	2E	056	&#46;	.	78	4E	116	&#78;	N	110	6E	156	&#110;	n
15	F	017	SI (shift in)	47	2F	057	&#47;	/	79	4F	117	&#79;	O	111	6F	157	&#111;	o
16	10	020	DLE (data link escape)	48	30	060	&#48;	0	80	50	120	&#80;	P	112	70	160	&#112;	p
17	11	021	DC1 (device control 1)	49	31	061	&#49;	1	81	51	121	&#81;	Q	113	71	161	&#113;	q
18	12	022	DC2 (device control 2)	50	32	062	&#50;	2	82	52	122	&#82;	R	114	72	162	&#114;	r
19	13	023	DC3 (device control 3)	51	33	063	&#51;	3	83	53	123	&#83;	S	115	73	163	&#115;	s
20	14	024	DC4 (device control 4)	52	34	064	&#52;	4	84	54	124	&#84;	T	116	74	164	&#116;	t
21	15	025	NAK (negative acknowledge)	53	35	065	&#53;	5	85	55	125	&#85;	U	117	75	165	&#117;	u
22	16	026	SYN (synchronous idle)	54	36	066	&#54;	6	86	56	126	&#86;	V	118	76	166	&#118;	v
23	17	027	ETB (end of trans. block)	55	37	067	&#55;	7	87	57	127	&#87;	W	119	77	167	&#119;	w
24	18	030	CAN (cancel)	56	38	070	&#56;	8	88	58	130	&#88;	X	120	78	170	&#120;	x
25	19	031	EM (end of medium)	57	39	071	&#57;	9	89	59	131	&#89;	Y	121	79	171	&#121;	y
26	1A	032	SUB (substitute)	58	3A	072	&#58;	:	90	5A	132	&#90;	Z	122	7A	172	&#122;	z
27	1B	033	ESC (escape)	59	3B	073	&#59;	;	91	5B	133	&#91;	[	123	7B	173	&#123;	{
28	1C	034	FS (file separator)	60	3C	074	&#60;	<	92	5C	134	&#92;	\	124	7C	174	&#124;	|
29	1D	035	GS (group separator)	61	3D	075	&#61;	=	93	5D	135	&#93;	]	125	7D	175	&#125;	}
30	1E	036	RS (record separator)	62	3E	076	&#62;	>	94	5E	136	&#94;	^	126	7E	176	&#126;	~
31	1F	037	US (unit separator)	63	3F	077	&#63;	?	95	5F	137	&#95;	_	127	7F	177	&#127;	DEL

Source: www.LookupTables.com

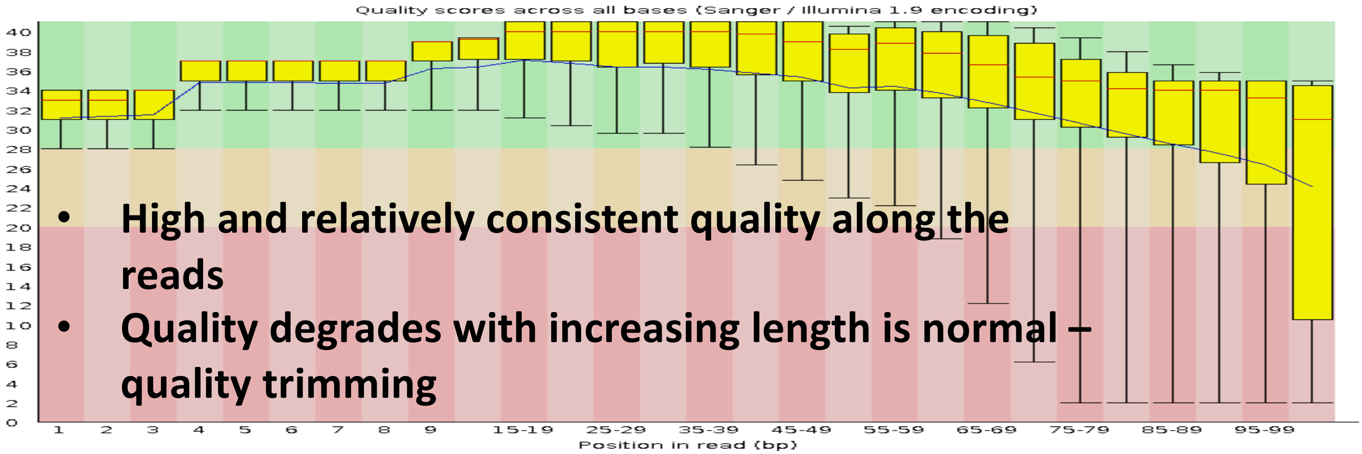
Read Quality Control

- Library construction could introduce bias
  - Fragmentation, ligation, amplification
  - GC bias
  - Over-amplification
  - Contamination
- Sequencing errors
  - Chemical, optical, computational

Platform	Primary error	Error rate (%)
Illumina	Substitution	0.1
PacBio	Indel	12 (consensus: 1)
Oxford Nanopore	Indel	3 - 20

Per base sequence quality - FastQC

- Range of quality values across all bases at each position



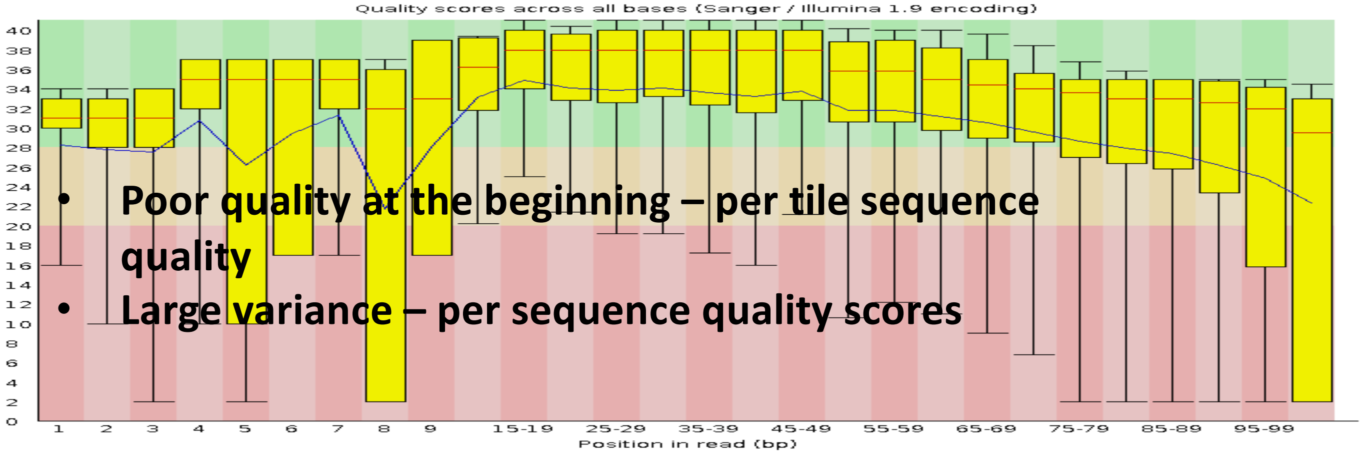
- High and relatively consistent quality along the reads
- Quality degrades with increasing length is normal – quality trimming

Green: >Q28, good

Orange: >Q20, reasonable

Red:<Q20, poor

Median > Q25



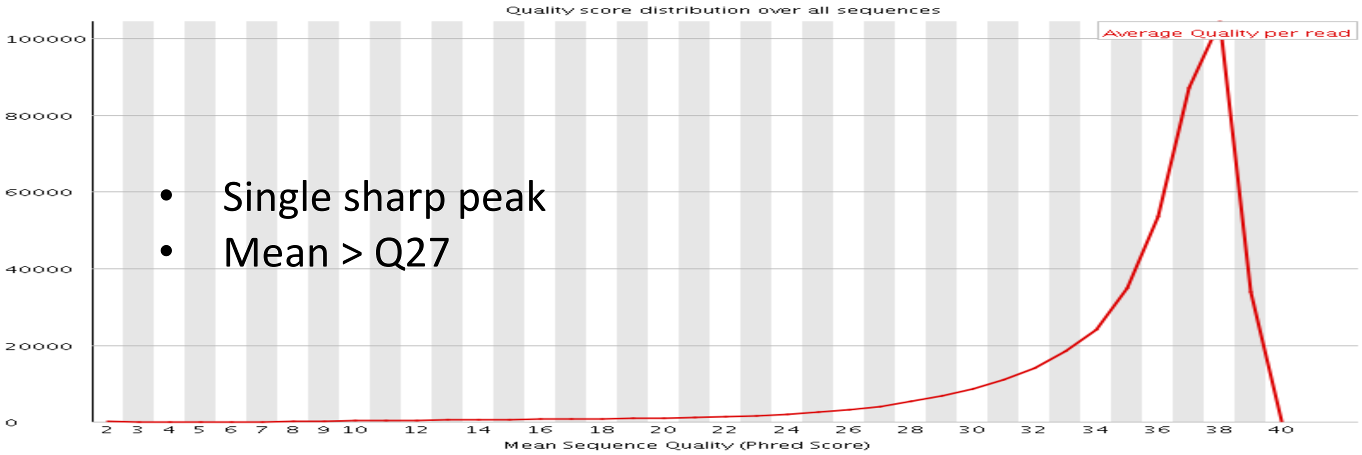
- Poor quality at the beginning – per tile sequence quality
- Large variance – per sequence quality scores

