RNA-seq Quality Control

Before the analysis begins

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KTH Royal Institute of Technology

Stockholm University

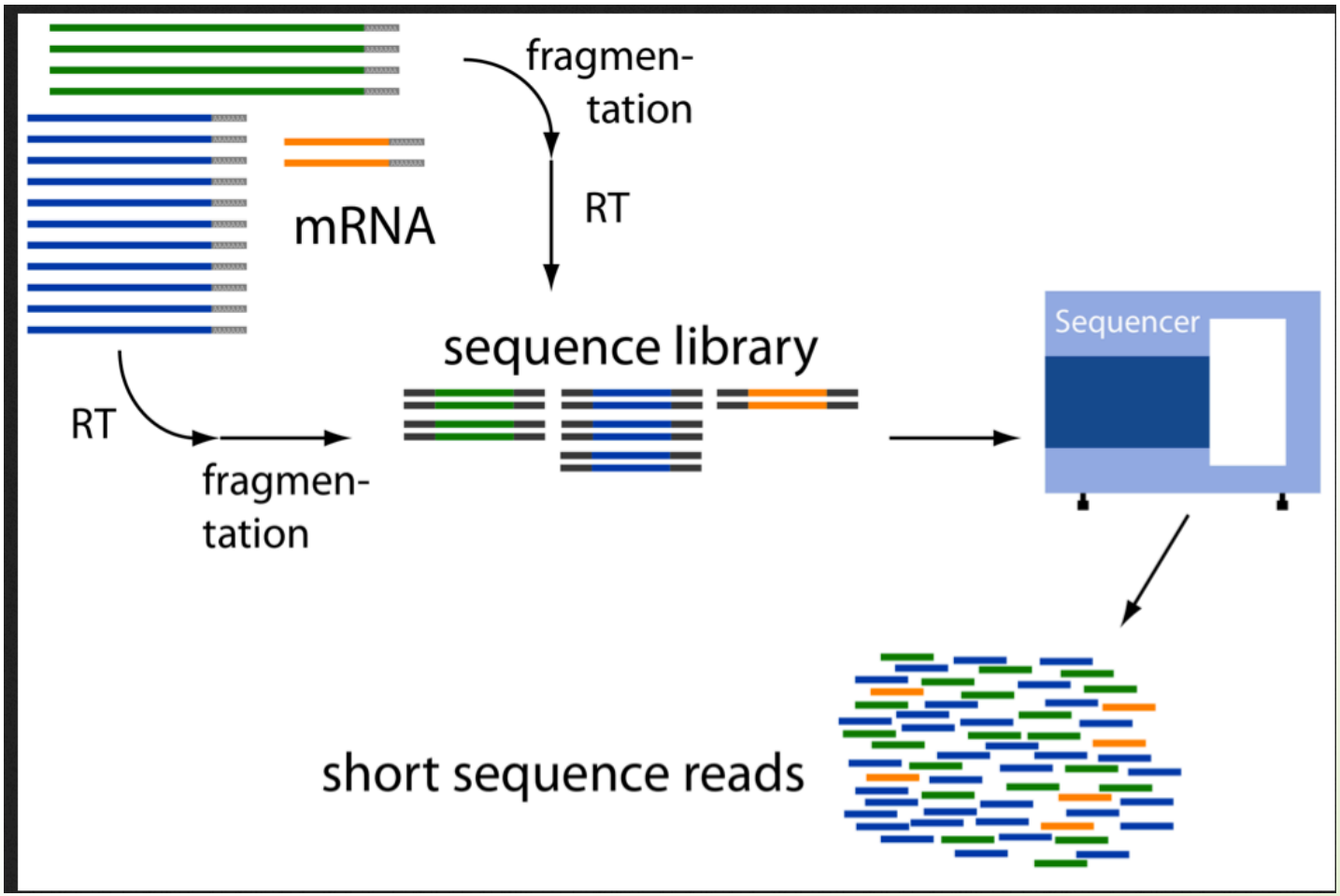
Uppsala Universitet

SciLifeLab

Overview

- Introduction
- FastQC – read based QC
- RseQC – mapping based QC
- PCA
- Spike-in controls
- Experimental design

RNA-seq libraries



What could go wrong?

What could go wrong?

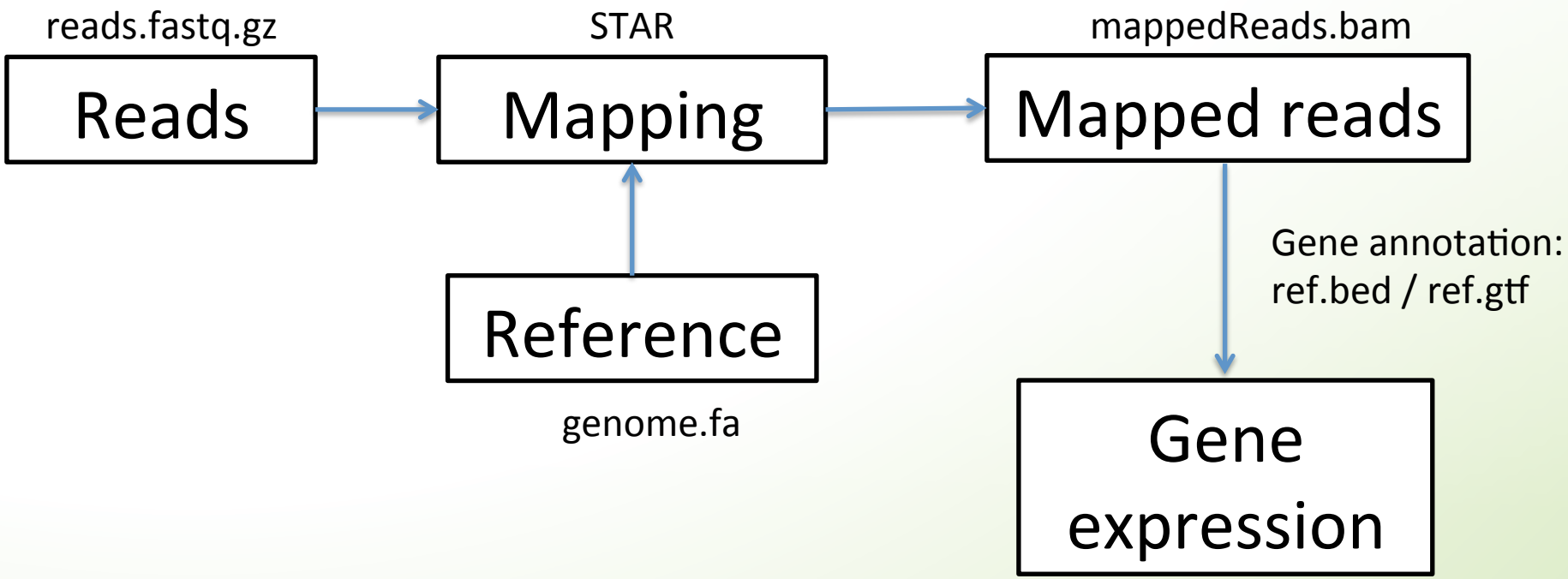
- RNA quality:
  - Degradation
  - Contaminations (pathogens or other sources)
  - GC-bias
  - Nuclear vs organellar reads
- Library prep:
  - Failed reactions
  - RNA / Adapter ratios – primer dimers
  - Clonal duplicates
  - Chimeric reads
  - Contaminations
- Sequencing:
  - Base calling errors
  - Uncalled bases
  - Low quality bases (3' end)
  - Contaminations
  - Sequence complexity

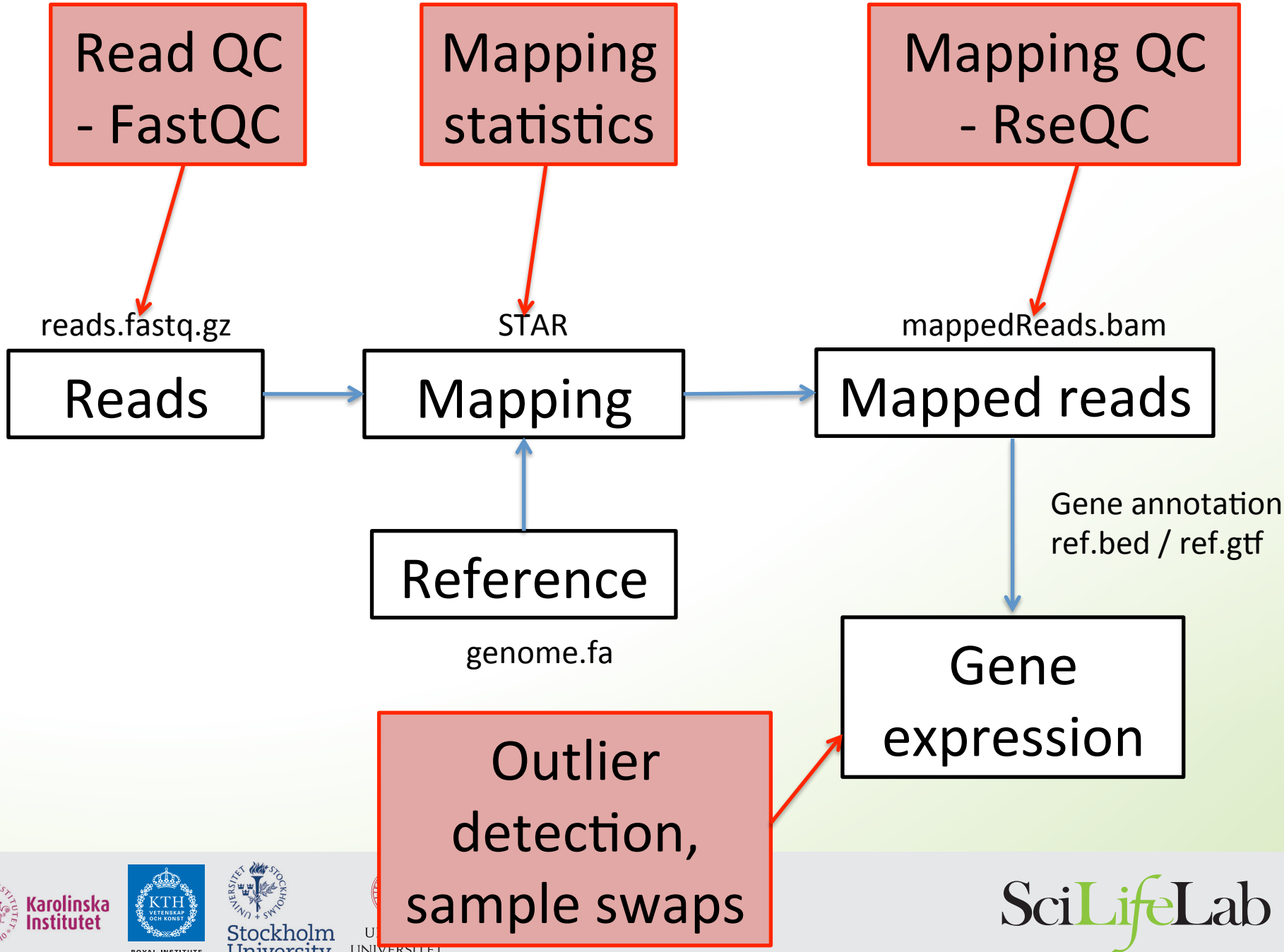
From samples to RNA to reads

-might not be what you think they are

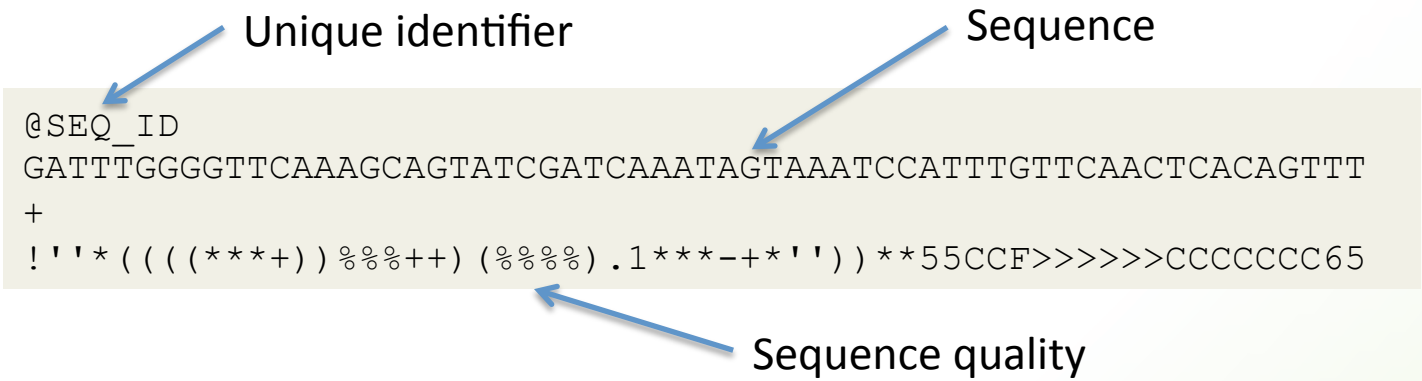
- Mixing samples
  - 30 samples with 5 steps from samples to reads has 24 300 000 potential mix ups of samples
  - Error rate 1/ 100 with 5 steps suggest that one of every 20 sample is mislabeled
- Experiments go wrong
  - 30 samples with 5 steps from samples to reads has 150 potential steps for errors
  - Error rate 1/100 with 5 steps suggest that one of every 20 samples the reads does not represent the sample
- Combine the two steps and approximately one of every 10 samples are wrong

