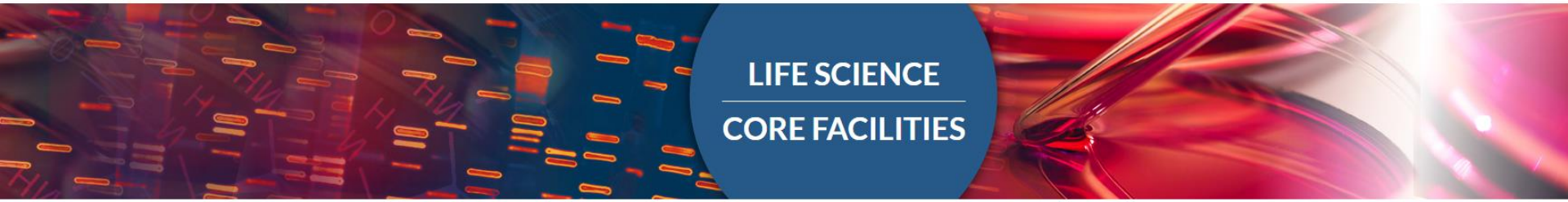
Gene Expression Analysis
Dena Leshkowitz

An introduction to deep-sequencing analysis for biologists
2019 - 2020

מכון ויצמן למדע
WEIZMANN INSTITUTE OF SCIENCE



Agenda

- Introduction & Experimental design
- Analysing Gene expression from RNA-Seq data
- Analysing Gene expression from bulk MARS-Seq data

RNA-Seq Potential

RNA-Seq: developed a decade ago has become an indispensable tool for transcriptome analysis

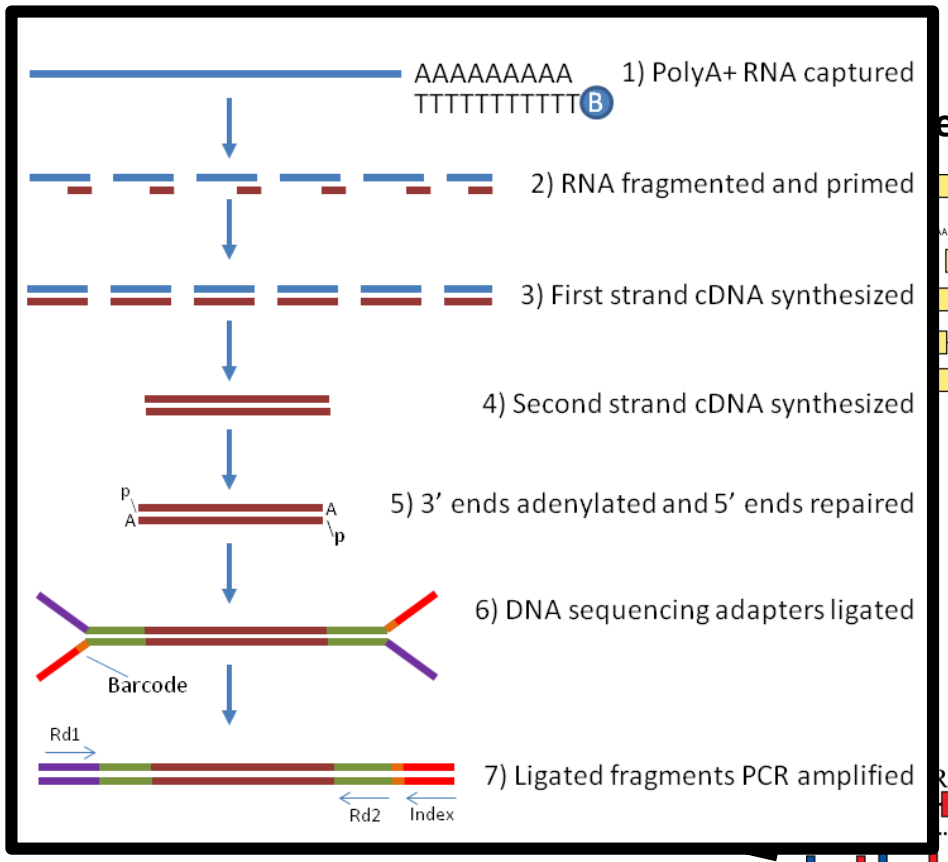
In theory RNA-Seq can be used to build a complete map of the transcriptome across all cell types, perturbations and states (Trapnell C. et al, Nature methods 6 469-477(2011))