Median < Q20

Per sequence quality scores - FastQC

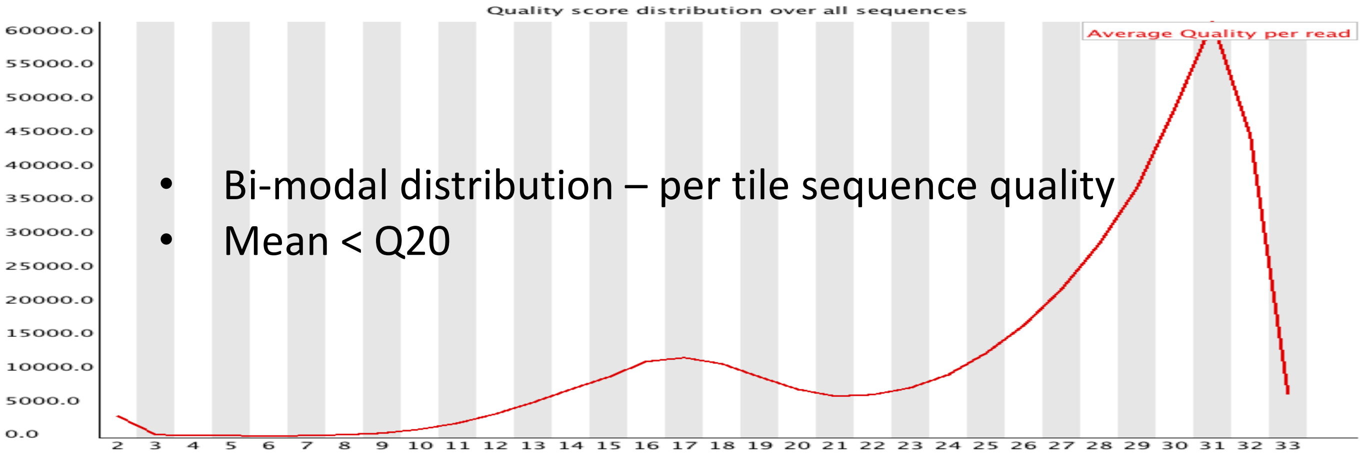
- Subset of sequences with universally low quality values

☑



- Single sharp peak
- Mean > Q27

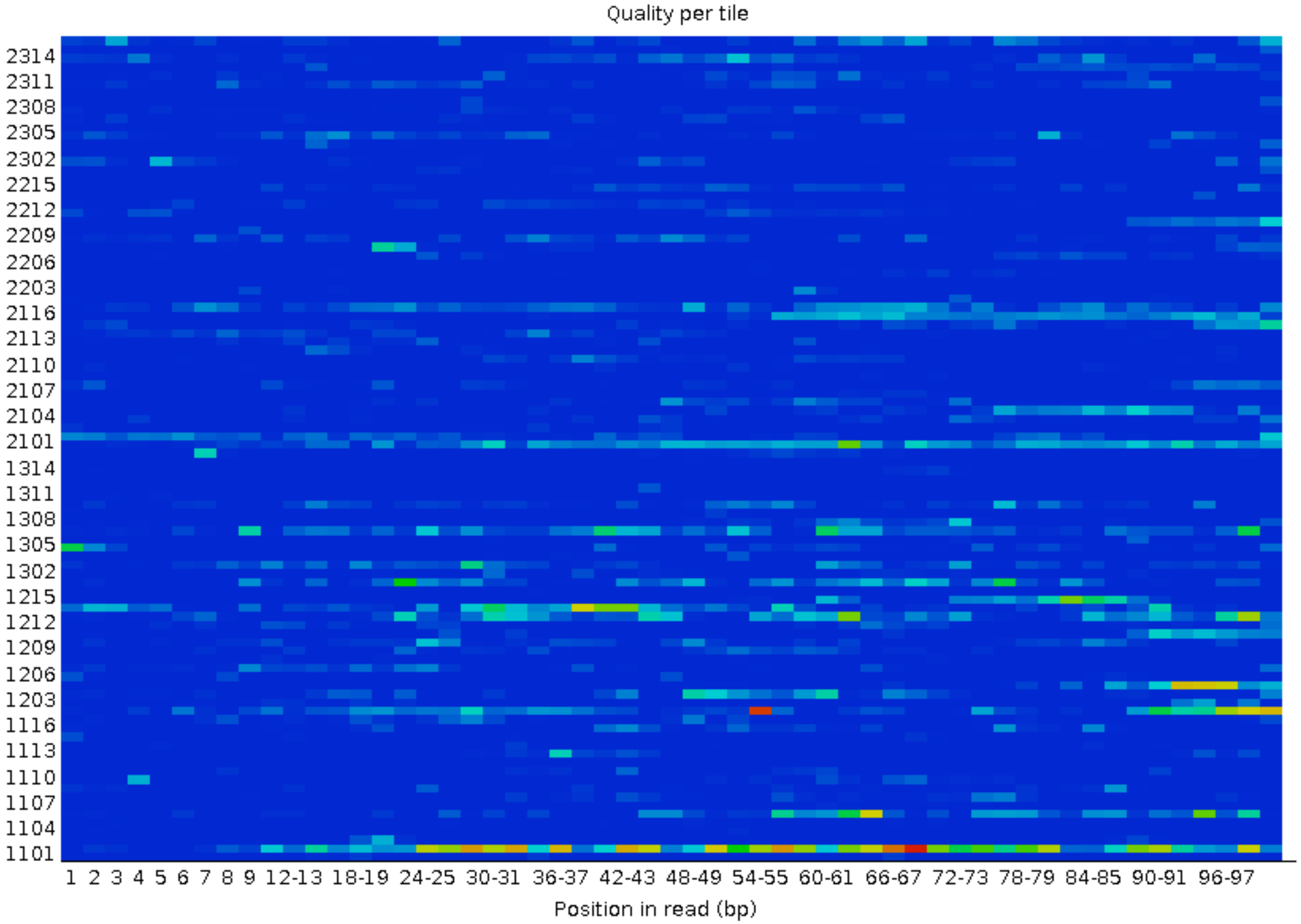
☒



- Bi-modal distribution – per tile sequence quality
- Mean < Q20

Per tile sequence quality - FastQC

- Quality scores from each tile across all bases - loss in quality associated with only one part of the flowcell