RNA-seq analysis workflow



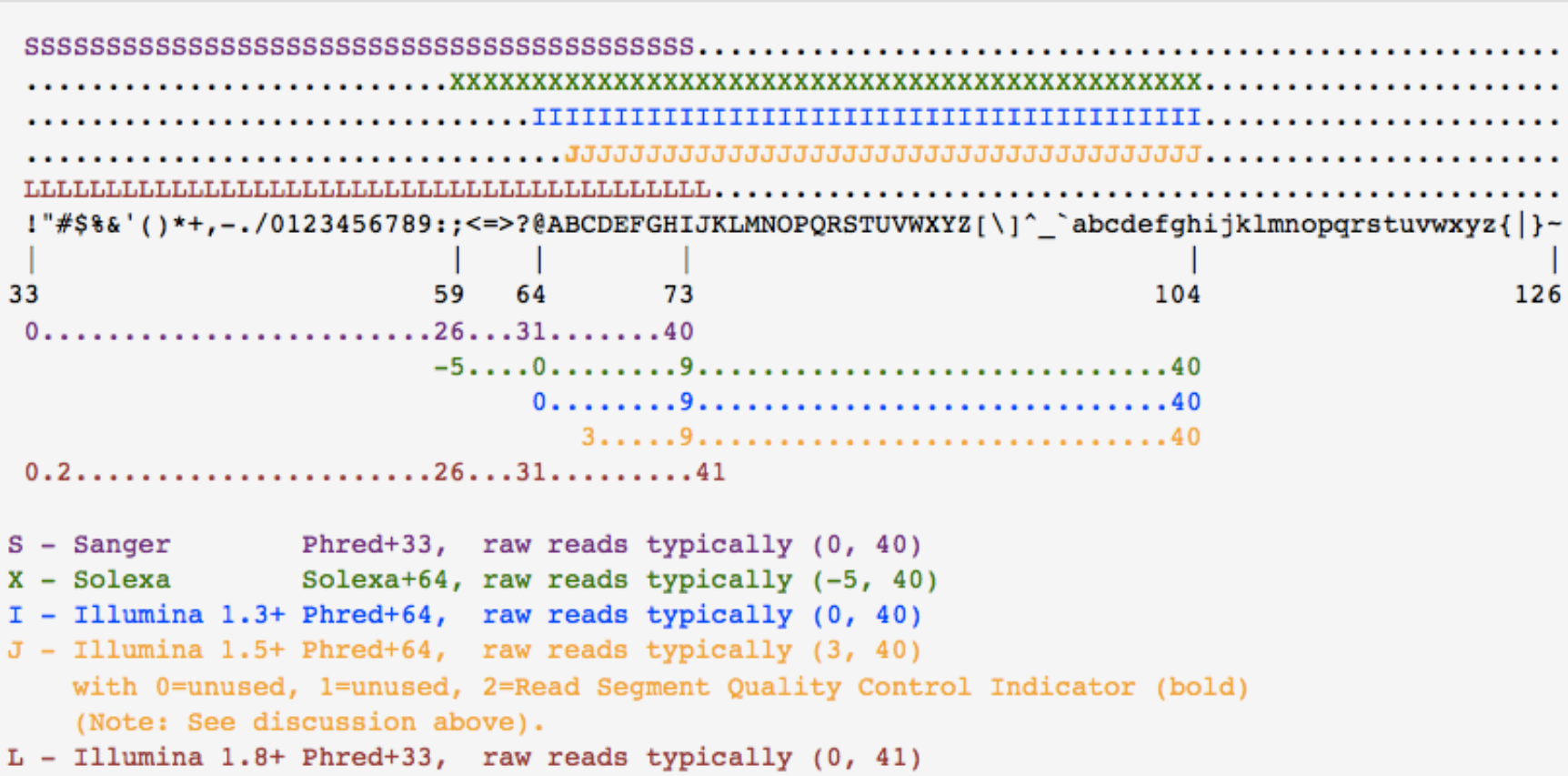

Read QC
- FastQC
Mapping
statistics
Mapping QC
- RseQC
reads.fastq.gz
STAR
mappedReads.bam
Reads
Mapping
Mapped reads
Gene annotation:
ref.bed / ref.gtf
Reference
genome.fa
Gene
expression
Outlier
detection,
sample swaps

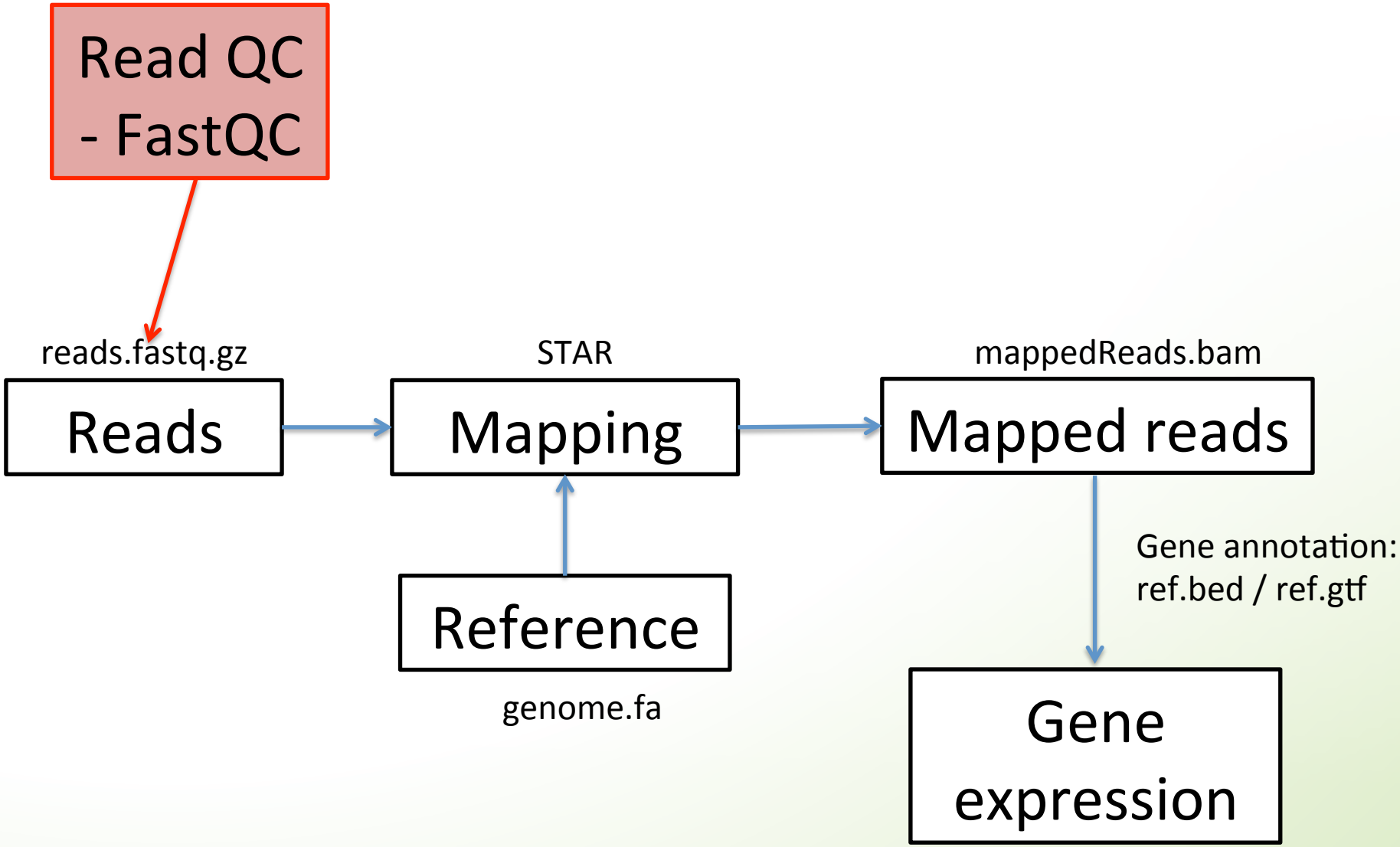
Fastq – read file format



Paired end data usually in format sampleX_1.fastq and sampleX_2.fastq with same SEQ_ID for both mate pairs, followed by /1 and /2 (or _f and _r)

Fastq – read file format

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Mapped reads
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expression

Basic read metrics with FastQC

A program that analyses some of the basic metrics on fastq raw read files.

- Quality
- Length
- Sequence bias
- GC content
- Repeated sequences
- Adapter contamination

Code

```
$ module load bioinfo-tools
$ module load FastQC/0.11.2

$ fastqc –o outdir seqfile.fastq
# multiple files:
$ fastqc –o outdir seqfile_*.fastq
```

http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

FastQC report

FastQC Report

Summary

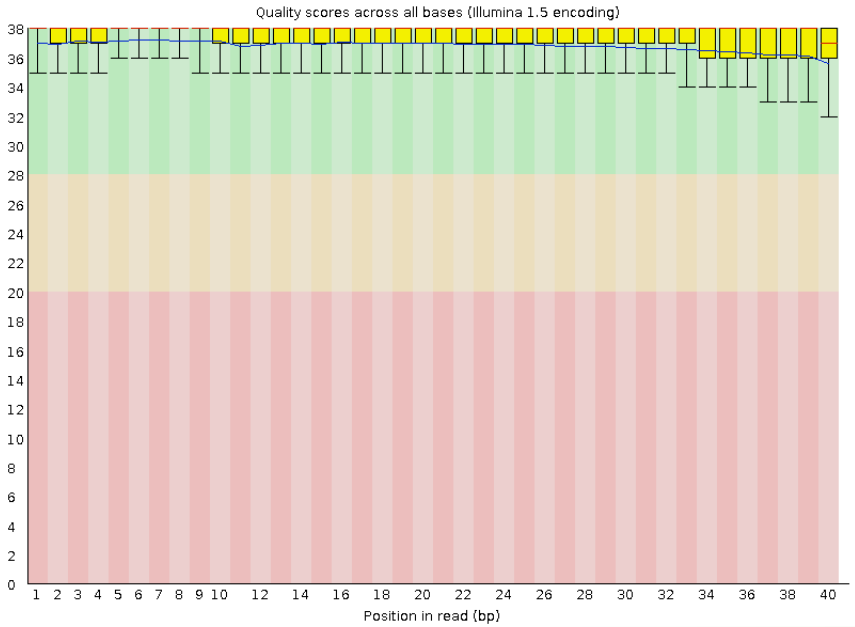
- Basic Statistics
- Per base sequence quality
- Per tile sequence quality
- Per sequence quality scores
- Per base sequence content
- Per sequence GC content
- Per base N content
- Sequence Length Distribution
- Sequence Duplication Levels
- Overrepresented sequences
- Adapter Content
- Kmer Content

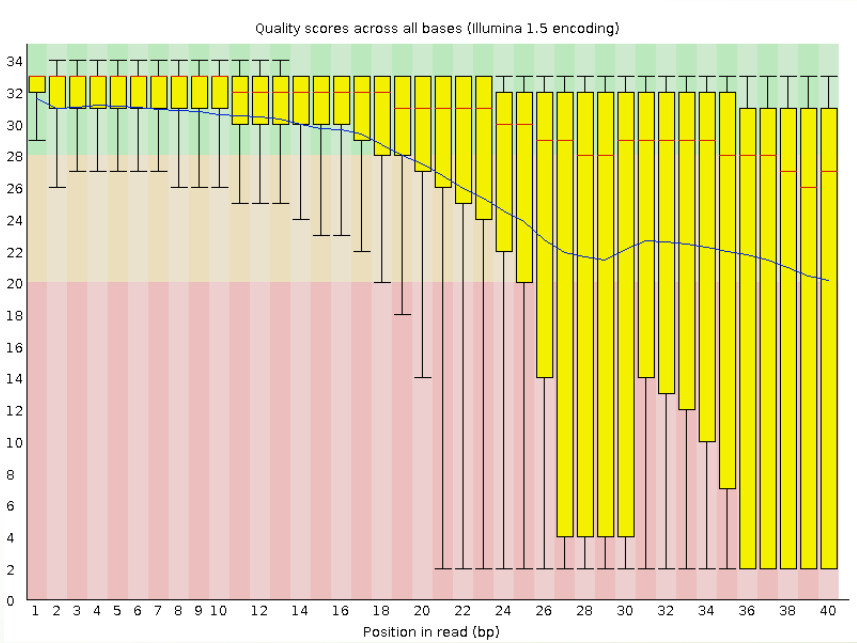
Basic Statistics

Measure	Value
Filename	bad_sequence.txt
File type	Conventional base calls
Encoding	Illumina 1.5
Total Sequences	395288
Sequences flagged as poor quality	0
Sequence length	40
%GC	47