RNA-Seq Applications

- Transcript level analysis :
  - Discover novel transcripts
  - Determine transcript structure,
  - Measure transcripts expression
  - Detect differentially expressed transcripts/isoforms between conditions, treatments…

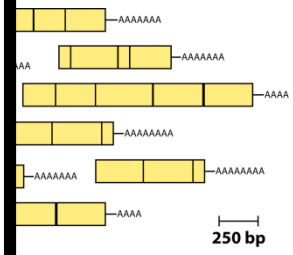
- Gene expression analysis for Model Organism:
  - Measure gene expression
  - Detect differentially expressed genes between conditions, treatments… based on known gene structures

RNA-Seq Workflow

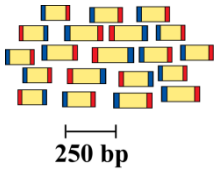


Quantify and detect differential expression

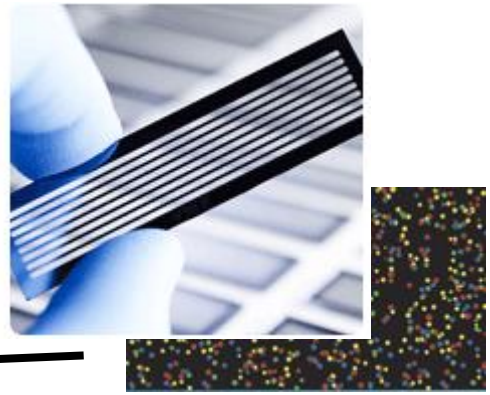
e RNAs

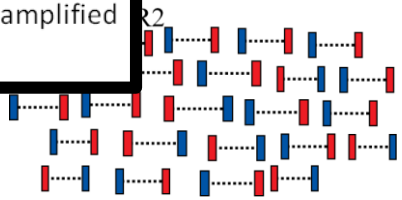


Converted to amplified small cDNA fragments with linkers at the ends



Sequence ends

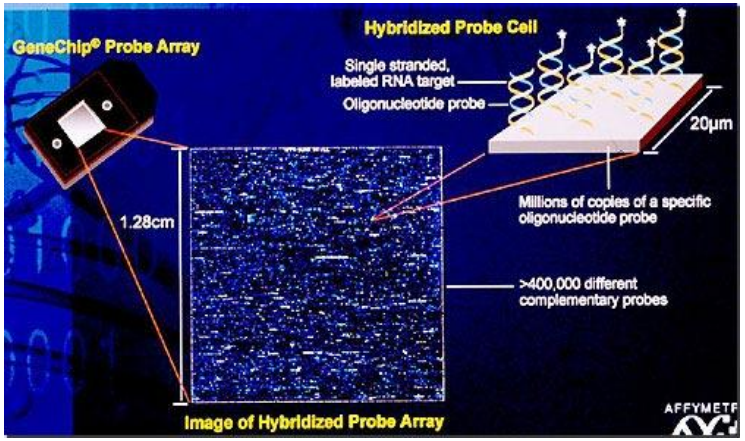




Million/billions of fragments are sequenced

Modified - www.med.nyu.edu/rcr/rcr/course/**RNA-seq**.pptx