Deviation from average quality

Cold colors: ≥ average

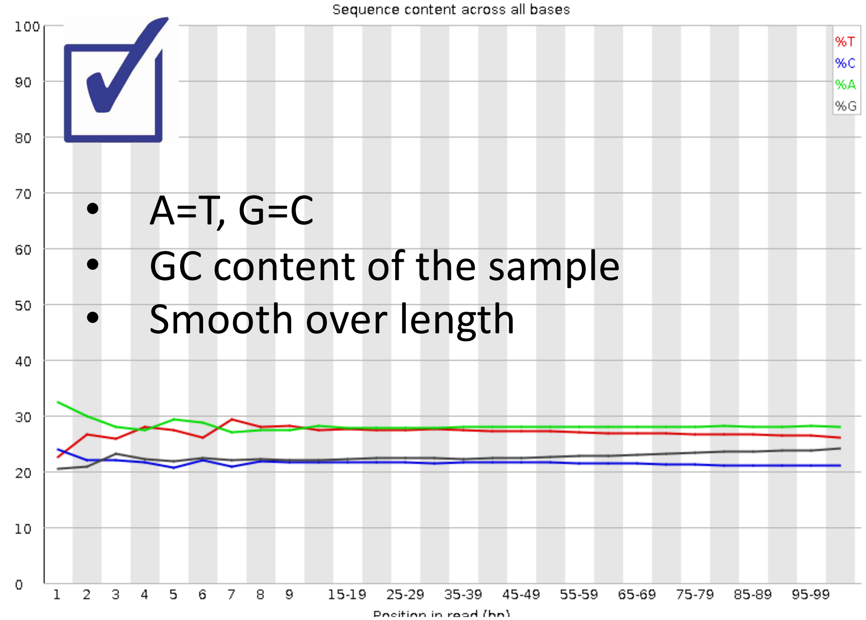
Hotter color: worse quality

Good: universal blue

Failure: < average - 5

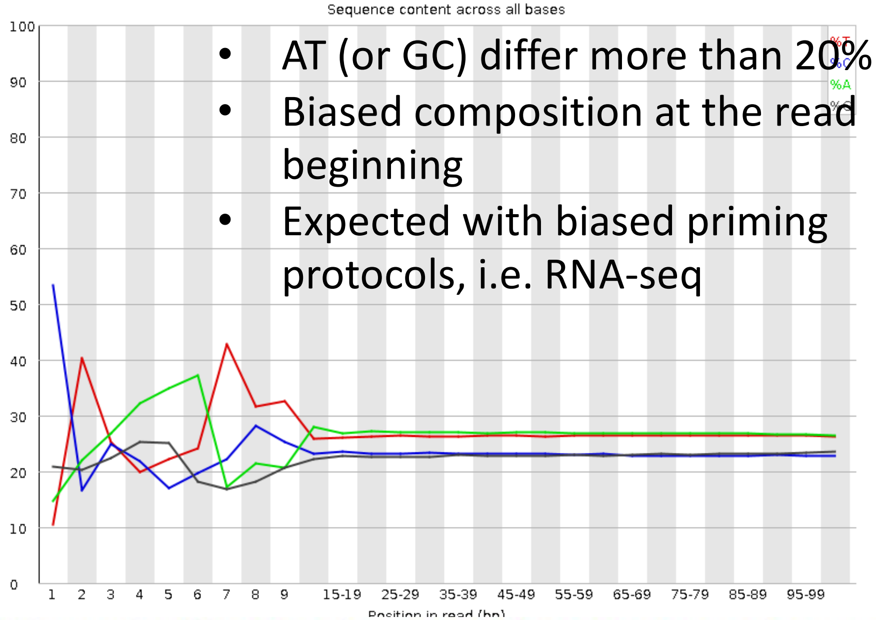
Per base sequence content - FastQC

- The portion of A, T, G, and C at each position

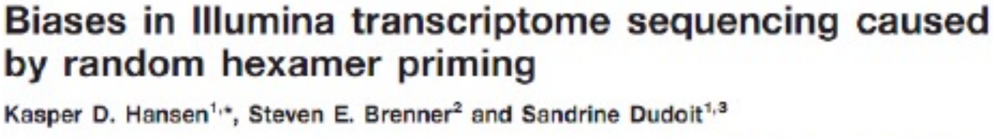


☑

- A=T, G=C
- GC content of the sample
- Smooth over length

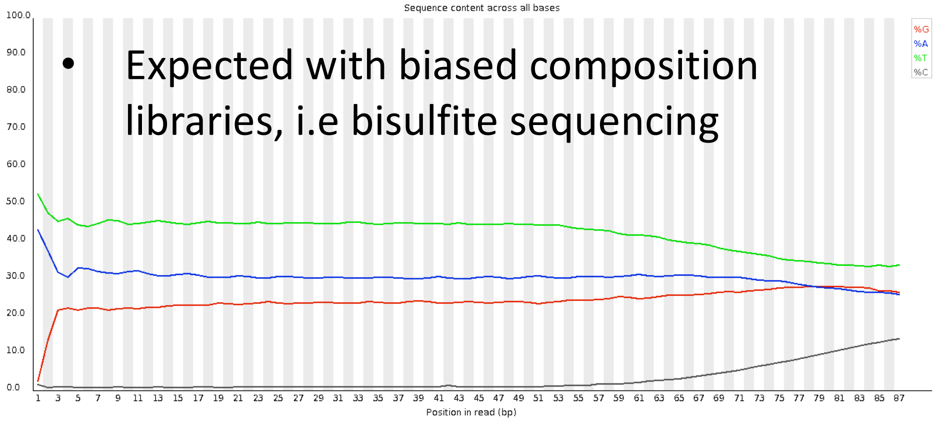


- AT (or GC) differ more than 20%
- Biased composition at the read beginning
- Expected with biased priming protocols, i.e. RNA-seq



Biases in Illumina transcriptome sequencing caused by random hexamer priming

Kasper D. Hansen[1,*], Steven E. Brenner[2] and Sandrine Dudoit[1,3]



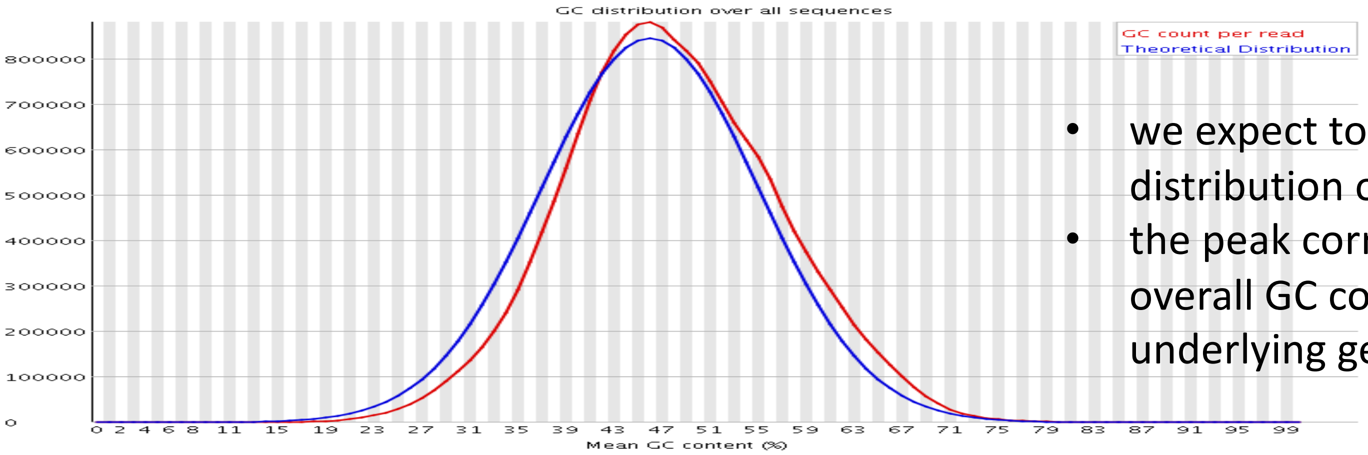
- Expected with biased composition libraries, i.e bisulfite sequencing

Treatment of DNA with bisulfite converts cytosine to uracil, but leaves methylated cytosine unaffected. Therefore, DNA that has been treated with bisulfite retains only methylated cytosines.

Per sequence GC content - FastQC

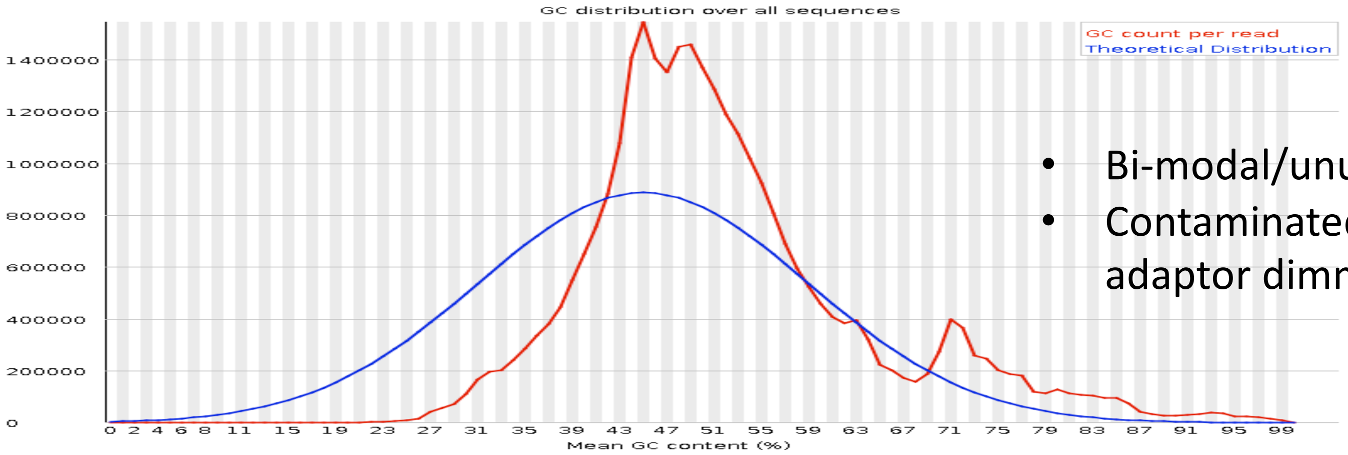
- Distribution of average GC in all reads

☑



- we expect to see a roughly normal distribution of GC content
- the peak corresponds to the overall GC content of the underlying genome