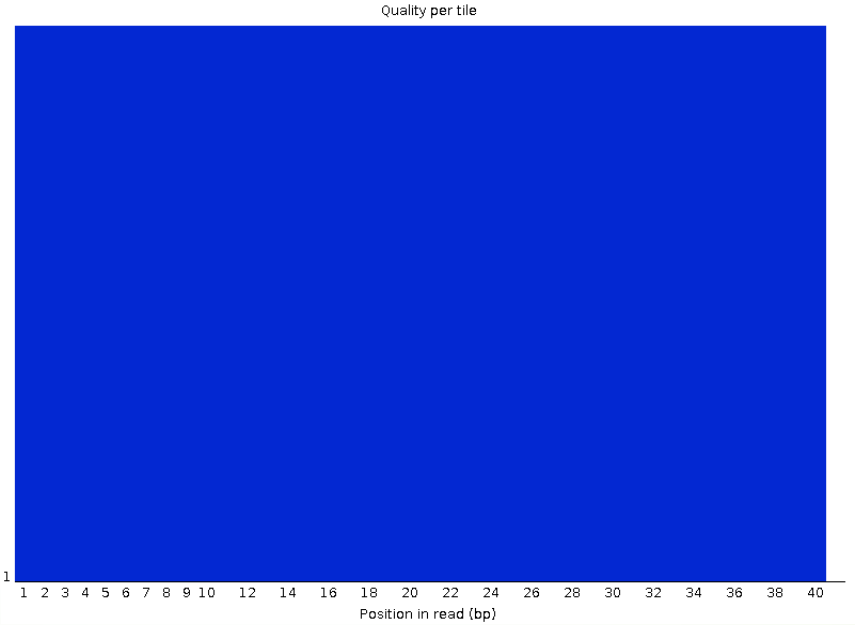
Per base sequence quality

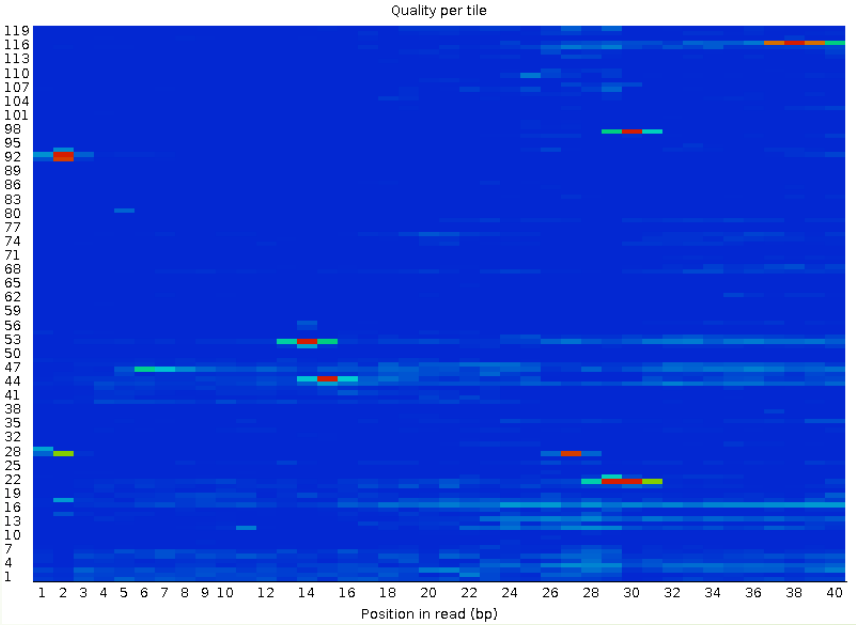
Per base sequence quality





Per tile sequence quality





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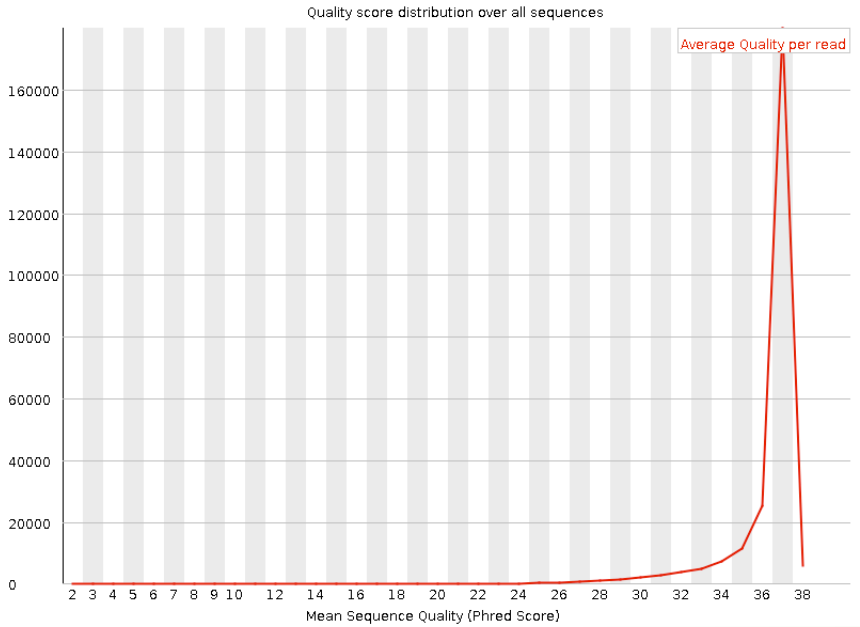
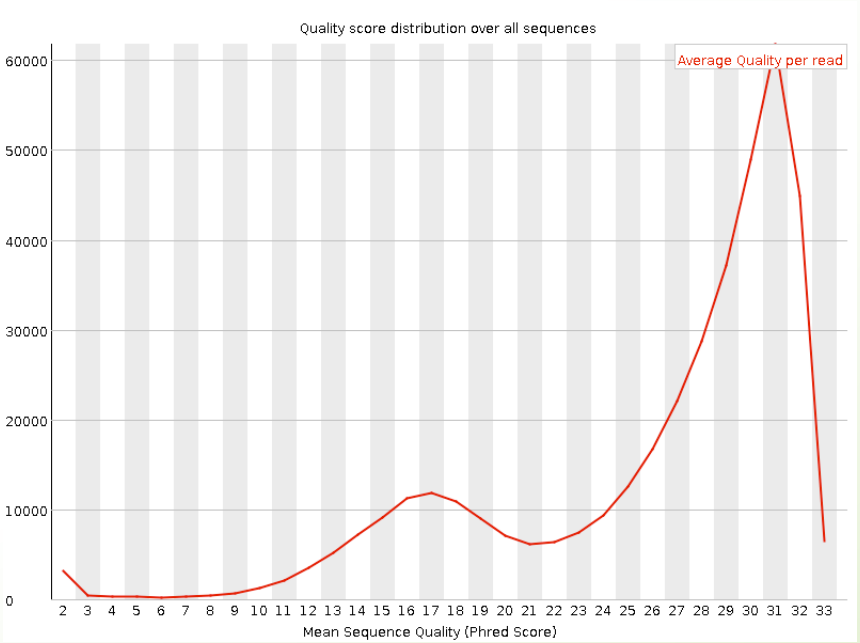
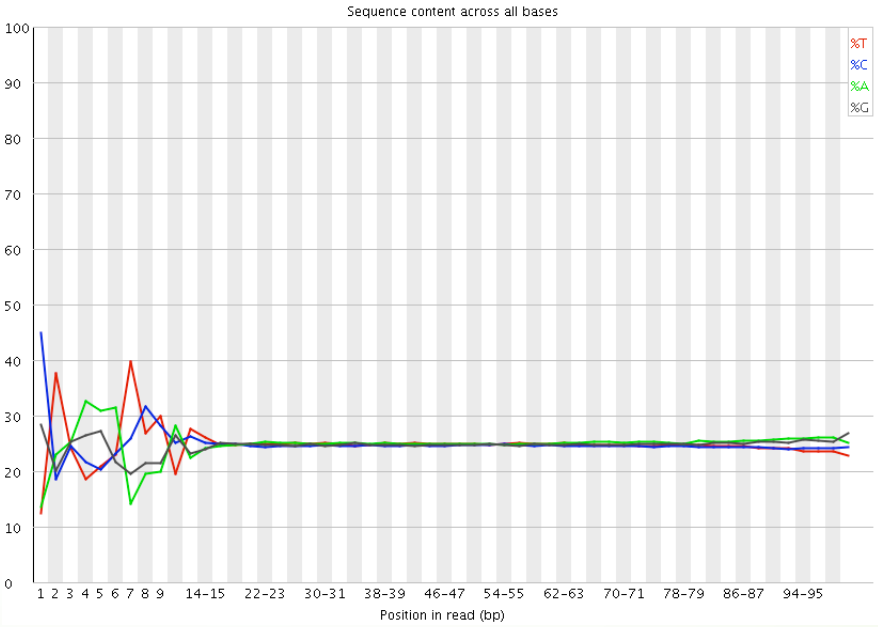
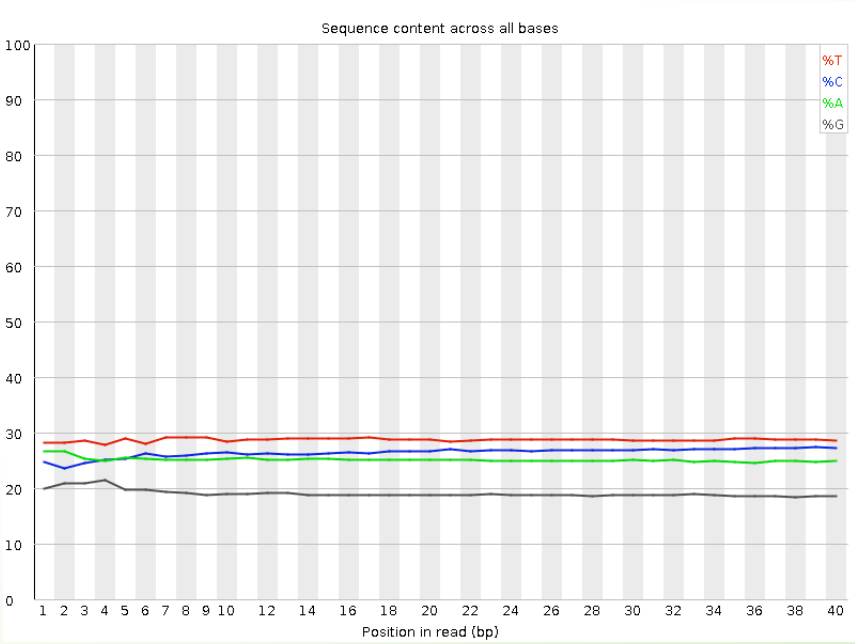
Per sequence quality scores