High Throughput Genomics



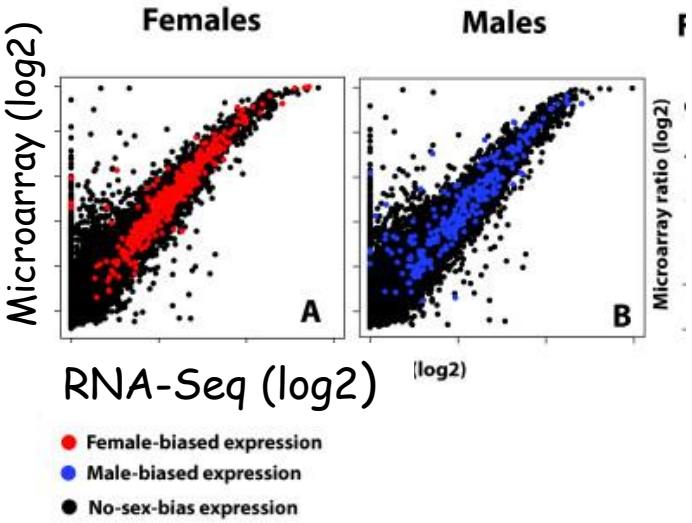
DNA Microarrays

Illumina
NovaSeq
NextSeq500
…

Microarrays vs RNA-Seq

Malone et al. BMC Biol. 2011; 9: 34.

- Both high throughput methods can profile the genes with similar performance
- Microarrays suffer from compression (saturation) at the high end
- Low expression is problematic in both platforms



Microarray & RNA-Seq Pros and Cons

	Microarrays	RNA-Seq Gene model organism/Transcript and *de novo* assembly
Cost	$-$$	$/$$$
Biases	Decade of research and solutions	Understanding is evolving
Data sizes	Mb -images	Gb- sequence data
Dynamic range	10^2	10^5
Transcript discovery , isoform identification & Transcript-chimeras	No	No/Yes
Genome required	Yes	Yes/No
Allele specific expression	No	No/Yes

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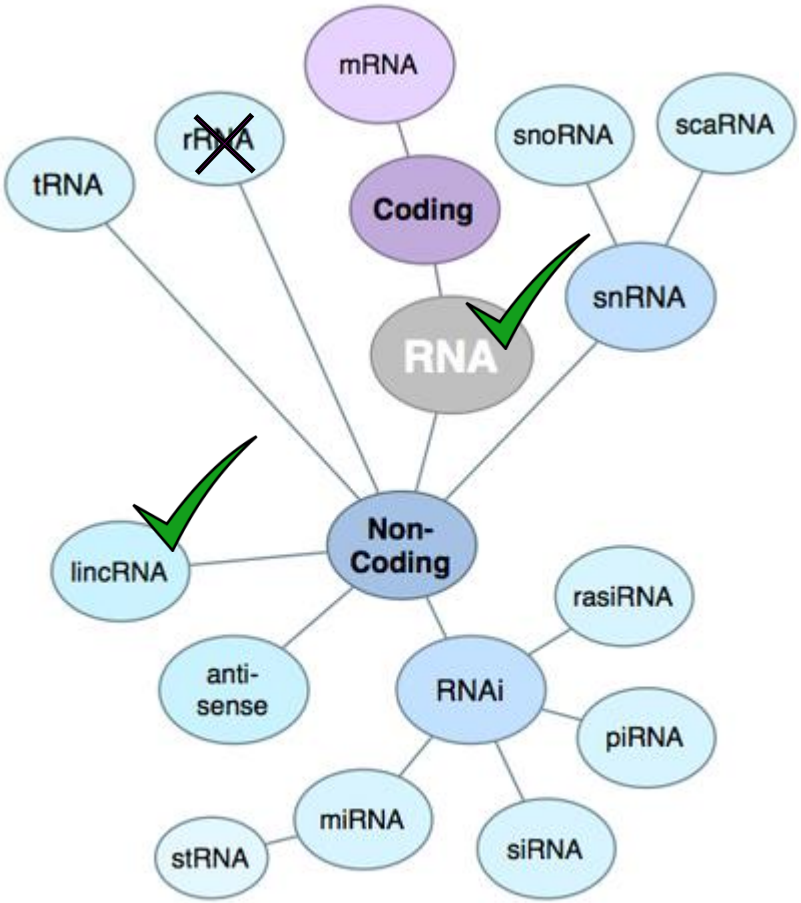
mRNA in the RNA "World"