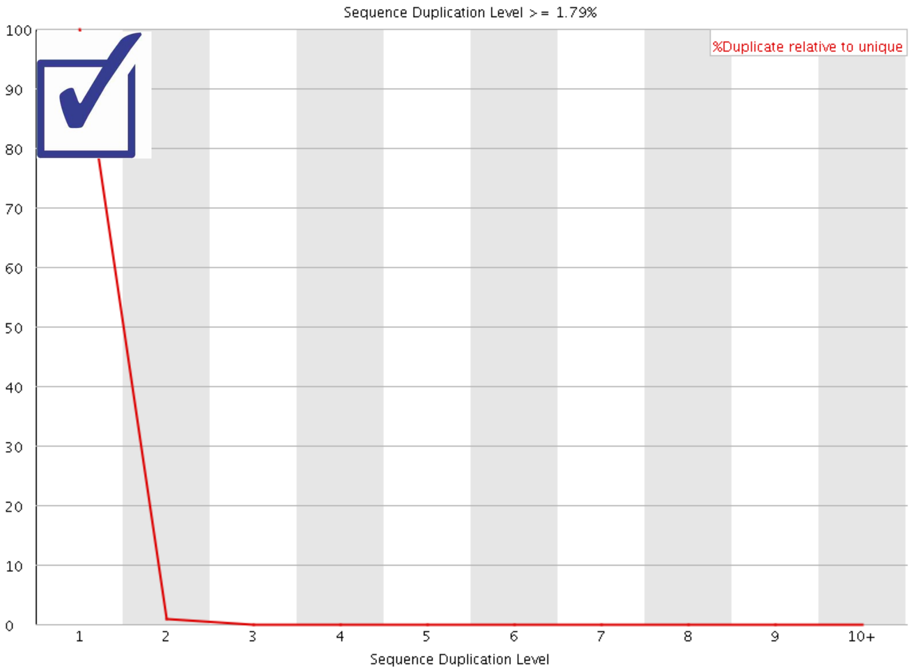
☒

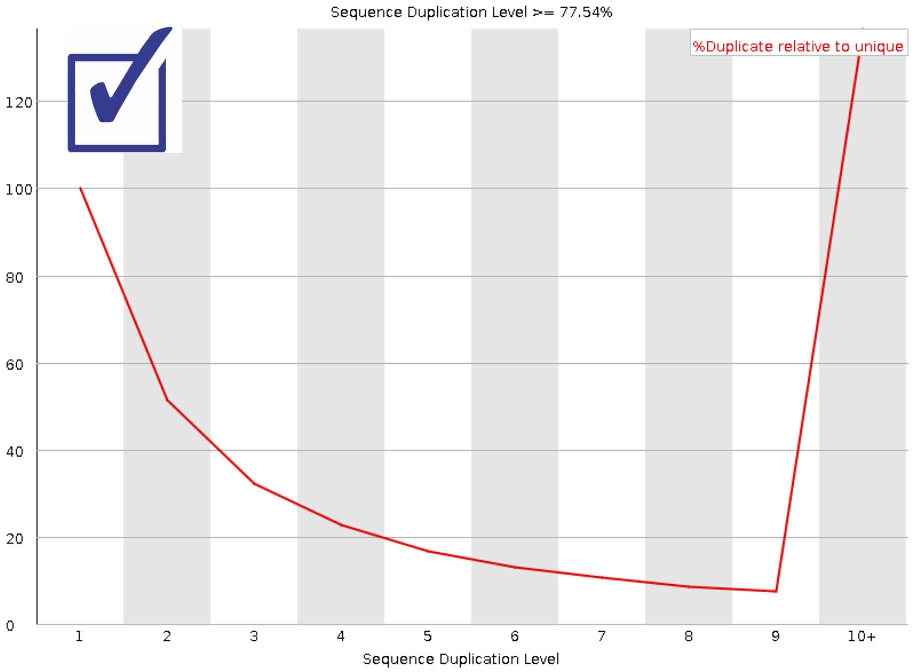


- Bi-modal/unusual distribution
- Contaminated/biased subset, i.e. adaptor dimmers, rRNA etc

Sequence duplication - FastQC

- Relative number of sequences with different degrees of duplication





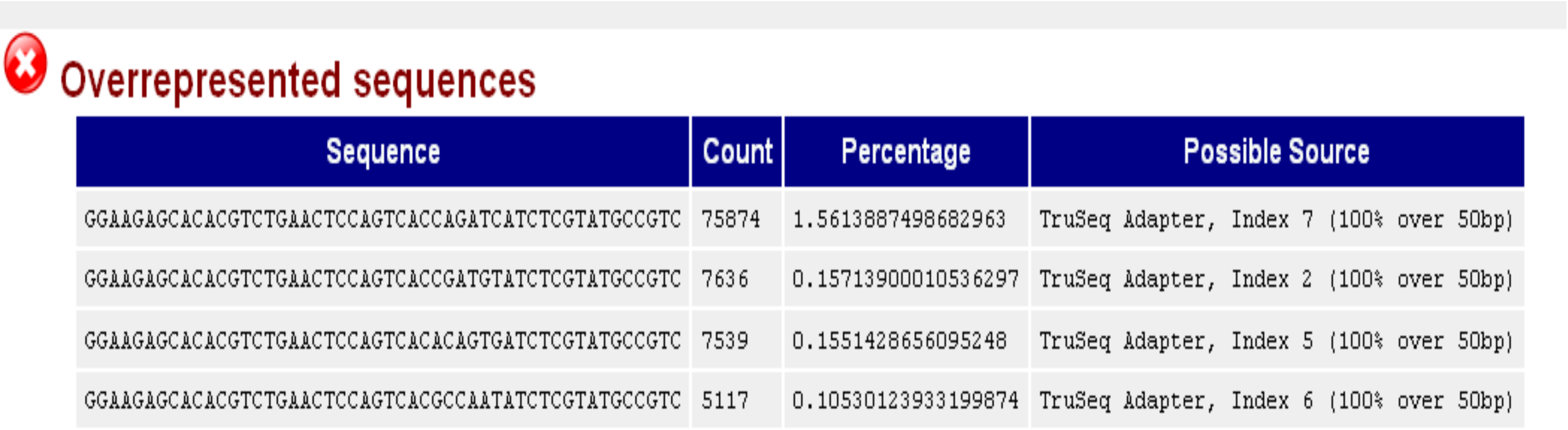
- Essentially no duplication

High duplication levels:

- DNA-seq: PCR over amplification, too little input material
- Normal in RNA-seq: high expression

Overrepresented sequences - FastQC

- Sequences make up >0.1 % of the total
- Compare those with a contamination database for finding contamination (i.e. adaptor dimmers)

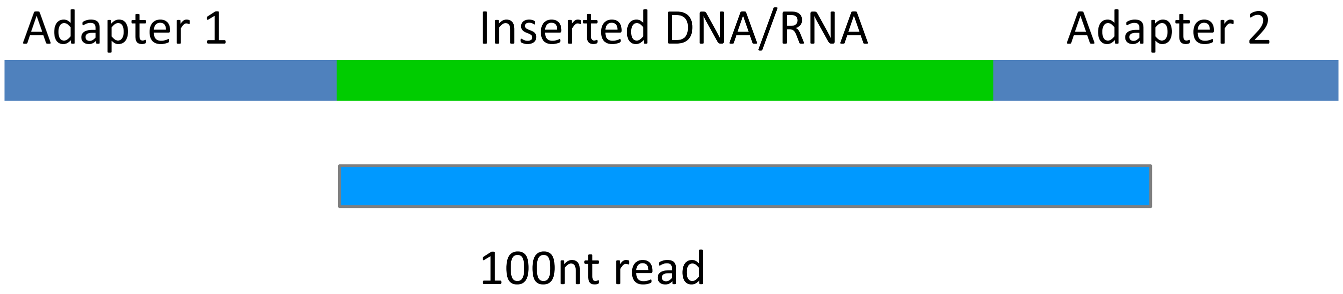


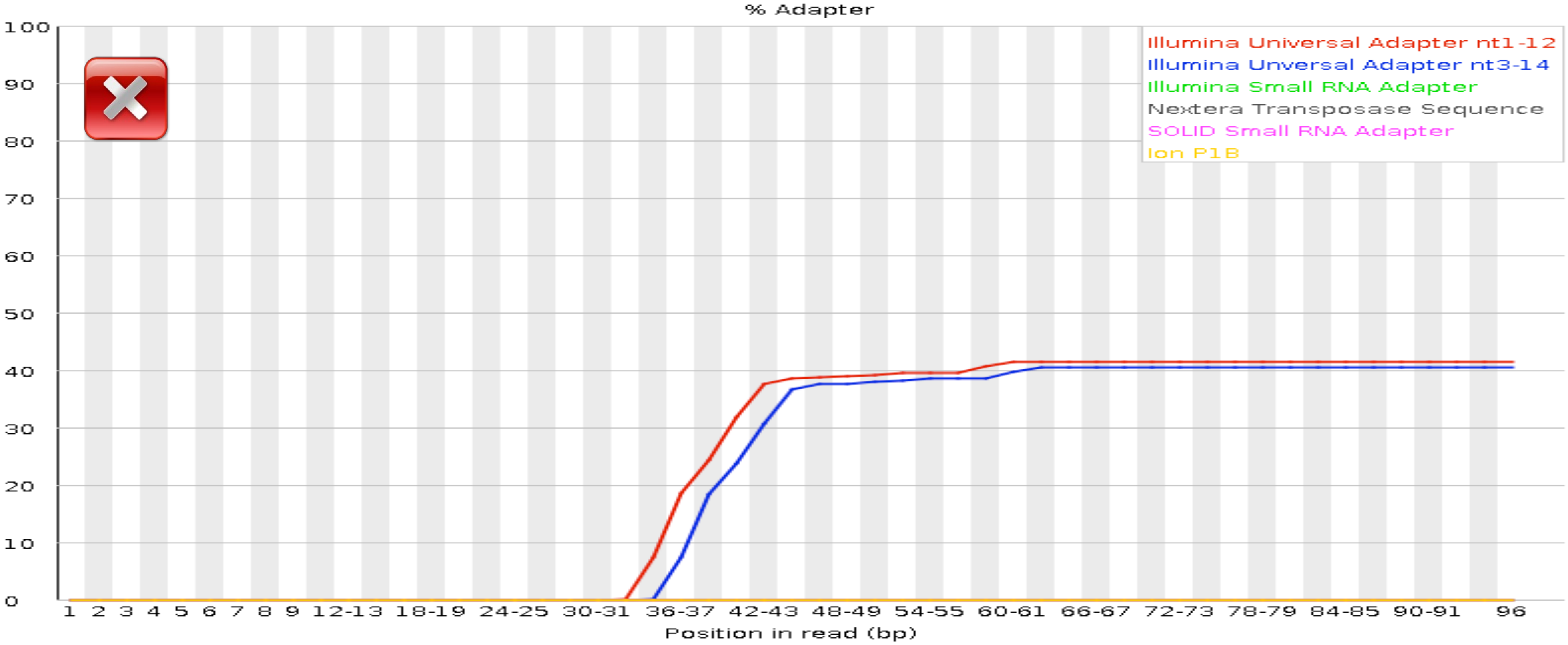
Overrepresented sequences

Sequence	Count	Percentage	Possible Source
GGAAGAGCACACGTCTGAACTCCAGTCACCAGATCATCTCGTATGCCGTC	75874	1.5613887498682963	TruSeq Adapter, Index 7 (100% over 50bp)
GGAAGAGCACACGTCTGAACTCCAGTCACCGATGTATCTCGTATGCCGTC	7636	0.15713900010536297	TruSeq Adapter, Index 2 (100% over 50bp)
GGAAGAGCACACGTCTGAACTCCAGTCACACAGTGATCTCGTATGCCGTC	7539	0.1551428656095248	TruSeq Adapter, Index 5 (100% over 50bp)
GGAAGAGCACACGTCTGAACTCCAGTCACGCCAATATCTCGTATGCCGTC	5117	0.10530123933199874	TruSeq Adapter, Index 6 (100% over 50bp)