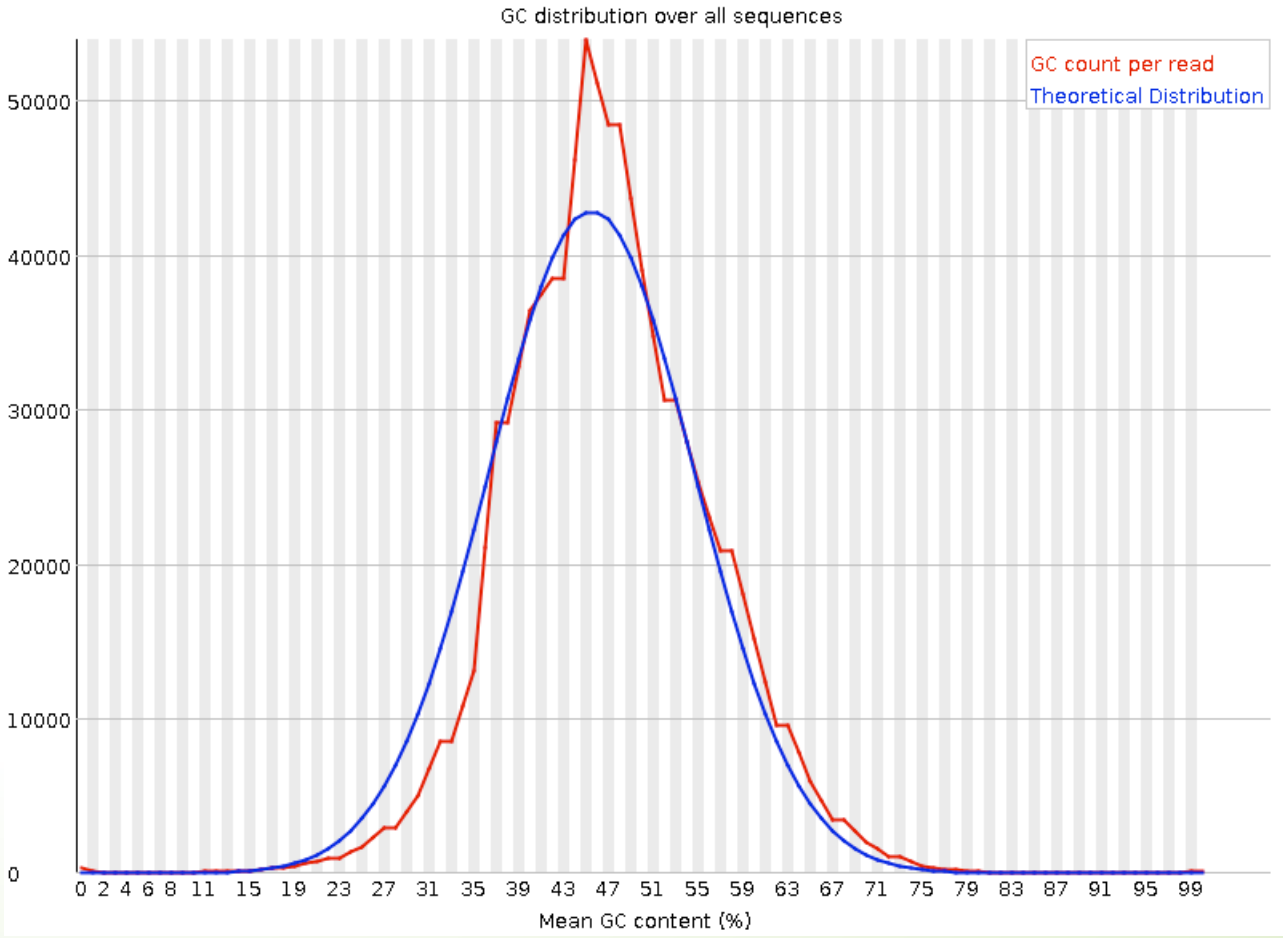


Per base sequence content

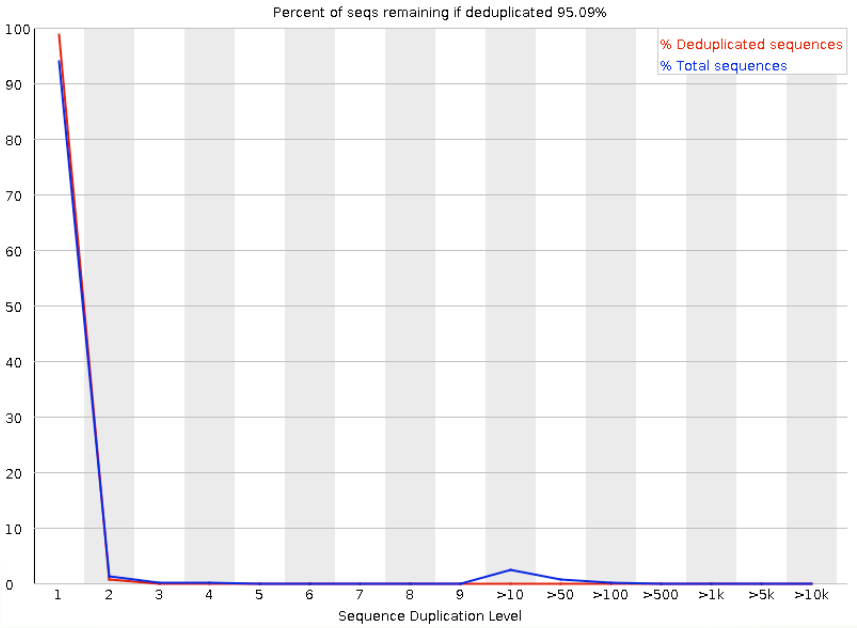


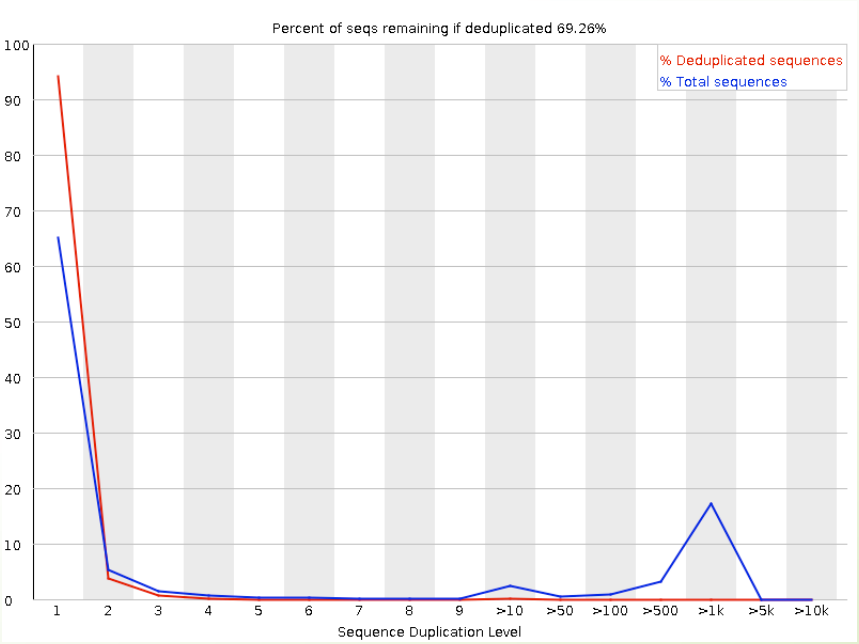


Per sequence GC content



Sequence Duplication Levels

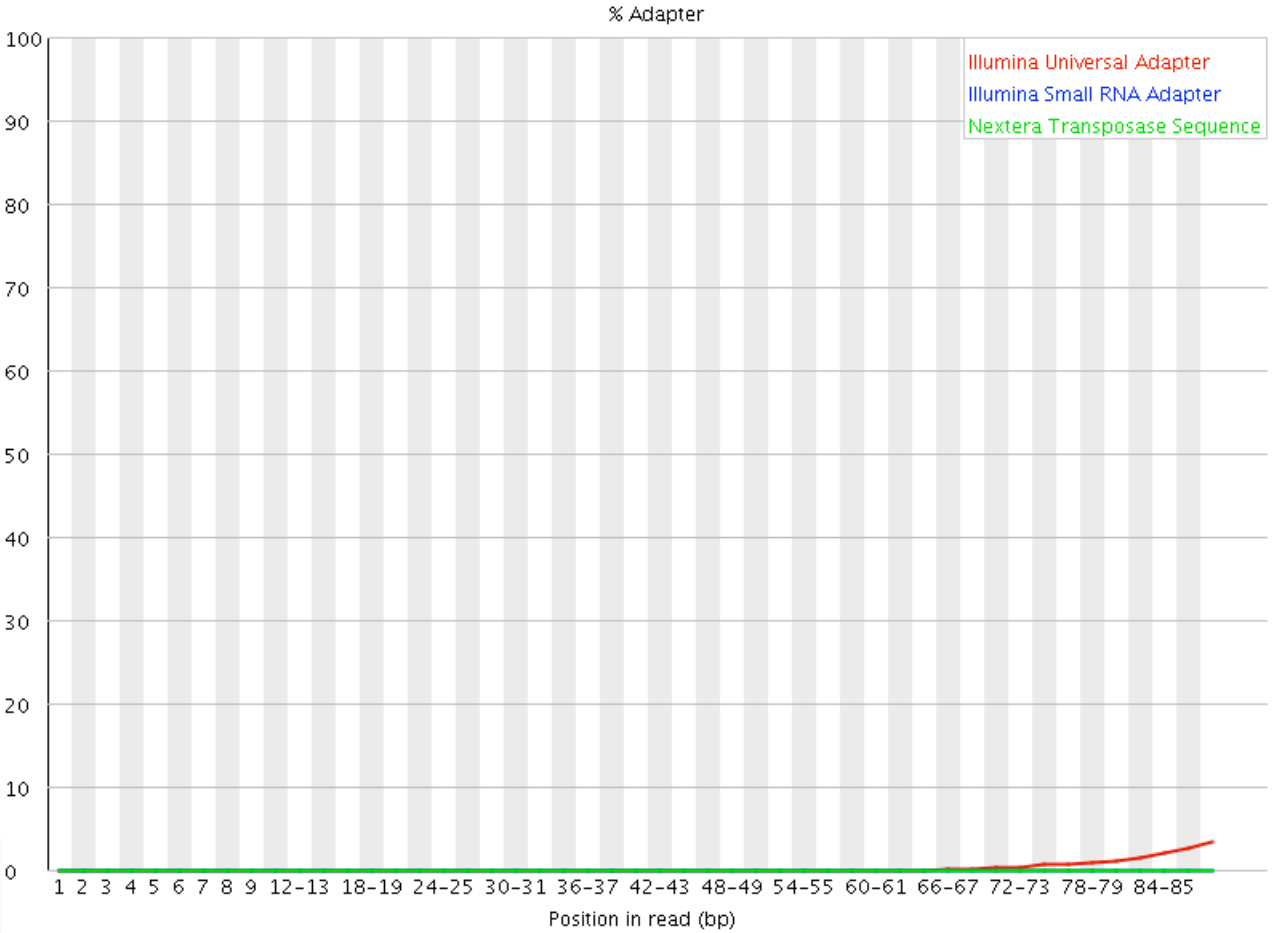




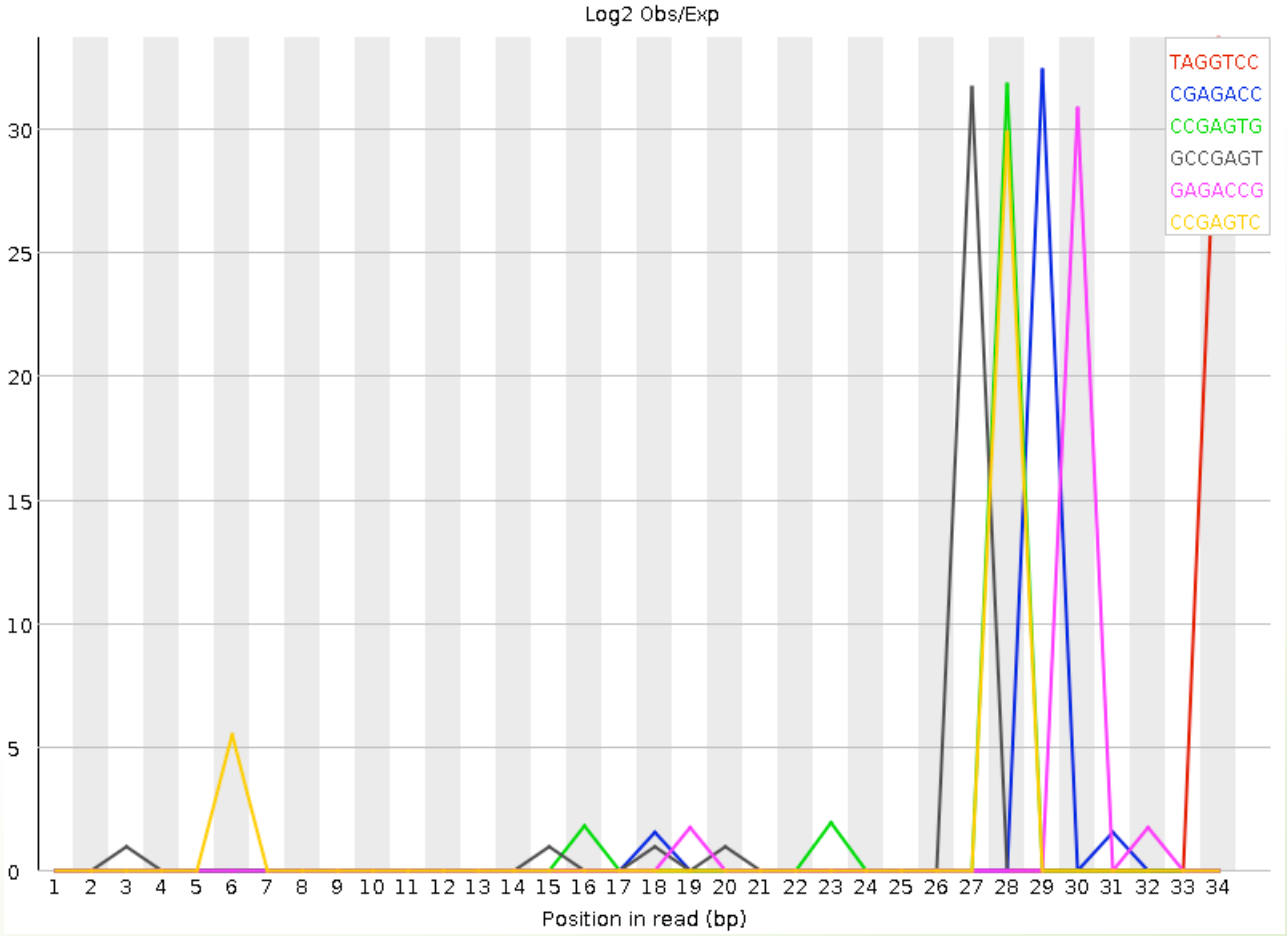
Overrepresented sequences

Sequence	Count	Percentage	Possible Source
AGAGTTTTATCGCTTCCATGACGCAGAAGTTAACACTTTC	2065	0.5224039181558763	No Hit
GATTGGCGTATCCAACCTGCAGAGTTTTATCGCTTCCATG	2047	0.5178502762542754	No Hit
ATTGGCGTATCCAACCTGCAGAGTTTTATCGCTTCCATGA	2014	0.5095019327680071	No Hit
CGATAAAAATGATTGGCGTATCCAACCTGCAGAGTTTTAT	1913	0.4839509420979134	No Hit
GTATCCAACCTGCAGAGTTTTATCGCTTCCATGACGCAGA	1879	0.47534961850600066	No Hit
AAAAATGATTGGCGTATCCAACCTGCAGAGTTTTATCGCT	1846	0.4670012750197325	No Hit
TGATTGGCGTATCCAACCTGCAGAGTTTTATCGCTTCCAT	1841	0.46573637449150995	No Hit
AACCTGCAGAGTTTTATCGCTTCCATGACGCAGAAGTTAA	1836	0.46447147396328753	No Hit
GATAAAAATGATTGGCGTATCCAACCTGCAGAGTTTTATC	1831	0.4632065734350651	No Hit
AAATGATTGGCGTATCCAACCTGCAGAGTTTTATCGCTTC	1779	0.45005160794155147	No Hit
ATGATTGGCGTATCCAACCTGCAGAGTTTTATCGCTTCCA	1779	0.45005160794155147	No Hit
AATGATTGGCGTATCCAACCTGCAGAGTTTTATCGCTTCC	1760	0.4452449859343061	No Hit
AAAATGATTGGCGTATCCAACCTGCAGAGTTTTATCGCTT	1729	0.4374026026593269	No Hit
CGTATCCAACCTGCAGAGTTTTATCGCTTCCATGACGCAG	1713	0.43335492096901496	No Hit
ATCCAACCTGCAGAGTTTTATCGCTTCCATGACGCAGAAG	1708	0.43209002044079253	No Hit

Adapter Content

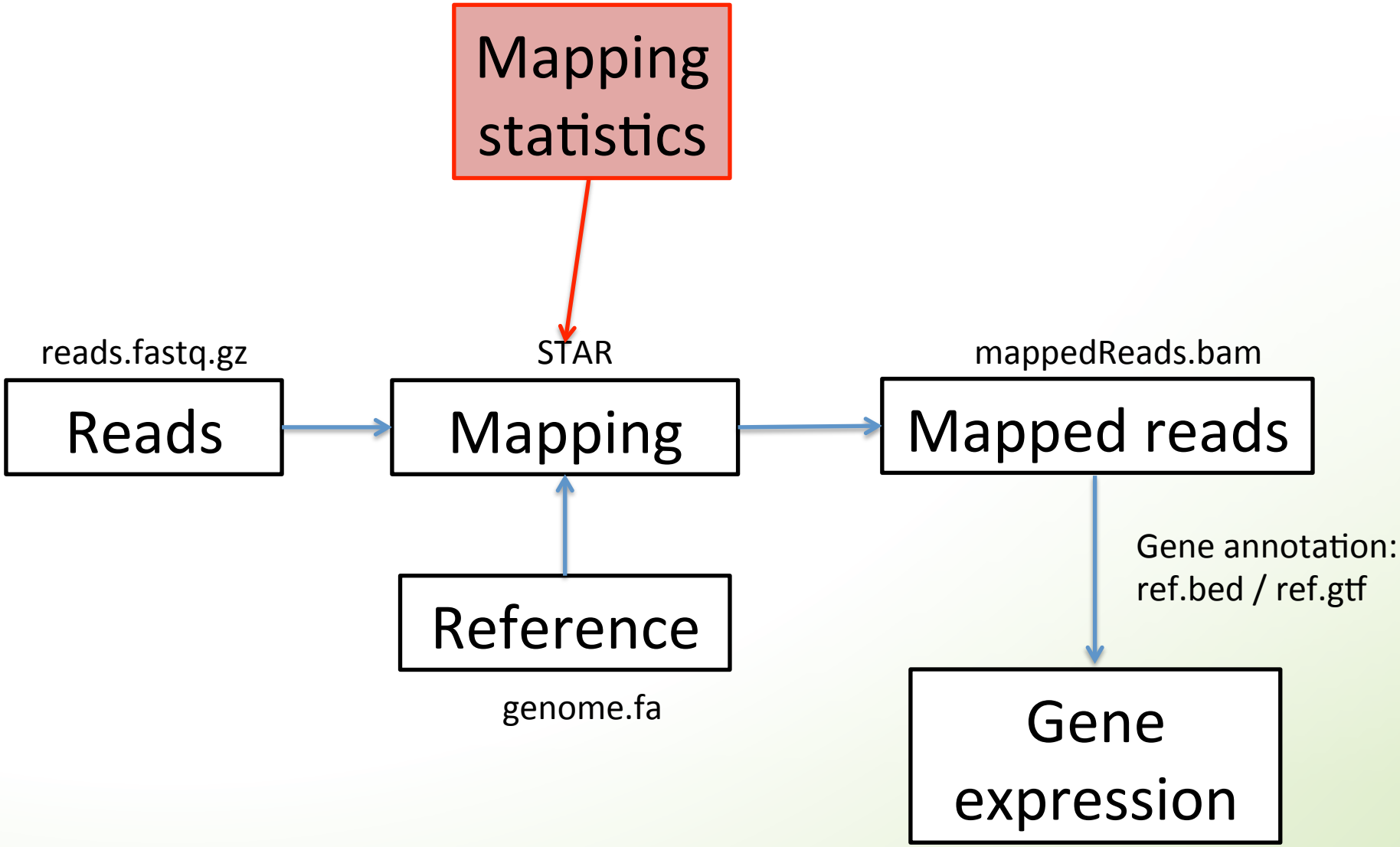


Kmer content



Failed FastQC – what to do?

- Try to figure out why
  - If problem seem to be related to problems during sequencing – resequence!
  - If problem is related to library prep – rerun if possible.
- You can filter out the low quality reads
  - Adapter trimming (cutadapt)
  - Filter low phred score reads (samtools, jaccard)
- If you have enough reads after filtering the data may still be useful.
- But be careful to do equal trimming on all samples!


Mapping statistics
reads.fastq.gz
STAR
mappedReads.bam
Reads
Mapping
Mapped reads
Gene annotation:
ref.bed / ref.gtf
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Gene expression

SAM format

```
HWI-ST1018:7:1101:1648:2188#0   99      chr1    115275270       255     1S100M  =       115275321       152
NTTCTATATTGGTTGCTCGCTCTAATTTGTCACGTCGGTCTGTTGAAATATTAAACCTAACATGGTCACCTTCCAGCAGGGTCACCTTGGATTTCGTATCT   BS
\ccceeeggeggghhhhbghhhhhhhhhhfhhhhhhhfhhhhhhhffhhhffhhhffgghhgg\Z^ddeeeedbdbdcacbabcbcccbbbcc^abbc]   NH:i:1
HI:i:1  AS:i:194        nM:i:0
HWI-ST1018:7:1101:1648:2188#0   147     chr1    115275321       255     101M    =       115275270       -152
AAACCTAACATGGTCACCTTCCAGCAGGGTCACCTTGGATTTCGTATCTTTGTCTCCAAAGGGAAGTTCTTTAGGGATCACAAAGTCNANTTTGNTNNGTC
BBccbdcccccccccbbccccccddcddeeeeccgggggghihiiiiifhihfgiiihhhhihhihiiiihhiihiiiiihihhihgd]RBRBec]QBQBBbbb   NH:i:1
HI:i:1  AS:i:194        nM:i:0
HWI-ST1018:7:1101:2039:2206#0   99      chr19   14574483        255     1S72M85N28M     =       14574529
232     NCCTTCCGCAACCCTGTCATTGAGAGGATTCCTCGGCTCCGACGGCAGAAGAAAATTTTCTCCAAGCAGCAAGGGAAGGCGTTCCAGCGTGCTAGGCAGAT
BP\ccceefgggghhighiiiiiiiiihhhiiiiihiiiiiiihiggeeeddddbbbcccbbccb^[`aacccccccccccX]accccc^acc]bc^b_a]   NH:i:1
HI:i:1  AS:i:203        nM:i:0  XS:A:+
HWI-ST1018:7:1101:2039:2206#0   147     chr19   14574529        255     26M85N75M       =       14574483
-232
GAAGAAAATTTTCTCCAAGCAGCAAGGGAAGGCGTTCCAGCGTGCTAGGCAGATGAACATCGATGTCGCCACGTGGGTGCGGCTGCTCCGGAGGCTCATCC   ]ccdcccb
bbacbcaccccbccccccccccccbcccaccccccccdd_dddeeeeeeeggggiiiiihihiiiiiiiihiihiiiiiiigfgggeeeeebbb   NH:i:1  HI:i:1
AS:i:203        nM:i:0  XS:A:+
```

More details on:
http://samtools.github.io/hts-specs/SAMv1.pdf
http://genome.sph.umich.edu/wiki/SAM

BAM/SAM file formats

- All mapped reads with location in genome, mapping information etc. (https://samtools.github.io/hts-specs/SAMv1.pdf)
- SAM (Sequence Alignment/Map) format – alignment.sam
- BAM is a compressed sam format – alignment.bam
- A bam-file (always) needs to be indexed and sorted - alignment.bam.bai
- Samtools – a simple program for converting between bam/sam, indexing, sorting, filtering, etc.