- Most abundant RNA is rRNA – 98%
- Illumina standard protocol enriches for mRNA by:
  - oligo(dT)-based affinity matrices
  - Sequence: rRNA capture beads (Ribo-Zero)

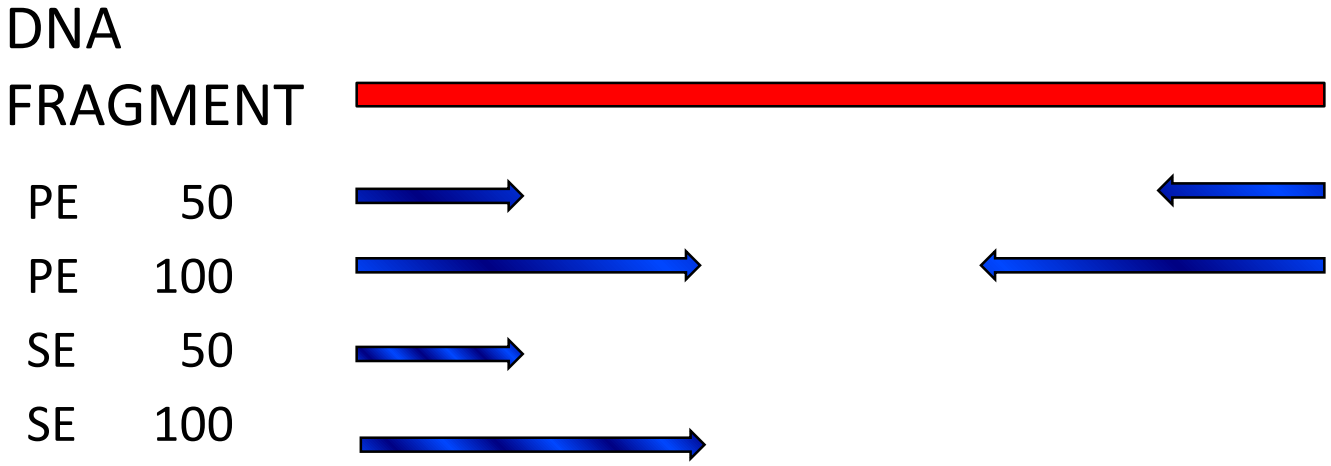


Sequencing Options

Illumina NextSeq/NovaSeq Sequencing options:

- Length of sequence (up to 300 bases)
- Paired-end (PE) or single-end (SE)

Both PE and longer length sequencing increase the sensitivity and specificity of the detection of the alternative splicing and novel transcripts