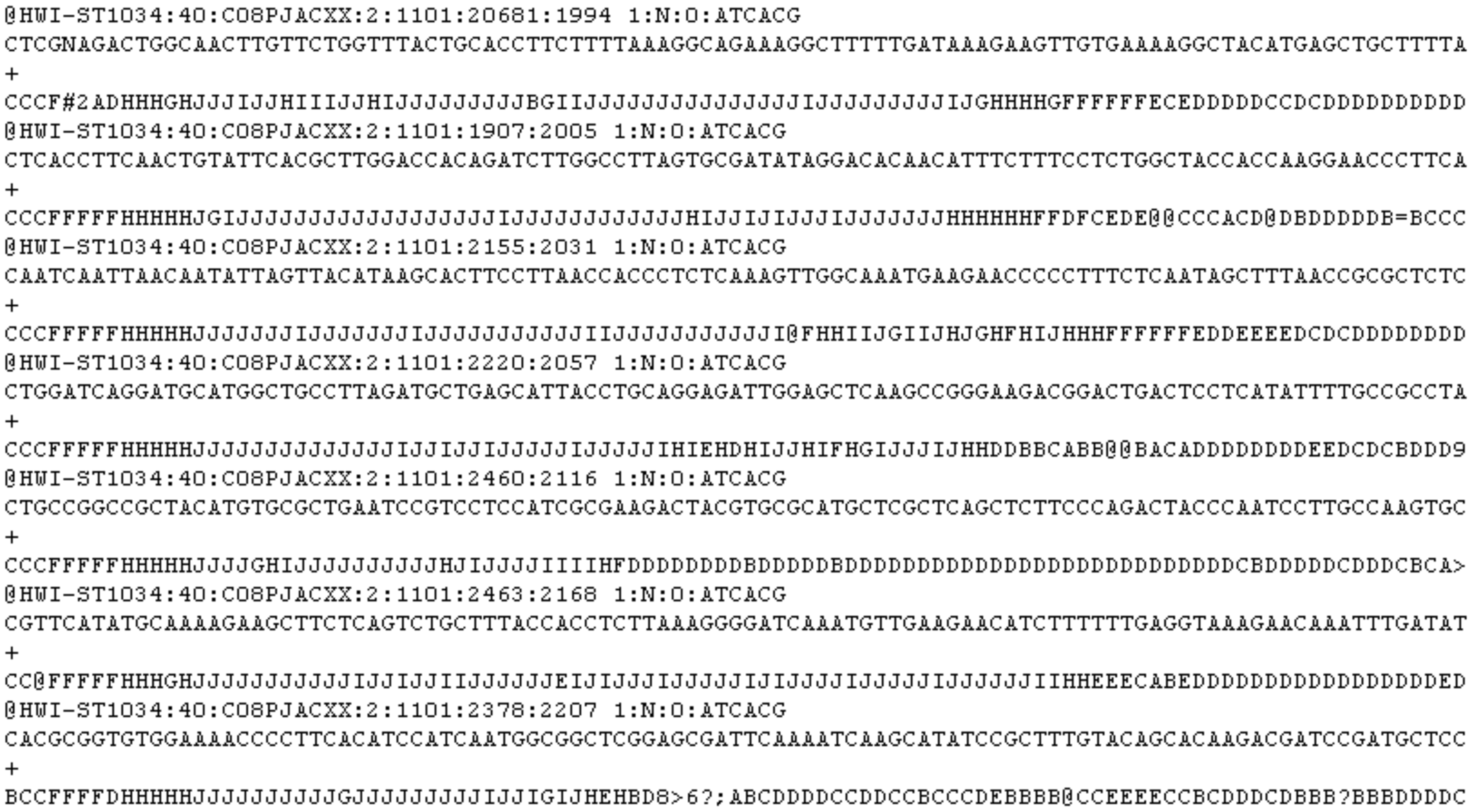
- Can be normal and biologically meaningful
  - highly expressed transcripts
  - high copy number repeats
  - Less diverse library (amplicons)