Code

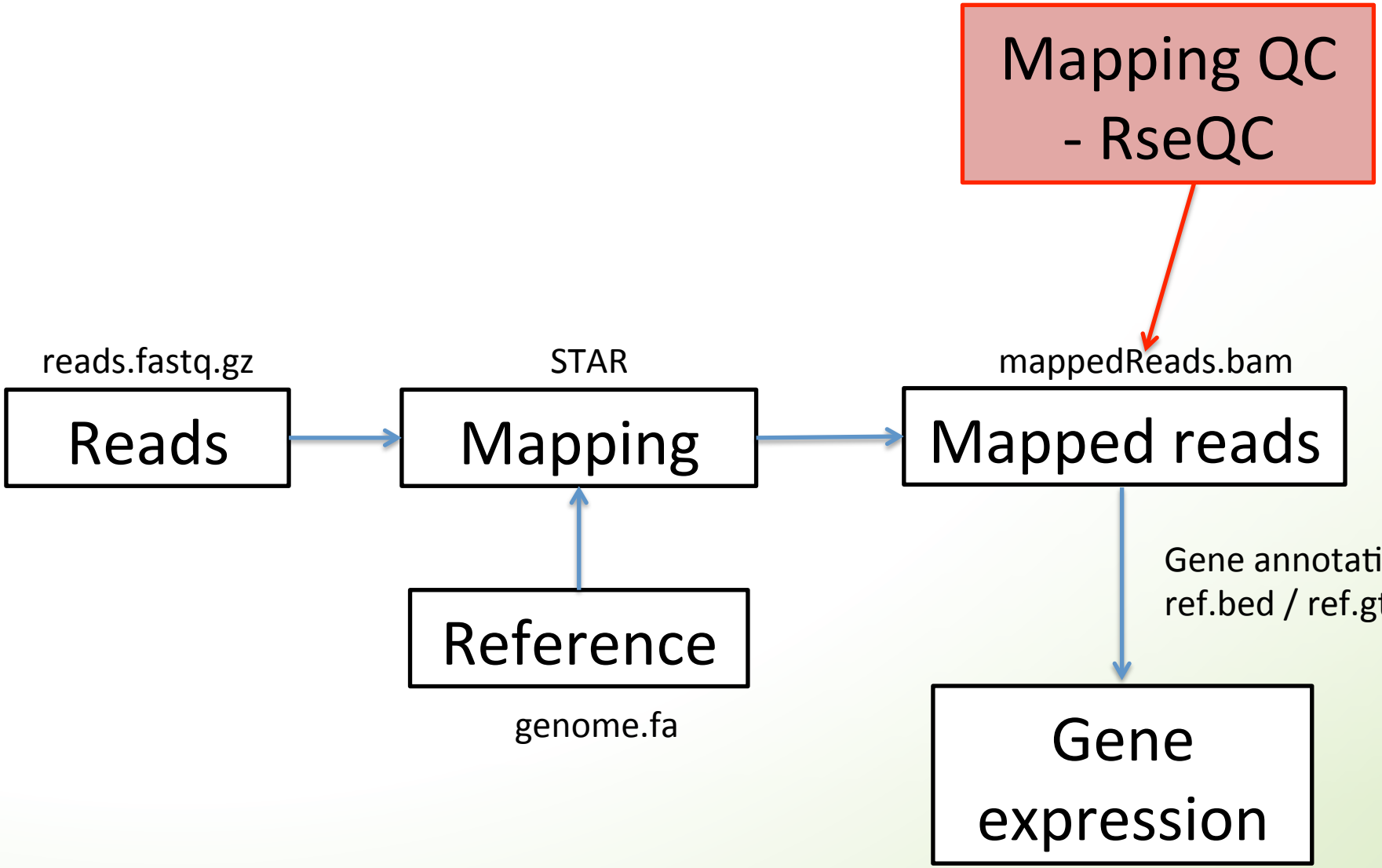
```
$ module load bioinfo-tools
$ module load samtools
```

Mapping logs – mapping efficiency

- Program specific how the output will be (STAR, Bowtie, BWA, Tophat…)
- Always gives:
  - % uniquely mapping – ideally around 90%
  - % multi-mapping – will depend on read length
  - % unmapped – could indicate contaminations, adaptors
- Also statistics on:
  - Mismatches / indels
  - Splice junctions

Bad mapping – what to do?

- First step – try to figure out why it failed. With the use of FastQC/RseQC/Mapping logs.
  - Perhaps also look for contaminant species
  - Redo library prep controlling for possible errors
- Low mapping, but not completely failed.
  - Figure out why!
  - Is it equal for all samples?
  - Could it introduce any bias in the data?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reads.fastq.gz
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Reference
genome.fa
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expression

After mapping - RseQC package

- General sequence QC:
  - sequence quality
  - nucleotide composition bias
  - PCR bias and
  - GC bias
- RNA-seq specific QC:
  - evaluate sequencing saturation
  - mapped reads distribution
  - coverage uniformity
  - strand specificity
  - Etc..
- Some tools for file manipulations

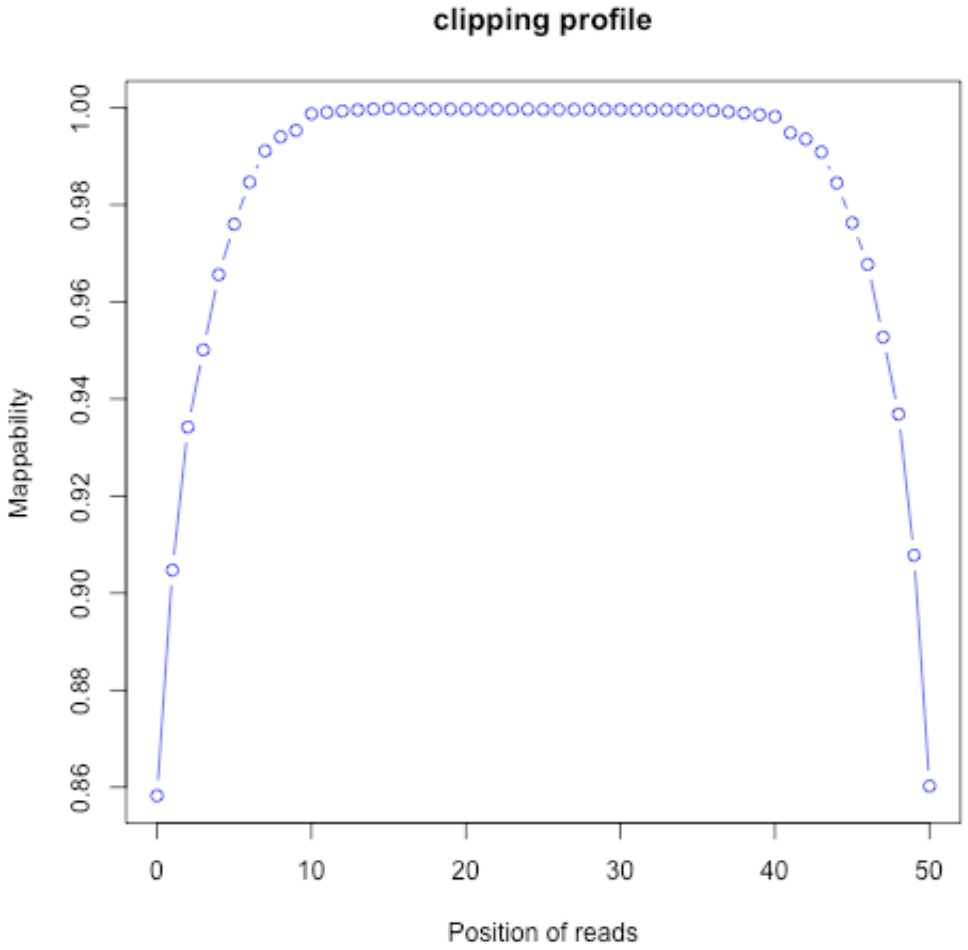
Code

```
$ module load bioinfo-tools
$ module load rseqc/2.4

$ geneBody_coverage.py –r
ref.bed12 –i mappedReads.bam -o
genecoverage
```