Experimental Design

Mammalian tissue

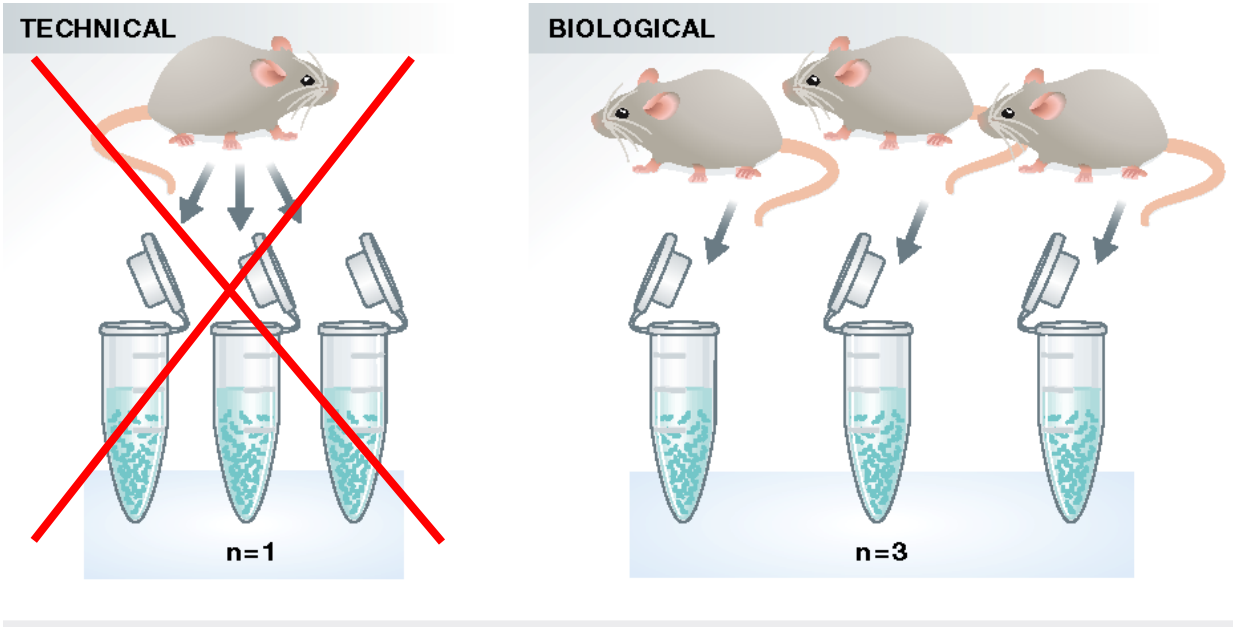
Liu Y. et al., 2014; ENCODE 2011 RNA-Seq

Differential gene expression profiling	**10-25M**	**50 base single-end**
Alternative splicing	50-100M	100 base paired-end
Allele specific expression	50-100M	100 base paired-end
De novo assembly	>100M	100 base paired-end

(5M Bulk MARS-Seq)

Biological Replicates

- Usually our goal in a RNA-Seq experiment is to detect Differentially Expressed Genes (DEGs) between groups.
- Each group contains several samples, which are also known as replicates
- Assessing biological variation requires biological replicates - (3) are a minimum, yet more are recommended



BIOINFORMATICS DISCOVERY NOTE

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doi:10.1093/bioinformatics/btt688

Gene expression

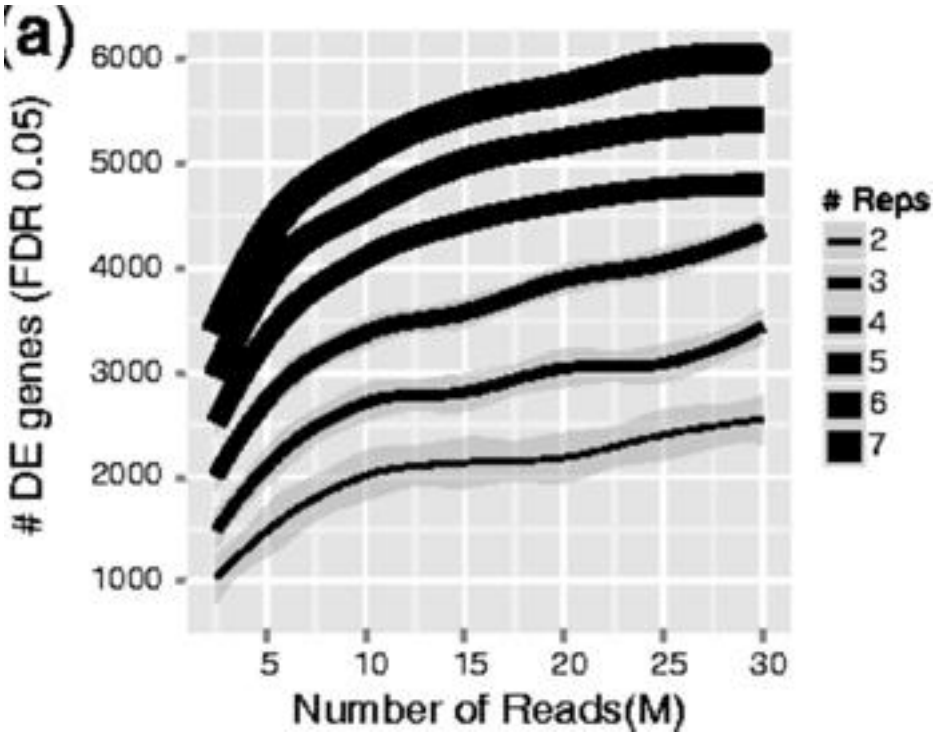
Advance Access publication December 6, 2013