Adapter Content - FastQC





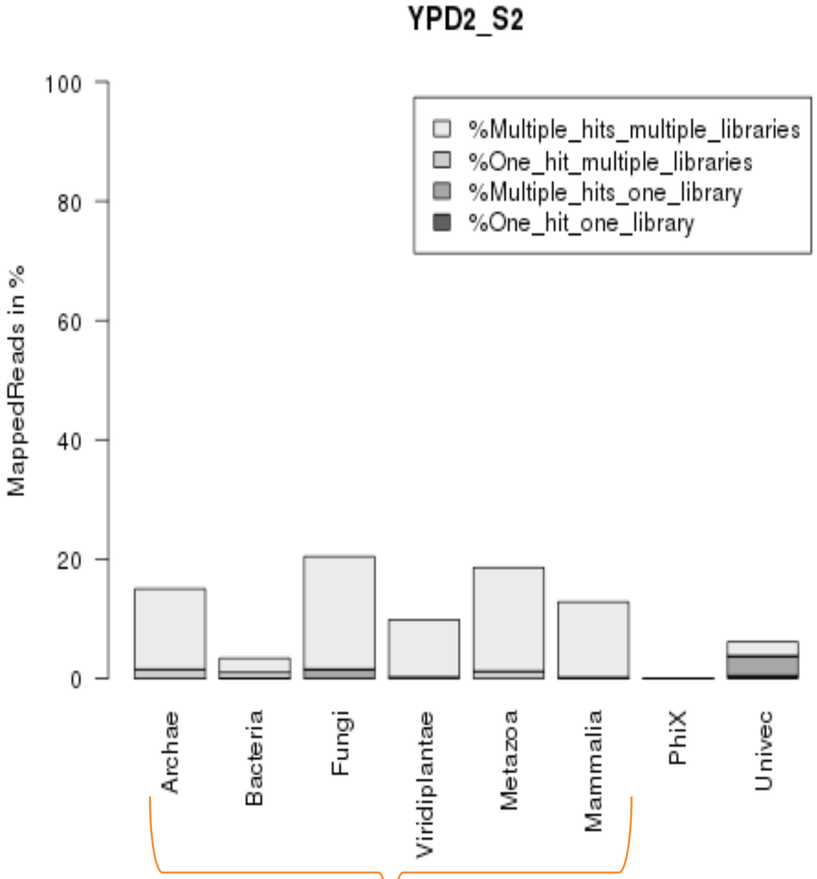
Millions of reads with base resolution



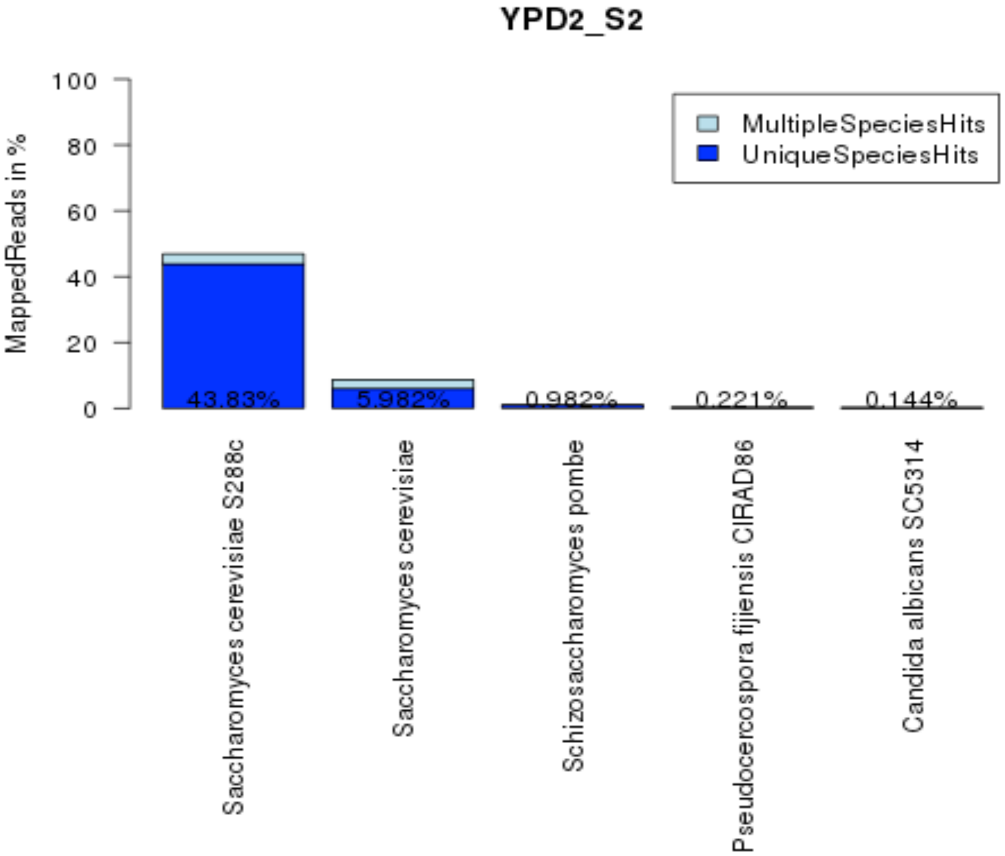
```
@HWI-ST1034:40:C08PJACXX:2:1101:20681:1994 1:N:0:ATCACG
CTCGNAGACTGGCAACTTGTTCTGGTTTACTGCACCTTCTTTTAAAGGCAGAAAGGCTTTTTGATAAAGAAGTTGTGAAAAGGCTACATGAGCTGCTTTTA
+
CCCF#2ADHHHGHJJJIJJHIIIJJHIJJJJJJJJJBGIIJJJJJJJJJJJJJJJJIJJJJJJJJJIJGHHHHGFFFFFFECEDDDDDCCDCDDDDDDDDDD
@HWI-ST1034:40:C08PJACXX:2:1101:1907:2005 1:N:0:ATCACG
CTCACCTTCAACTGTATTCACGCTTGGACCACAGATCTTGGCCTTAGTGCGATATAGGACACAACATTTCTTTCCTCTGGCTACCACCAAGGAACCCTTCA
+
CCCFFFFFHHHHHJGIJJJJJJJJJJJJJJJJJJJJIJJJJJJJJJJJJHIJJIJIJJJIJJJJJJJHHHHHHFFDFCEDE@@CCCACD@DBDDDDDB=BCCC
@HWI-ST1034:40:C08PJACXX:2:1101:2155:2031 1:N:0:ATCACG
CAATCAATTAACAATATTAGTTACATAAGCACTTCCTTAACCACCCTCTCAAAGTTGGCAAATGAAGAACCCCCTTTCTCAATAGCTTTAACCGCGCTCTC
+
CCCFFFFFHHHHHJJJJJJIJJJJJJJIJJJJJJJJJJJIIJJJJJJJJJJJI@FHHIIJGIIJHJGHFHIJHHHFFFFFFEDDEEEEDCDCDDDDDDDD
@HWI-ST1034:40:C08PJACXX:2:1101:2220:2057 1:N:0:ATCACG
CTGGATCAGGATGCATGGCTGCCTTAGATGCTGAGCATTACCTGCAGGAGATTGGAGCTCAAGCCGGGAAGACGGACTGACTCCTCATATTTTGCCGCCTA
+
CCCFFFFFHHHHHJJJJJJJJJJJJJJIJJIJJIJJJJJIJJJJJIHIEHDHIJJHIFHGIJJJIJHHDDBBCABB@@BACADDDDDDDDEEDCDCBDDD9
@HWI-ST1034:40:C08PJACXX:2:1101:2460:2116 1:N:0:ATCACG
CTGCCGGCCGCTACATGTGCGCTGAATCCGTCCTCCATCGCGAAGACTACGTGCGCATGCTCGCTCAGCTCTTCCCAGACTACCCAATCCTTGCCAAGTGC
+
CCCFFFFFHHHHHJJJGHIJJJJJJJJJJHJIJJJJIIIIHFDDDDDDDDBDDDDDBDDDDDDDDDDDDDDDDDDDDDDDDDDDCBDDDDDCDDDCBCA>
@HWI-ST1034:40:C08PJACXX:2:1101:2463:2168 1:N:0:ATCACG
CGTTCATATGCAAAAGAAGCTTCTCAGTCTGCTTTACCACCTCTTAAAGGGGATCAAATGTTGAAGAACATCTTTTTTGAGGTAAAGAACAAATTTGATAT
+
CC@FFFFFHHHGHJJJJJJJJJJJIJJIJJIIJJJJJJEIJIJJJIJJJJJIJIJJJJIJJJJJIJJJJJJIIHHEEECABEDDDDDDDDDDDDDDDDED
@HWI-ST1034:40:C08PJACXX:2:1101:2378:2207 1:N:0:ATCACG
CACGCGGTGTGGAAAACCCCTTCACATCCATCAATGGCGGCTCGGAGCGATTCAAAATCAAGCATATCCGCTTTGTACAGCACAAGACGATCCGATGCTCC
+
BCCFFFFFDHHHHHJJJJJJJJJGJJJJJJJJJIJJIGIJHEHBD8>6?;ABCDDDDCCDDCCBCCCDEBBBB@CCEEEECCBCDDDCDBBB?BBBDDDDC
```

- How accurate was the sequencing → Fastqc
- Are these reads the intented ones → FastqScreen

Contamination Check - FastqScreen



rRNA-Content (Silva)



Mapping to RefSeq mRNAs
(all species)

Data preprocessing common tasks

1. Trimming: remove bad bases from the ends of the reads
    - Adapter sequence
    - Low quality bases

2. Filtering: remove bad reads
    - Low quality reads
    - Contaminating sequences
    - Low complexity reads (repeats)
    - Short (<20bp) reads – they slow down mapping software

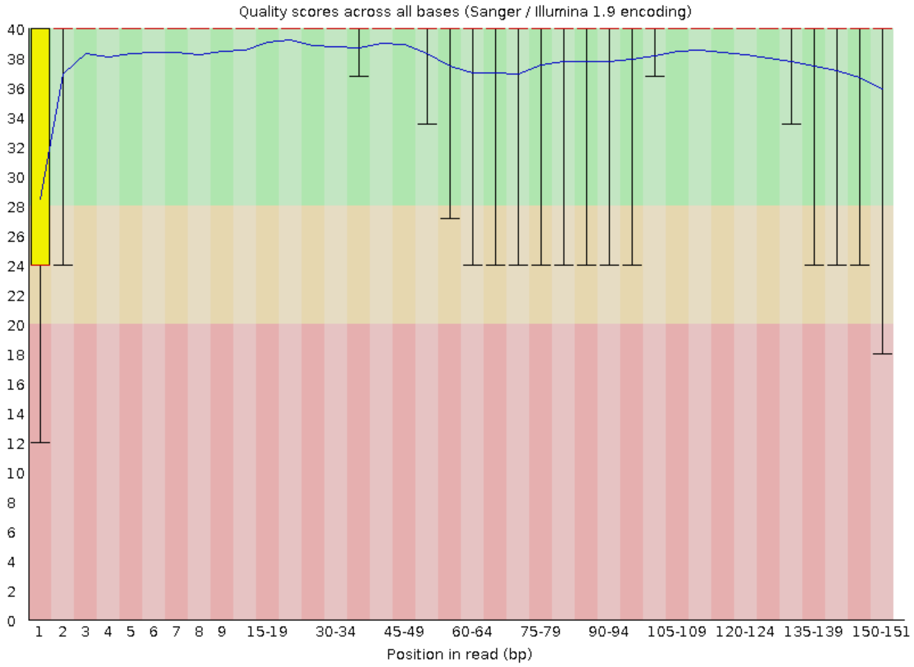
Data preprocessing software

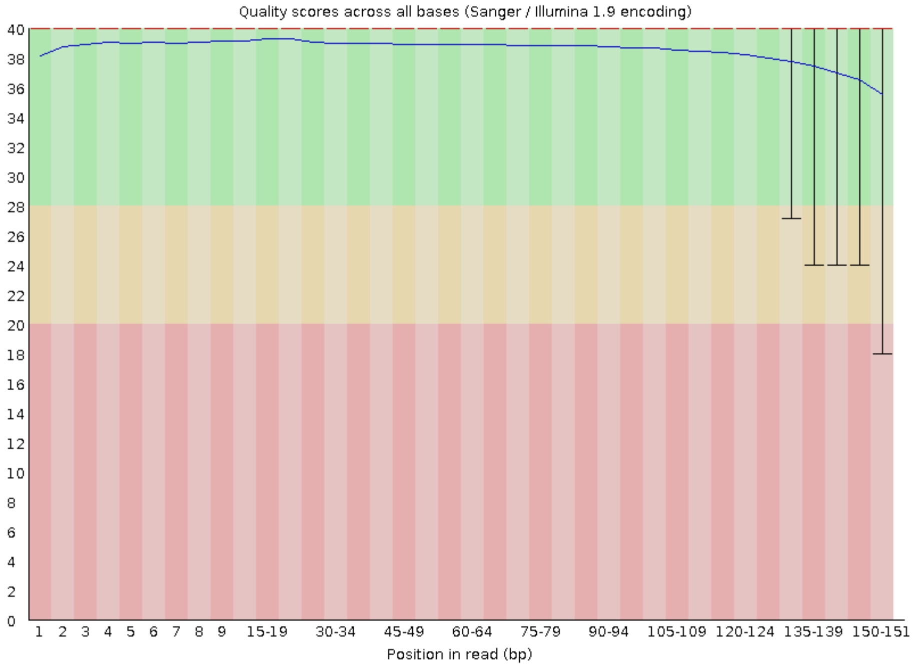
- fastp
  - https://github.com/OpenGene/fastp
  - Adapter trimming, quality trimming &filtering, ...
- Trimmomatic
  - https://github.com/usadellab/Trimmomatic
  - Adapter trimming, quality trimming &filtering, ...
- FlexBar (FAR)
  - https://github.com/seqan/flexbar
  - Flexible barcode detection and adapter removal
- cutadapt
  - https://cutadapt.readthedocs.io/en/v4.0/index.html
  - remove adaptors and types of wanted sequence
- TagCleaner
  - http://tagcleaner.sourceforge.net
  - Trim MIDs or adaptors, demultiplexing
- DeconSeq
  - http://deconseq.sourceforge.net
  - Remove potential contaminants