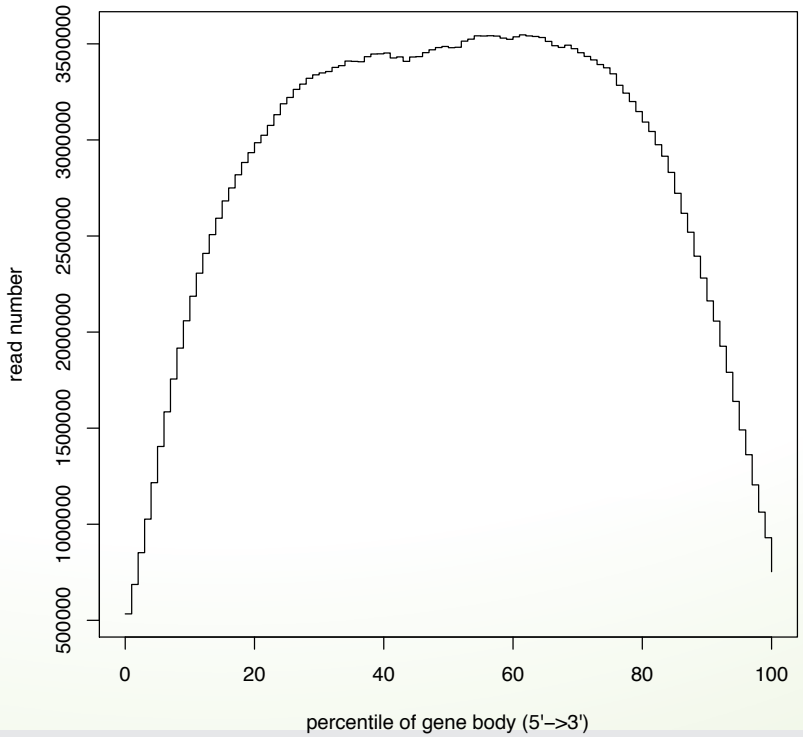
http://rseqc.sourceforge.net/

Soft clipping - clipping_profile.py

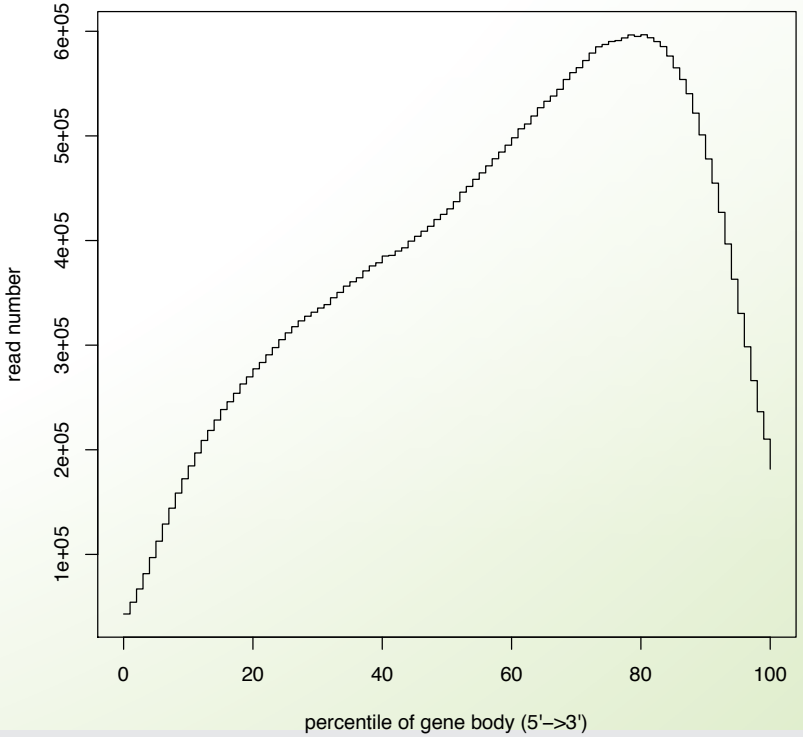


Gene coverage - geneBody_coverage.py

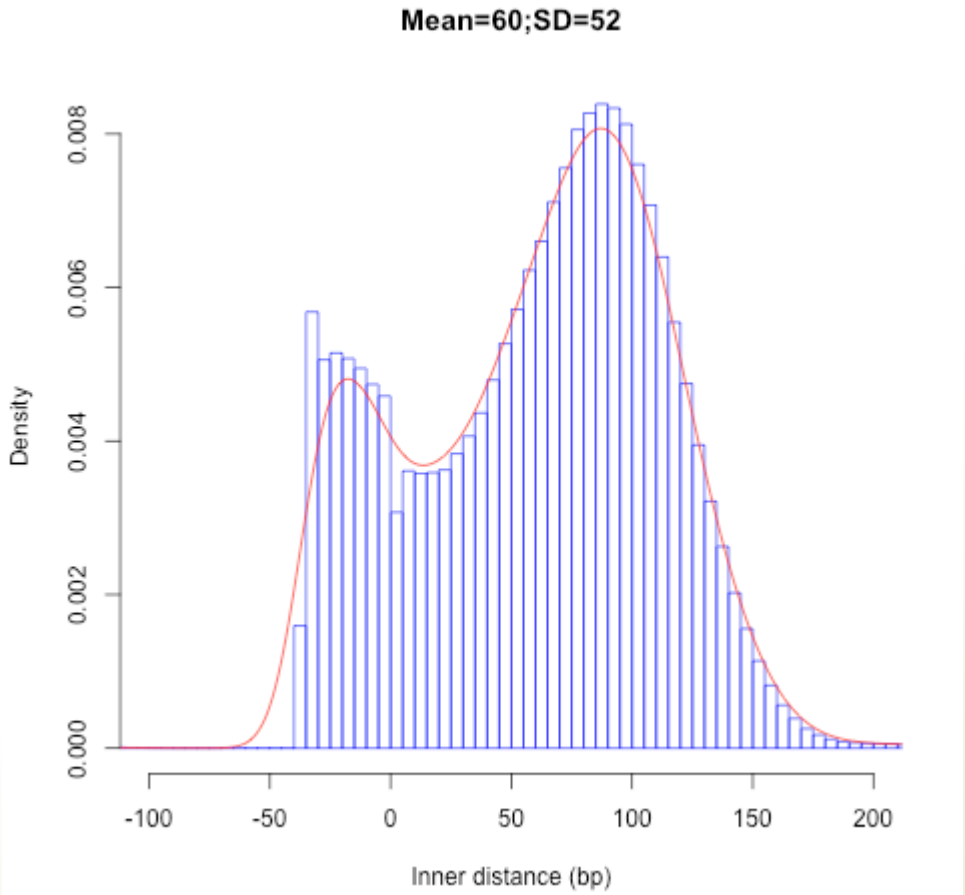
Not degraded



Degraded



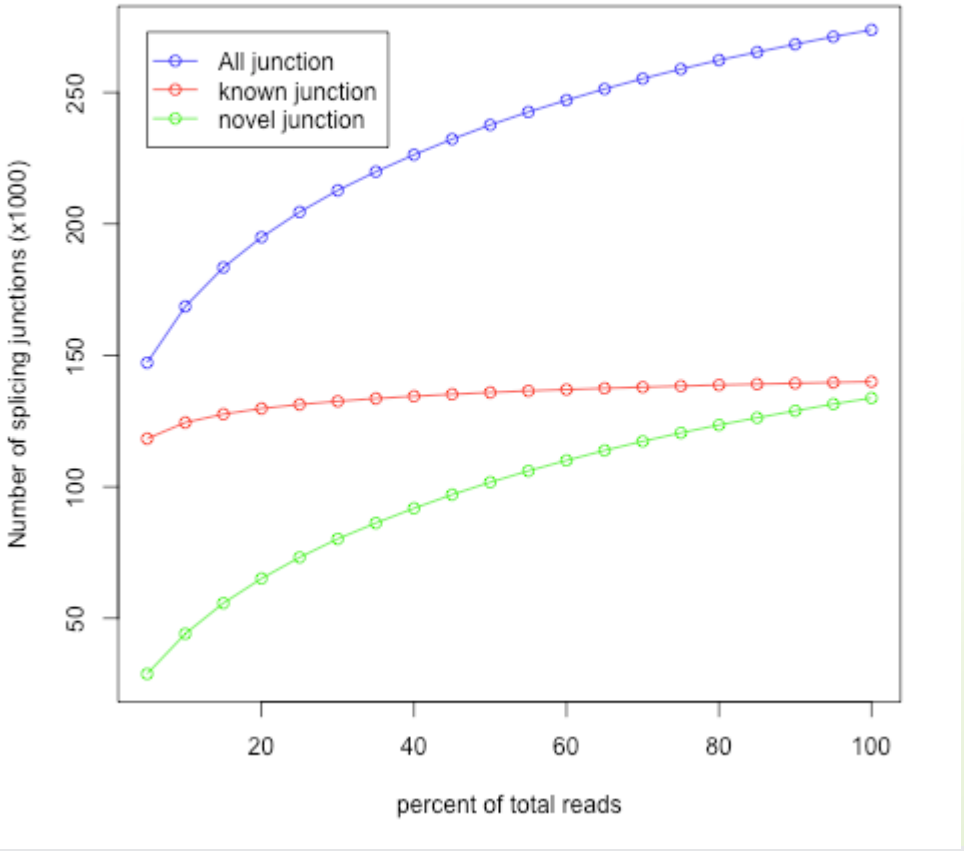
Distance between PE-reads - inner_distance.py



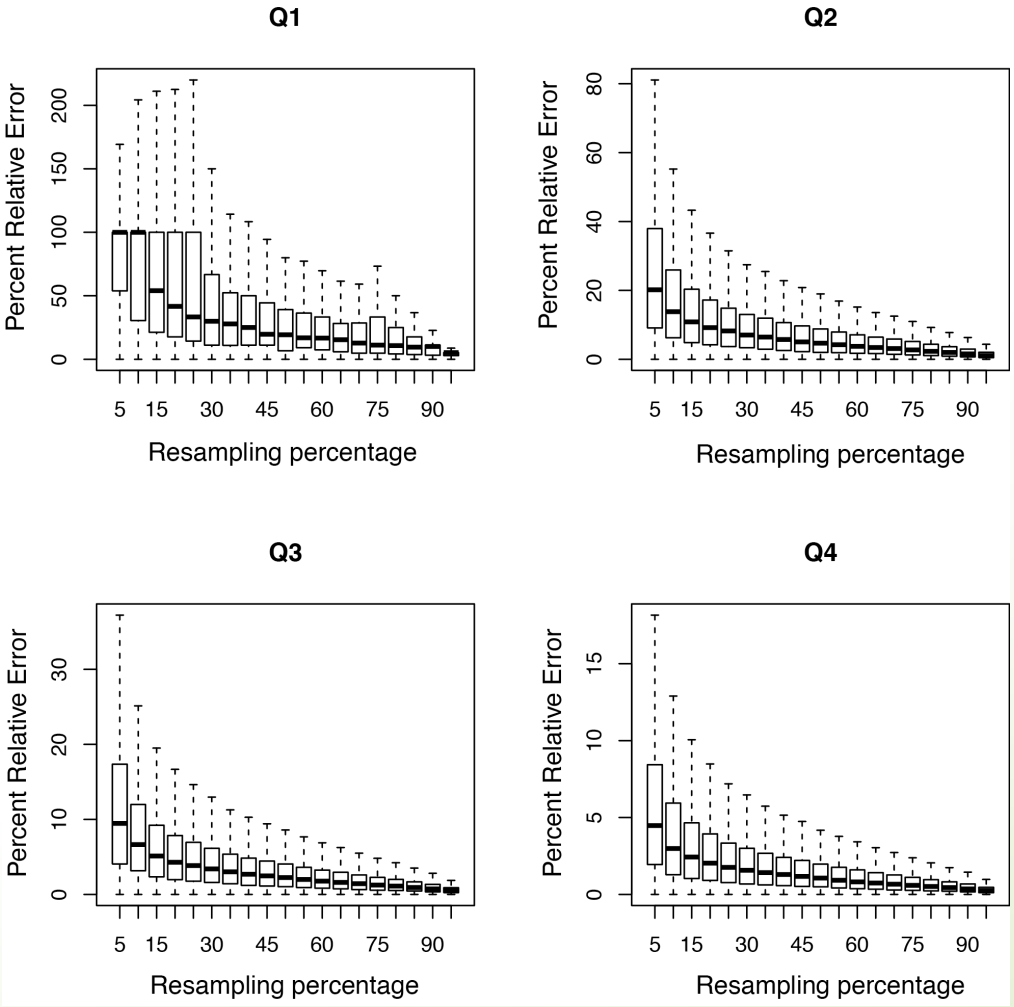
Where in the genome do your reads map? - read_distribution.py

Group	Total_bases	Tag_count	Tags/Kb
CDS_Exons	33302033	20002271	600.63
5'UTR_Exons	21717577	4408991	203.01
3'UTR_Exons	15347845	3643326	237.38
Introns	1132597354	6325392	5.58
TSS_up_1kb	17957047	215331	11.99
TSS_up_5kb	81621382	392296	4.81
TSS_up_10kb	149730983	769231	5.14
TES_down_1kb	18298543	266161	14.55
TES_down_5kb	78900674	729997	9.25
TES_down_10kb	140361190	896882	6.39

Known and novel splice junctions – junction_saturation.py or junction_annotation.py

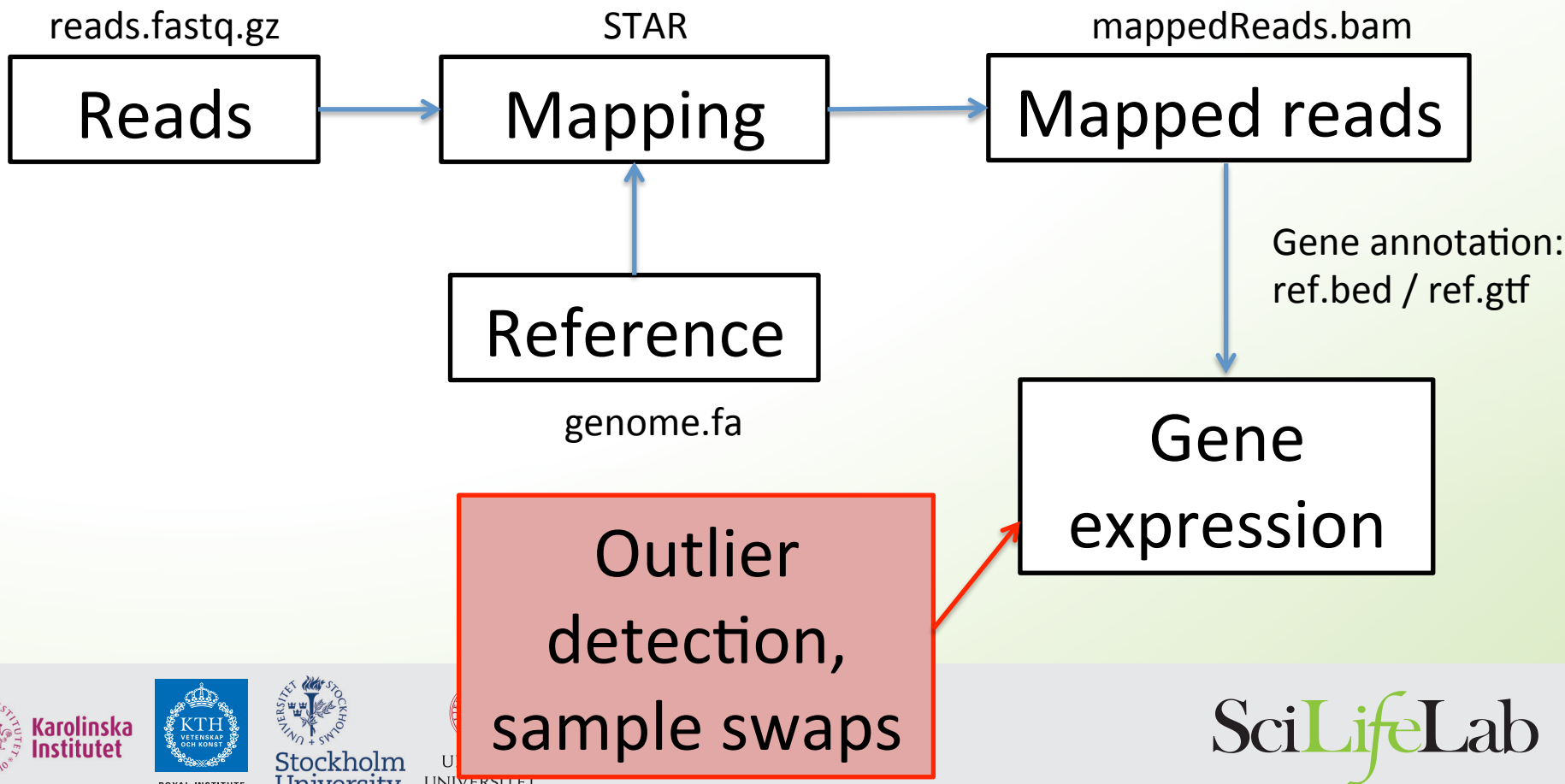


Gene detection subsampling - RPKM_saturation.py
How deep do you need to sequence?



Bad RseQC output – what to do?

- Try to figure out what went wrong.
  - Redo library prep controlling for possible errors
  - Is it equal for all samples?
  - Could it introduce any bias in the data?
- RNA-degradation in some samples
  - Possible to use a region at 3' end for expression estimates.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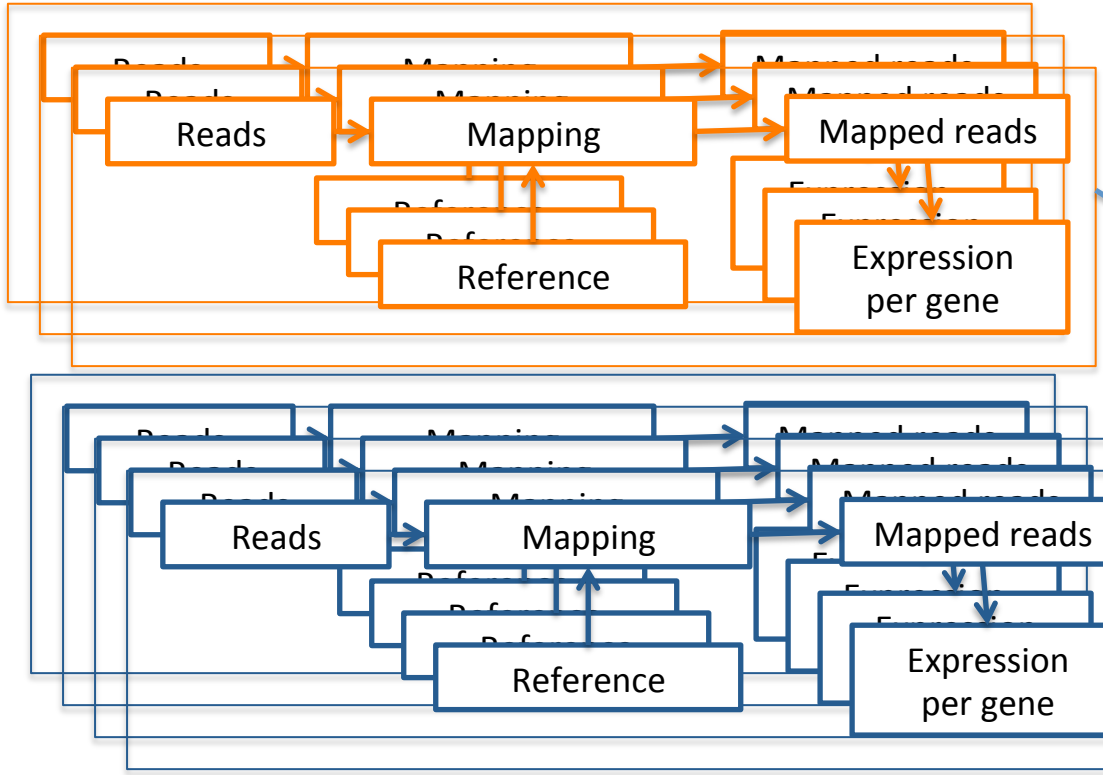
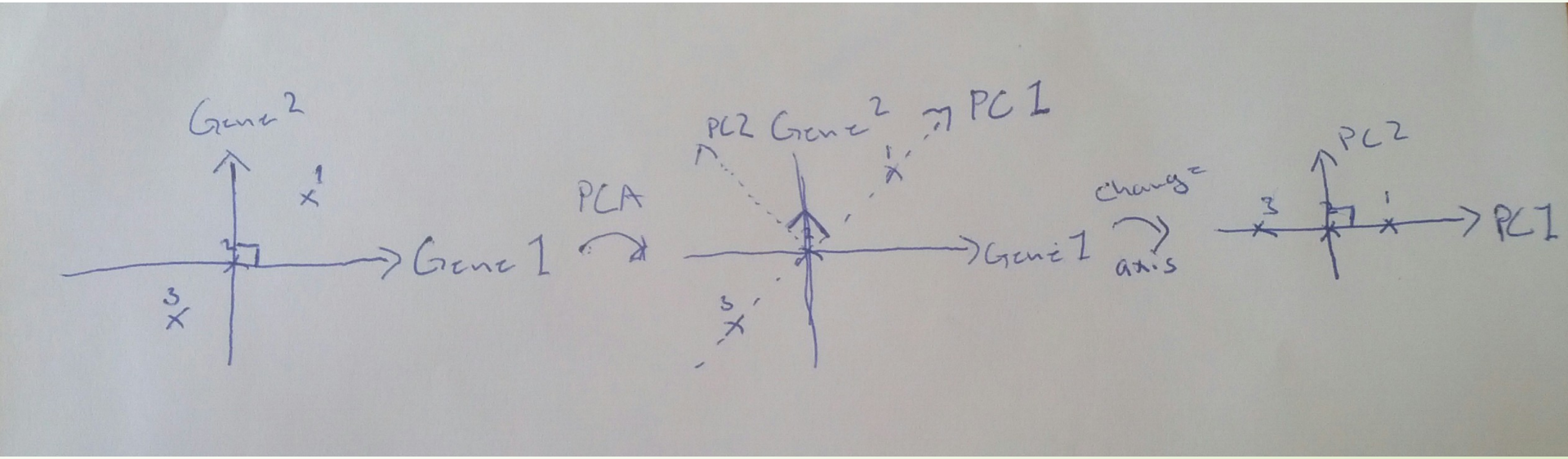