Example: Batch effect

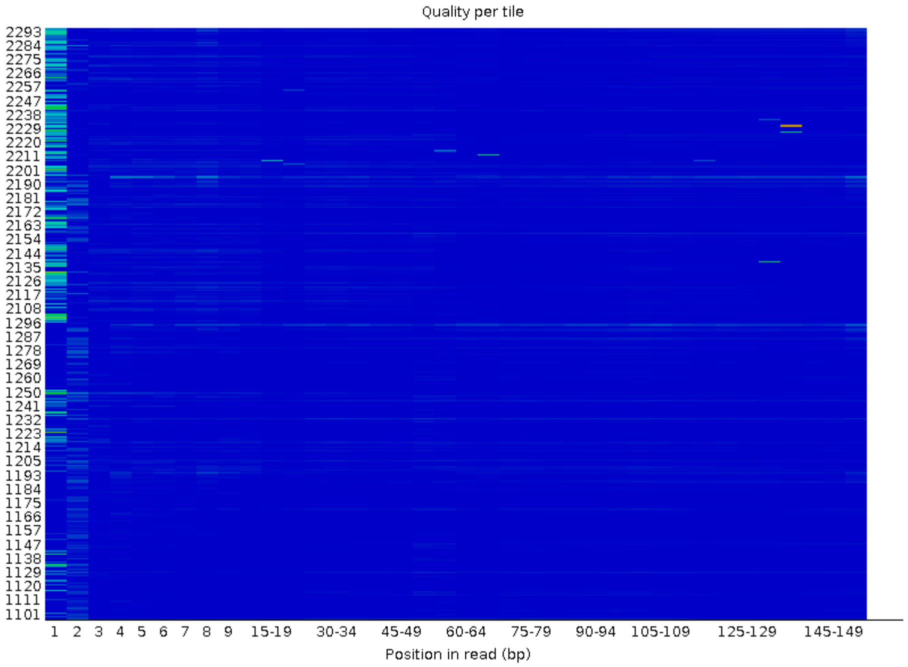
- The Batch effect is visible in the reads of RNA-seq samples done in two batches
- The Batch effect has no impact on final gene expression analysis