Table with counts, rpkms, fpkms or similar

Sample swaps and outliers can be identified using PCA

Differences in read distribution between samples can be identified using Principal Component Analysis (PCA)

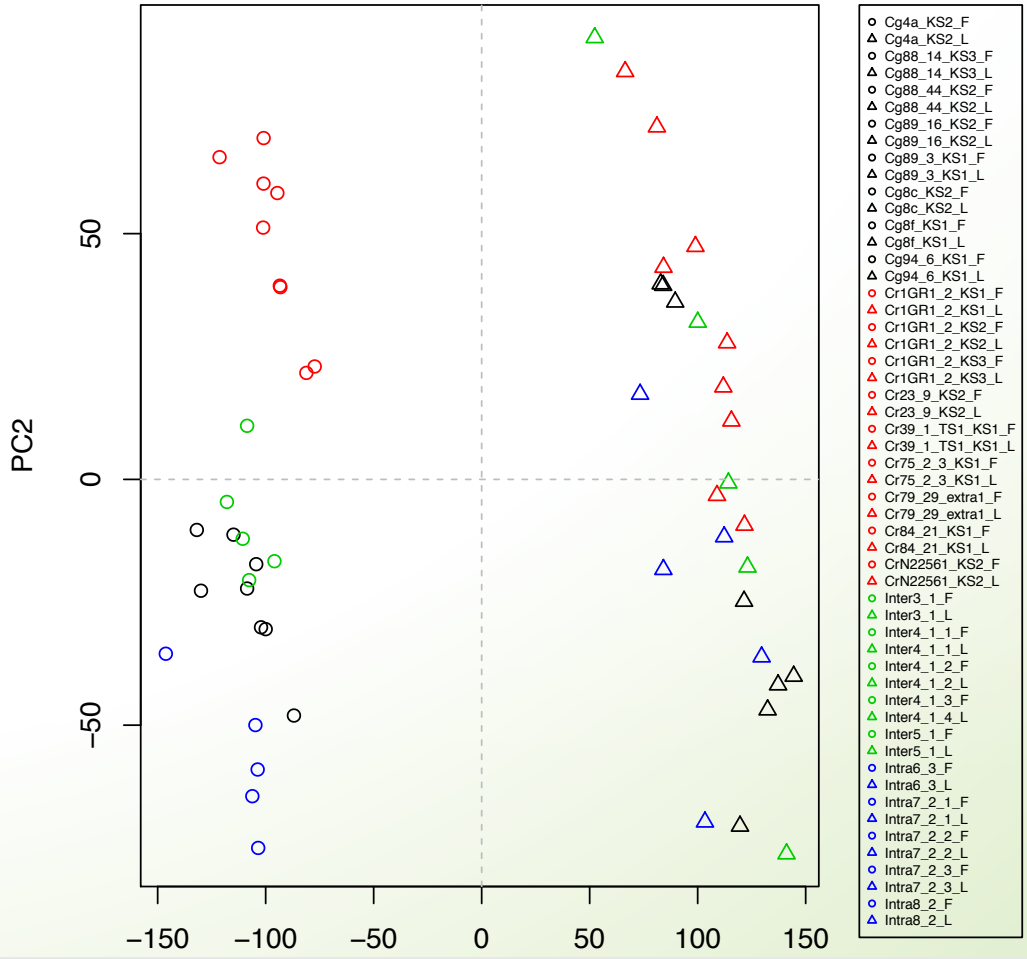


QC test case 1

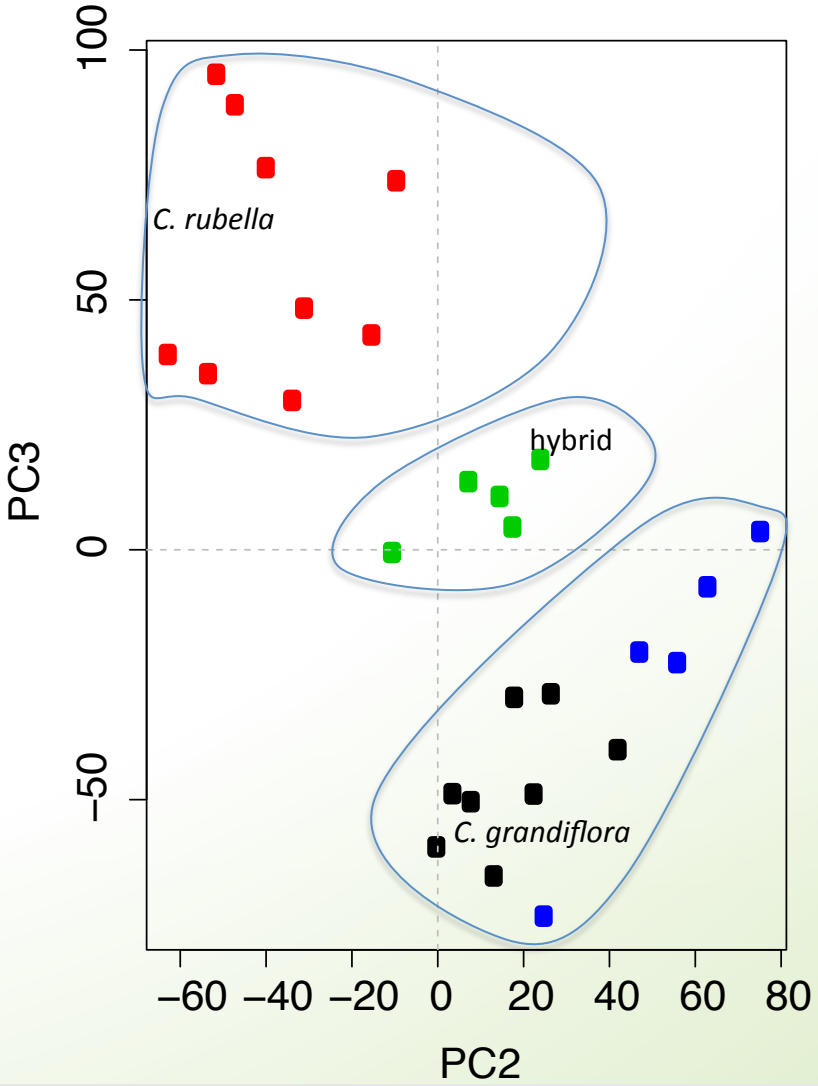
Samples from three different species

1. C.rubella
   - Small flowers
   - Normal leaves
   - Genome is sequenced
2. C. grandiflora
   - Large flowers
   - Normal leaves
3. Hybrid
   - Intermediate flowers
   - Normal leaves

Principal component 1 separates samples from flowers and leaves



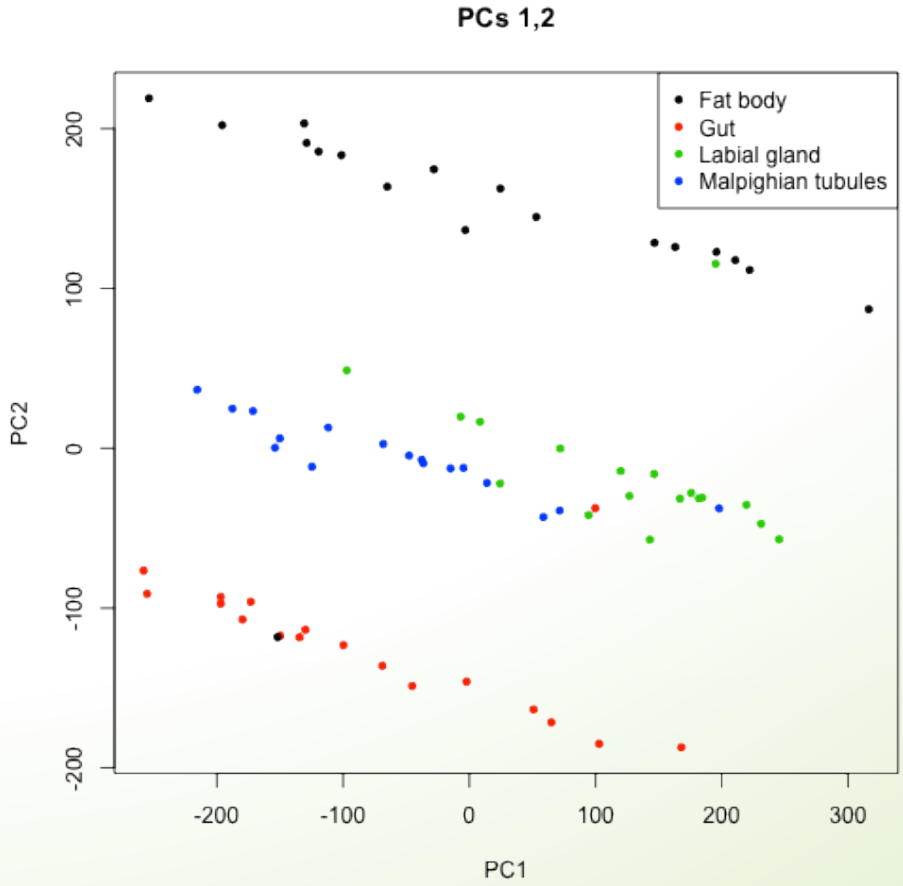
Principal component 2 and 3 separates the different species



QC test case 2

- 4 Tissues
  - Fat body
  - Gut
  - Labial gland
  - Malphighian tubules
- 3 Phylogenetic groups
- >70 samples

PCA analysis detected potential sample swaps



My PCA looks strange – what to do?

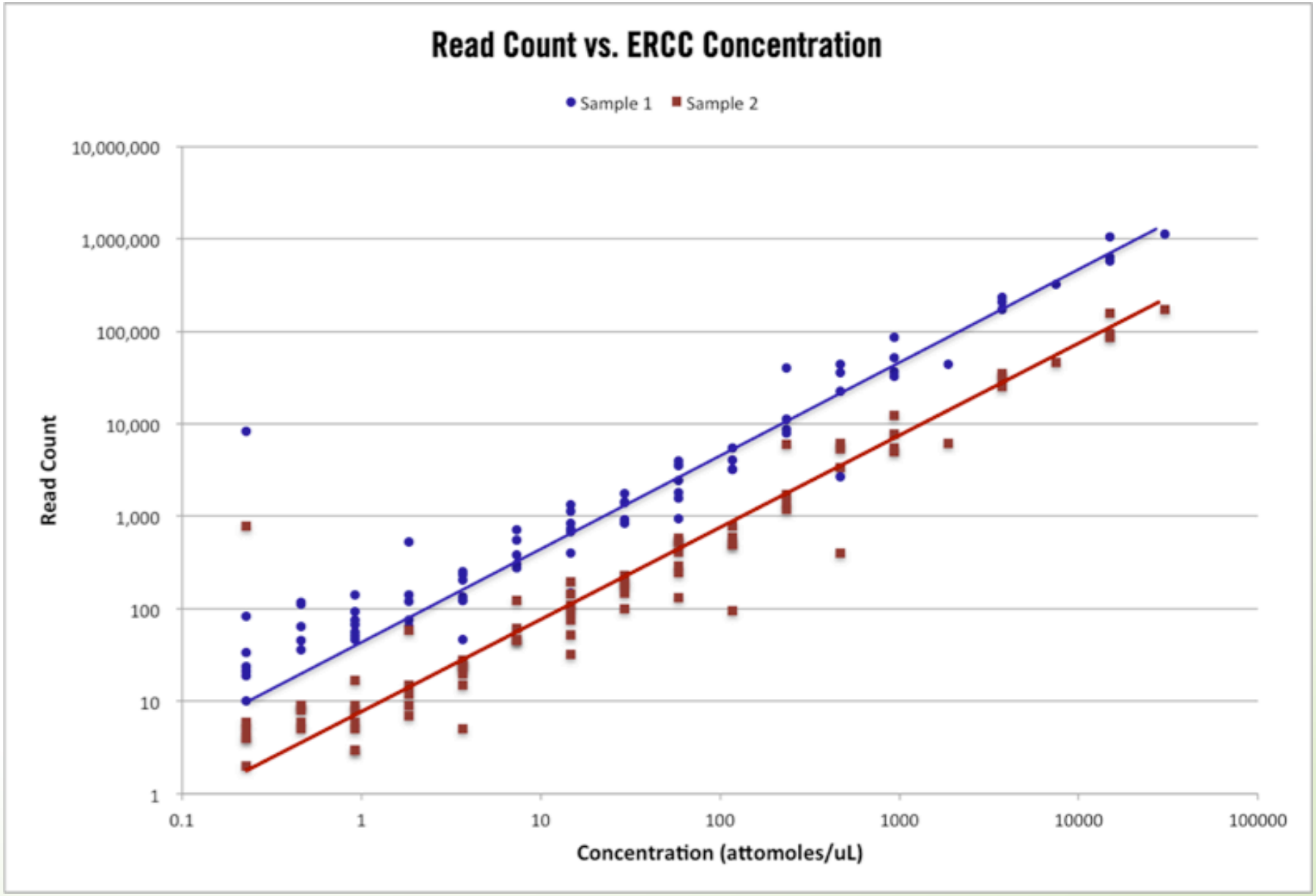
- Clear sample swaps
  - Check sequence indices, lab logs etc. to verify new classification.
  - If you have enough replicates, remove instead of changing labels if you are uncertain.
- Outliers
  - Figure out why they are outliers
  - Do not remove samples only because they do not fit your expectation – Bad science!
- PCA does not group my sample sets
  - Try different methods of dimensionality reduction / clustering
  - Perhaps technical/biological variation is higher than your expected effect -> Batch normalization

Sources of variation

- Biological variation
  - Patient to patient variation
  - Sex
  - Time points of samples taken
  - Etc…..
- Technical variation
  - At each step of RNA extraction and library preparation

Spike-in control RNA

- Addition of external RNA molecules into the samples before library prep
- Will give estimate of technical variation:
  - Sensitivity / detection
  - Accuracy
  - Specific biases
- Also used to estimate amount of RNA in the samples
- Most commonly ERCC - pool of 48 or 96 synthetic mRNAs with various lengths and GC content, at 17 different concentrations
- Allows for cross comparison of datasets



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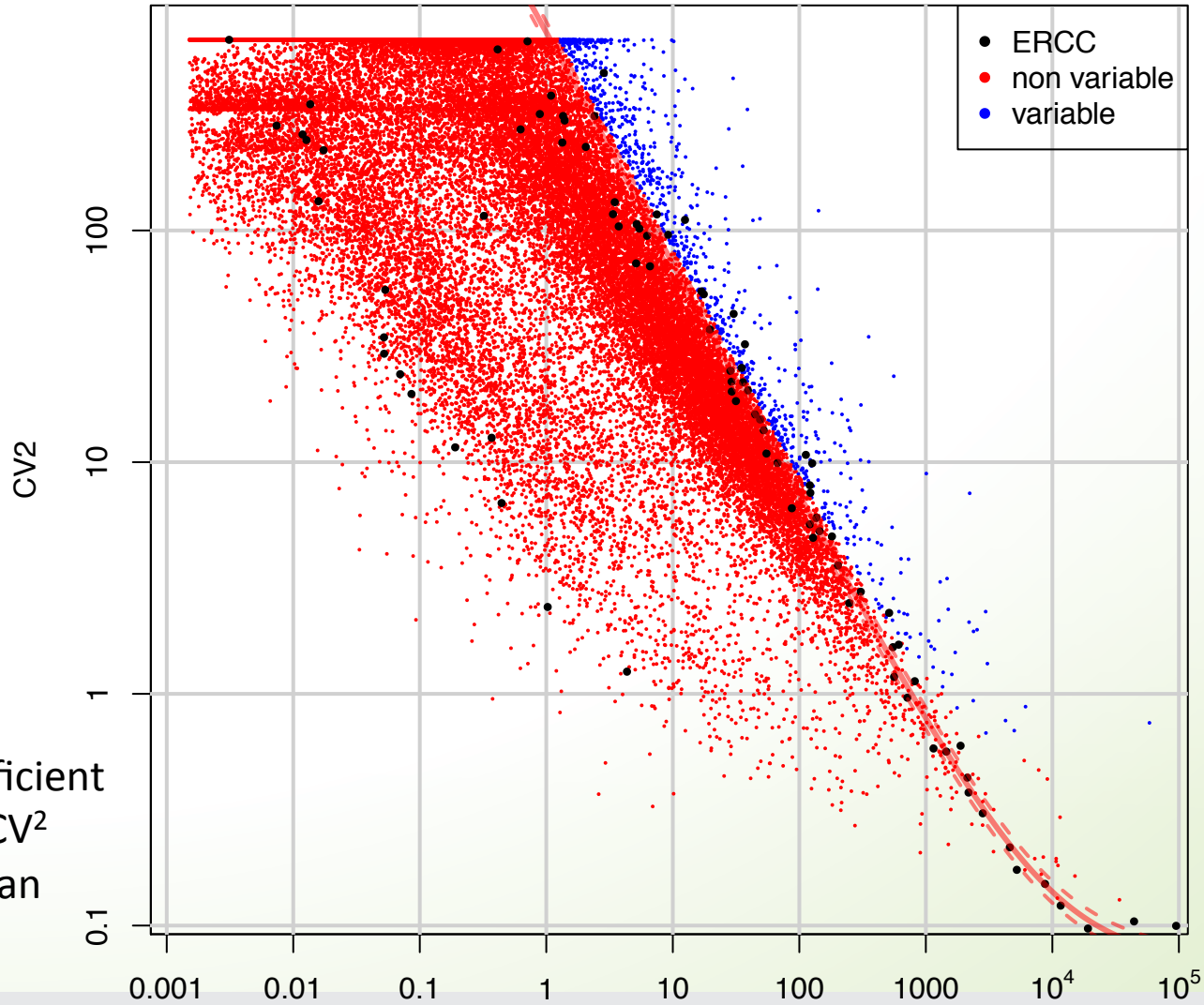
(https://cofactorgenomics.com)

Technical noise / Biological variation

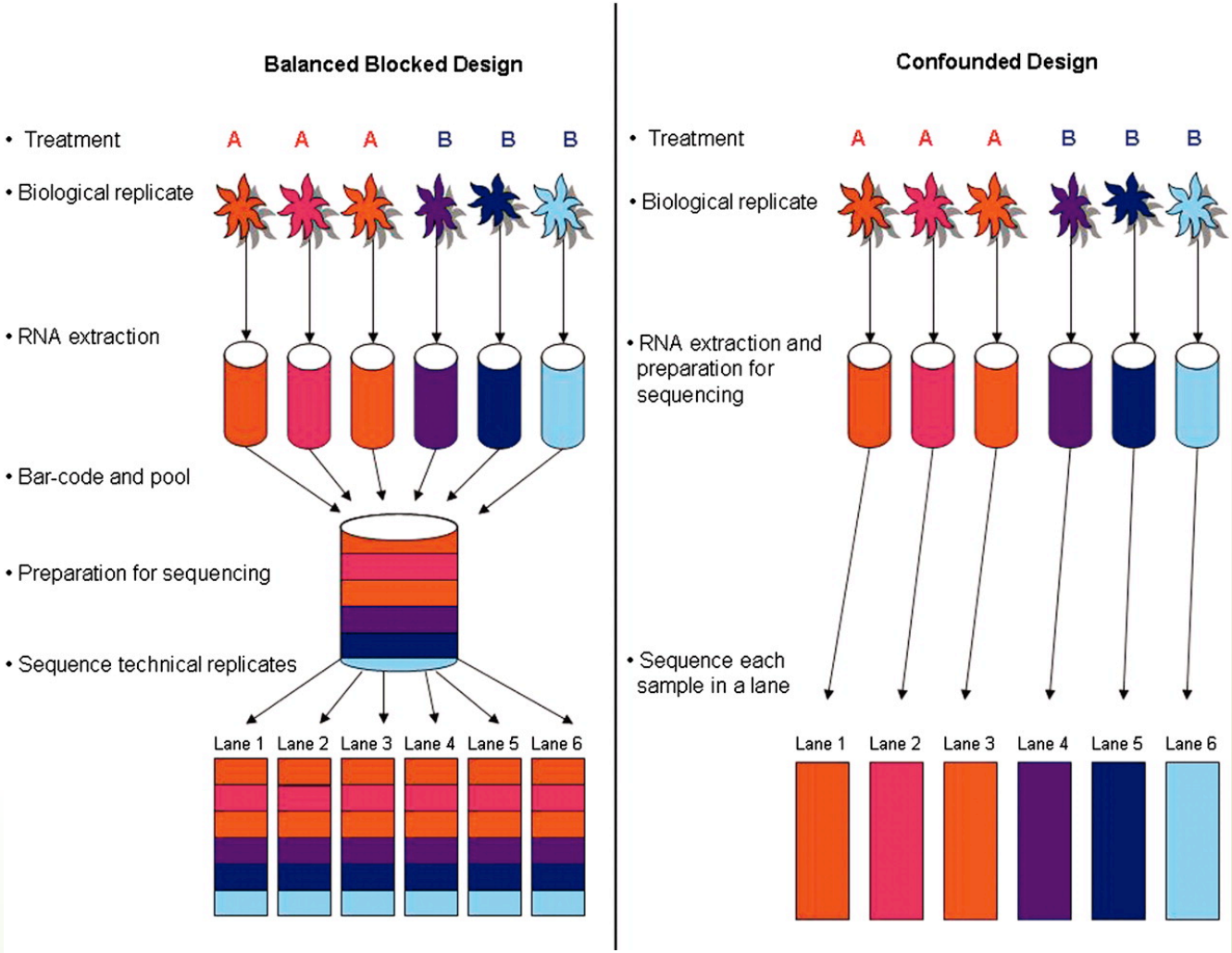


Squared coefficient of variation: CV^2
CV = std / mean

Replicates, replicates, replicates

- Technical replicates
- Biological replicates
- If you have enough material, always do extra replicates in case you want to remove low quality samples.

Experimental Design



DOI: 10.1534/genetics.110.114983

Statistical Design and Analysis of RNA Sequencing Data

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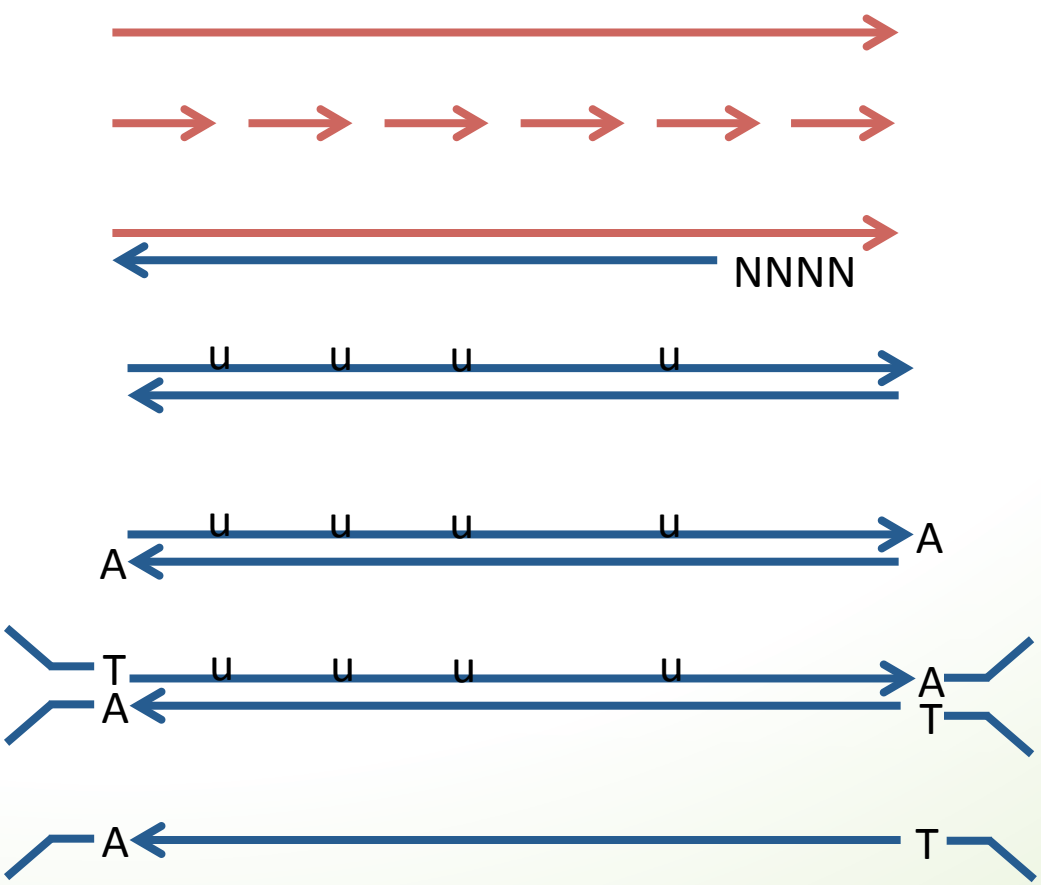
Manuscript received January 31, 2010
Accepted for publication March 15, 2010

Conclusions

- Good quality data is the first step in any RNA-seq experiment
- The reason for low quality samples may require some detective work
- More replicates allows you to filter out low quality libraries without losing statistical power
- Depending on where you sequence, some of the QC steps will be performed at the platform.

Questions?

RNA-seq libraries



rRNA depleted mRNA

Fragment

Random prime + RT

2nd strand synthesis (+ U)

A-tailing

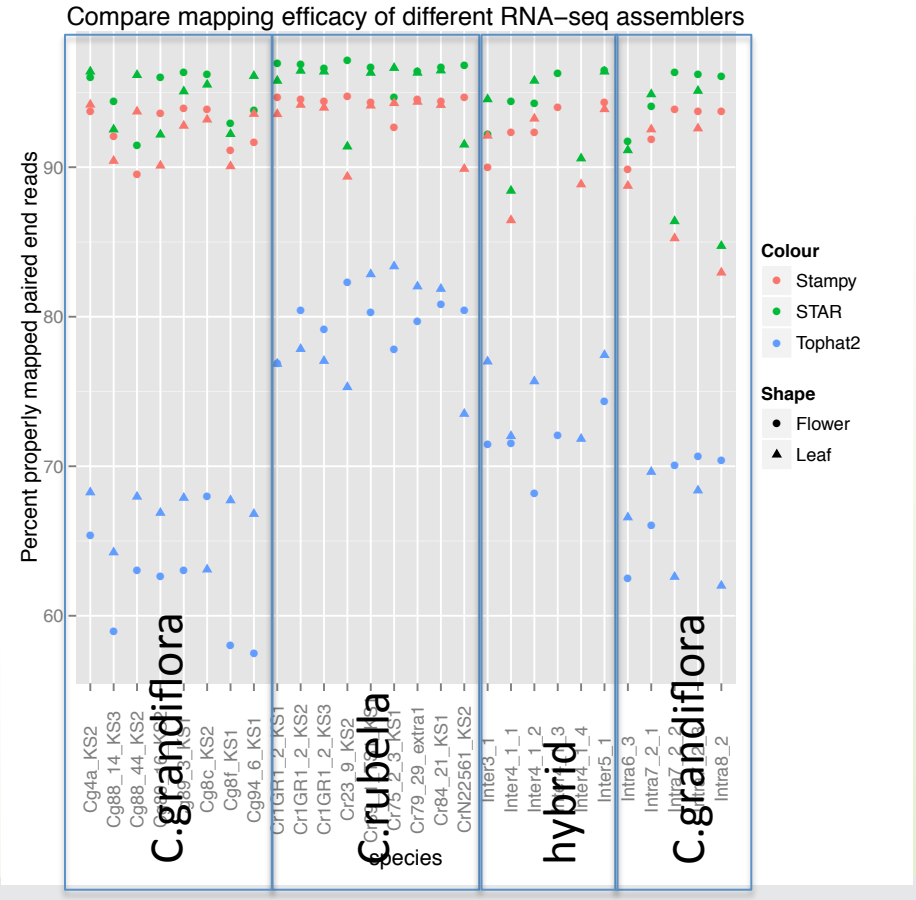
Adapter Ligation

(U strand degradation)

Sequencing

(copied from Simon Andrews)

More mapping bias when using top hat 2 with default settings than when using STAR or Stampy with default setting



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PCR duplicates – read_duplication.py