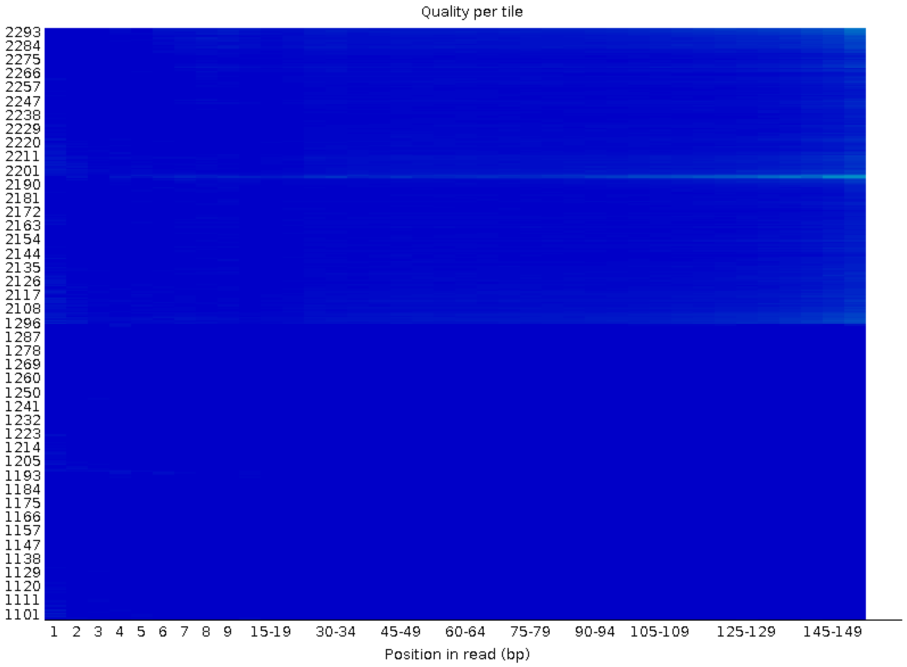
Per-base quality



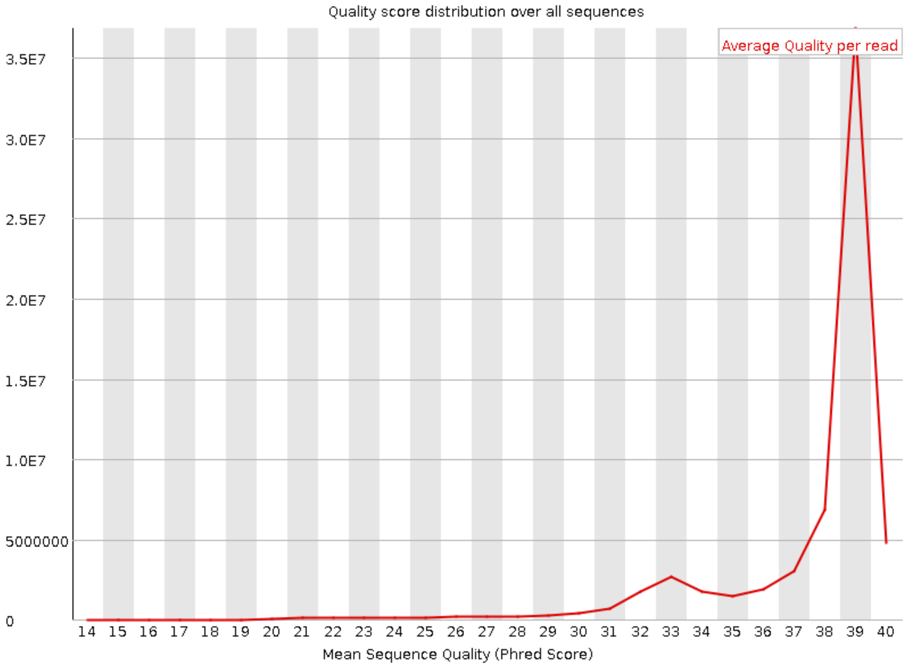


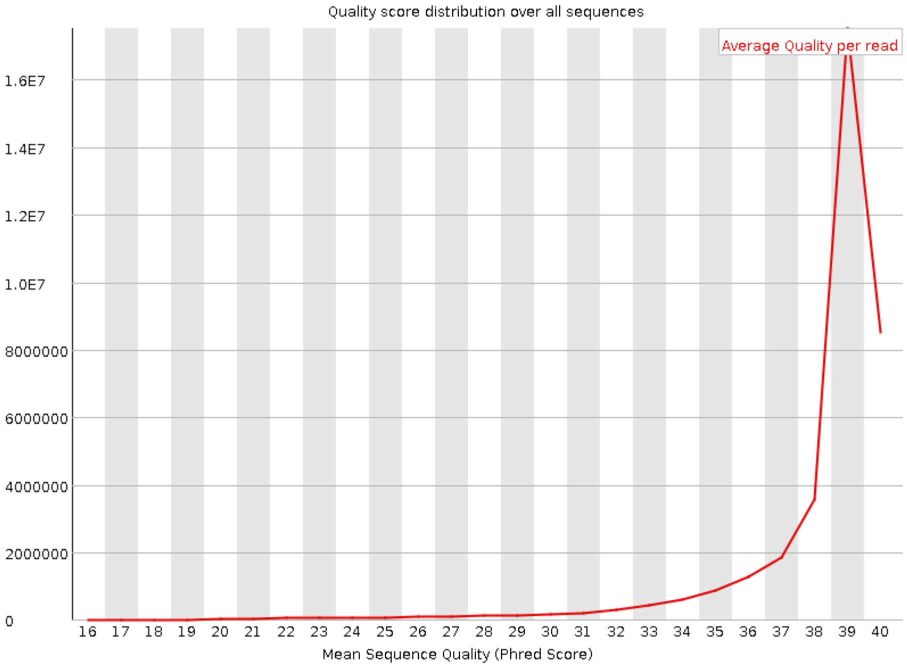
Per tile sequence quality



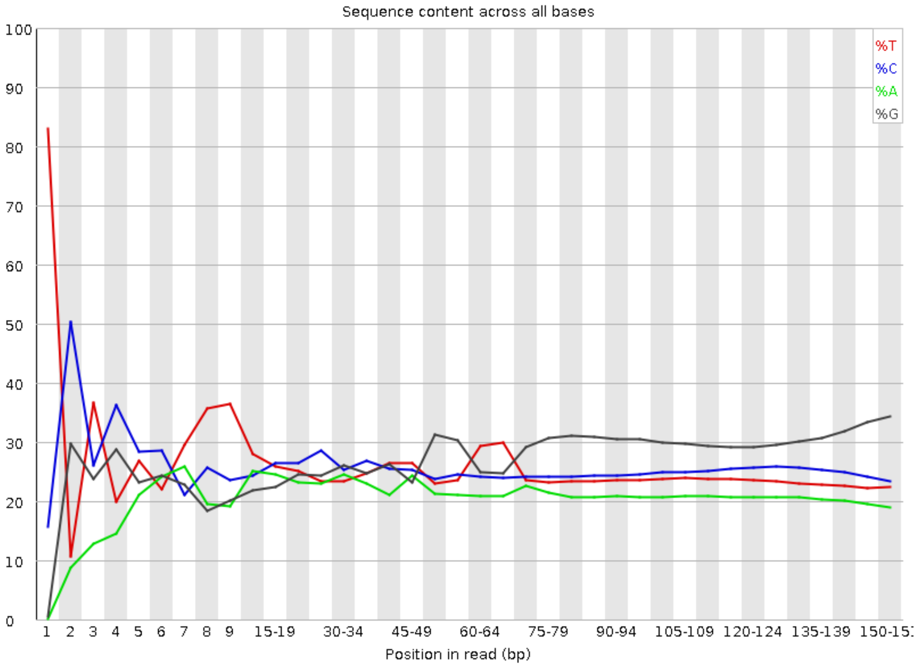


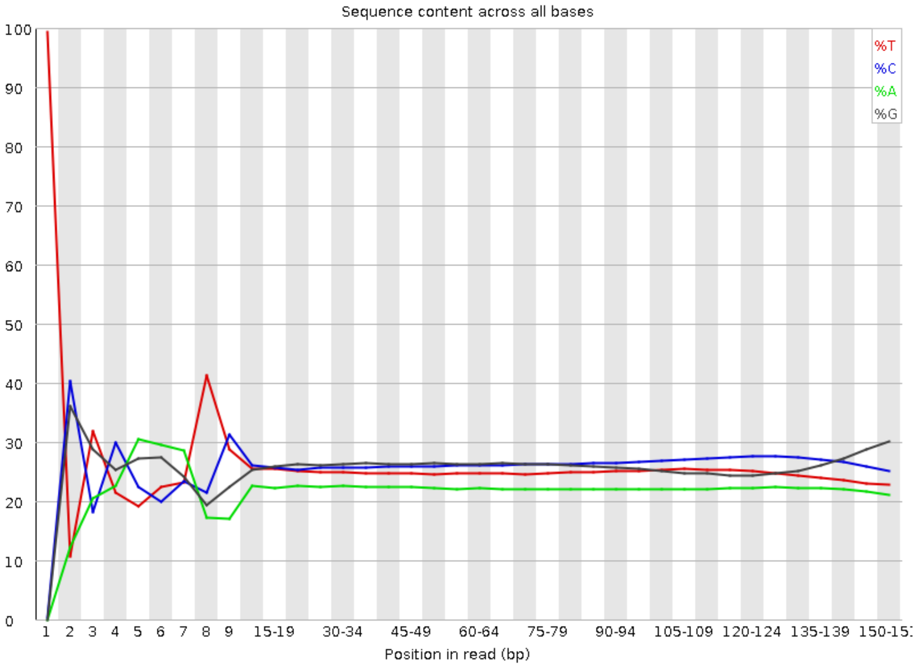
Per Sequence Quality





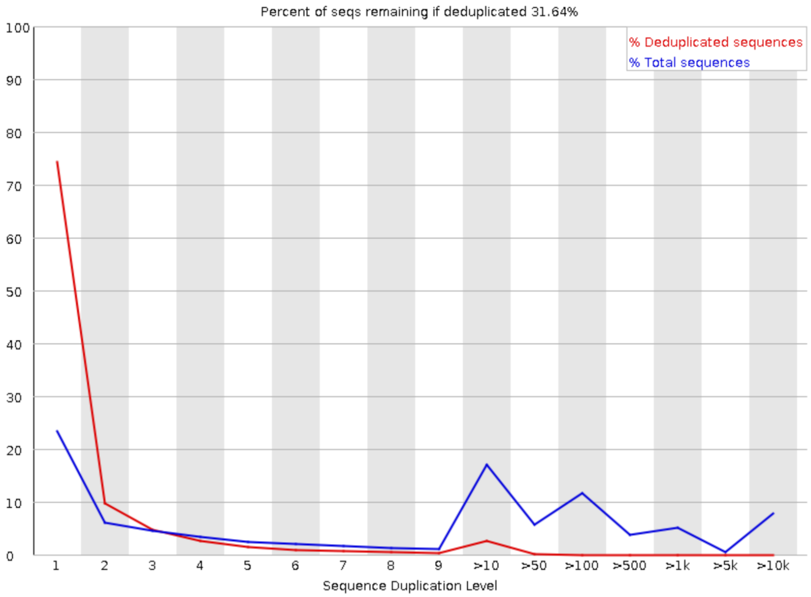
Per-base sequence content



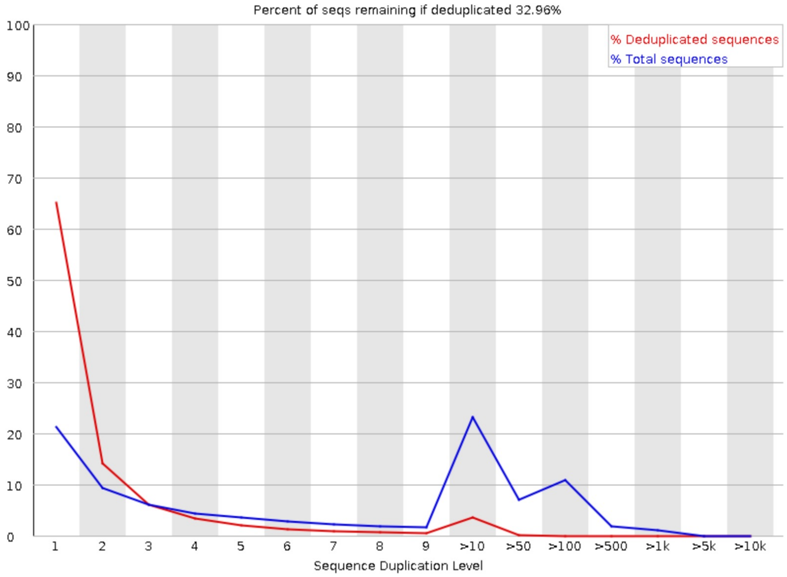


Duplication Levels

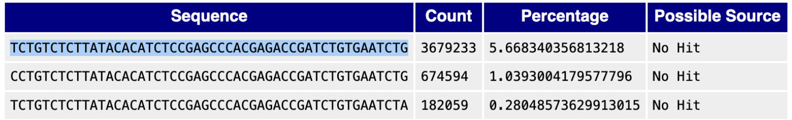
✗ **Sequence Duplication Levels**



✗ **Sequence Duplication Levels**



✗ **Overrepresented sequences**

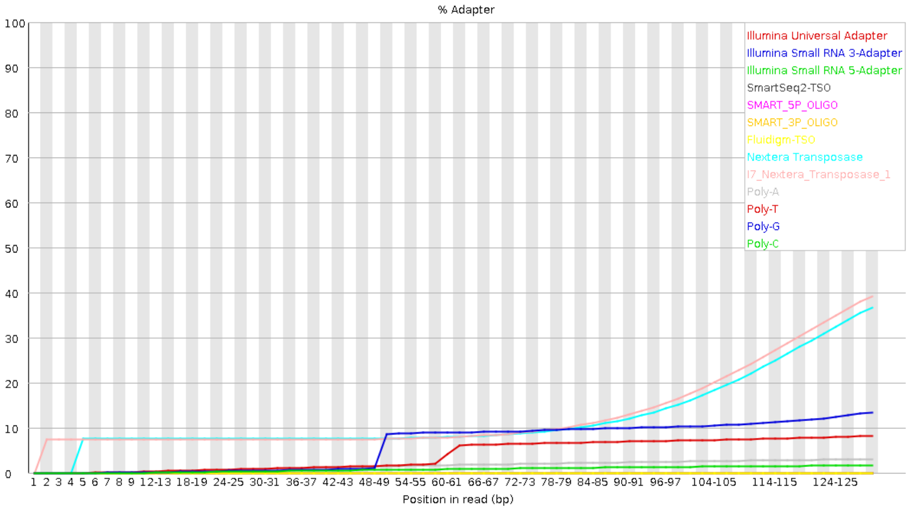


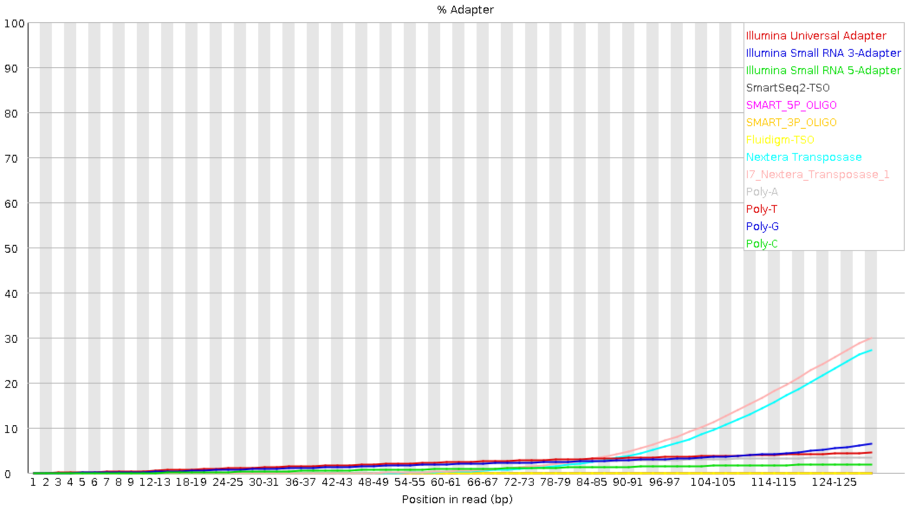
Sequence	Count	Percentage	Possible Source
TCTGTCTCTTATACACATCTCCGAGCCCACGAGACCGATCTGTGAATCTG	3679233	5.668340356813218	No Hit
CCTGTCTCTTATACACATCTCCGAGCCCACGAGACCGATCTGTGAATCTG	674594	1.0393004179577796	No Hit
TCTGTCTCTTATACACATCTCCGAGCCCACGAGACCGATCTGTGAATCTA	182059	0.28048573629913015	No Hit

✓ **Overrepresented sequences**

No overrepresented sequences

Adapter Content





Recommendations

- Always generate quality control plots visualizing key characteristics for all libraries
- Trim and/or filter data if needed
- Applications where erroneous reads are of concern:
  - de novo assembly
  - low coverage variant calling
- Applications that are more tolerant to low quality bases
  - RNA-seq