RNA-seq differential expression studies: more sequence or more replication?

Yuwen Liu[1,2], Jie Zhou[1,3] and Kevin P. White[1,2,3,*]

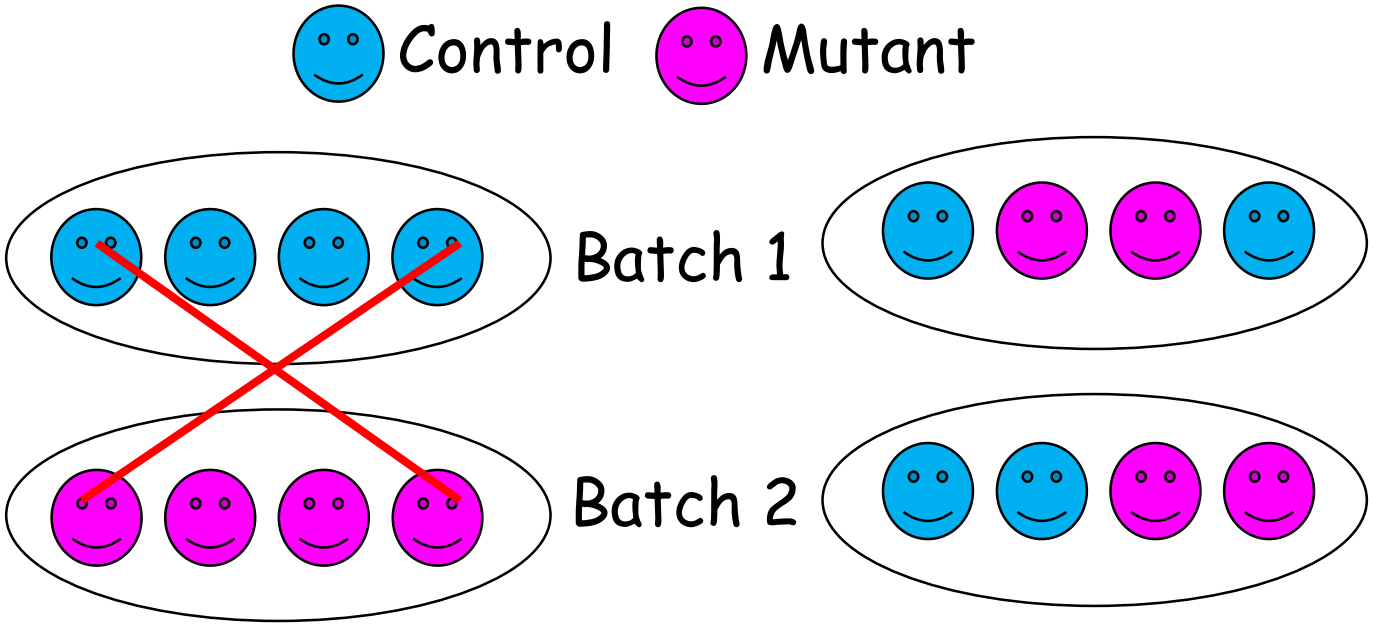
[1]Institute of Genomics and Systems Biology, [2]Committee on Development, Regeneration, and Stem Cell Biology and [3]Department of Human Genetics, University of Chicago, Chicago, IL 60637, USA

Associate Editor: Janet Kelso



ENCODE consortium's *Standards, Guidelines and Best Practices for RNA-Seq*

Proper Experimental Design



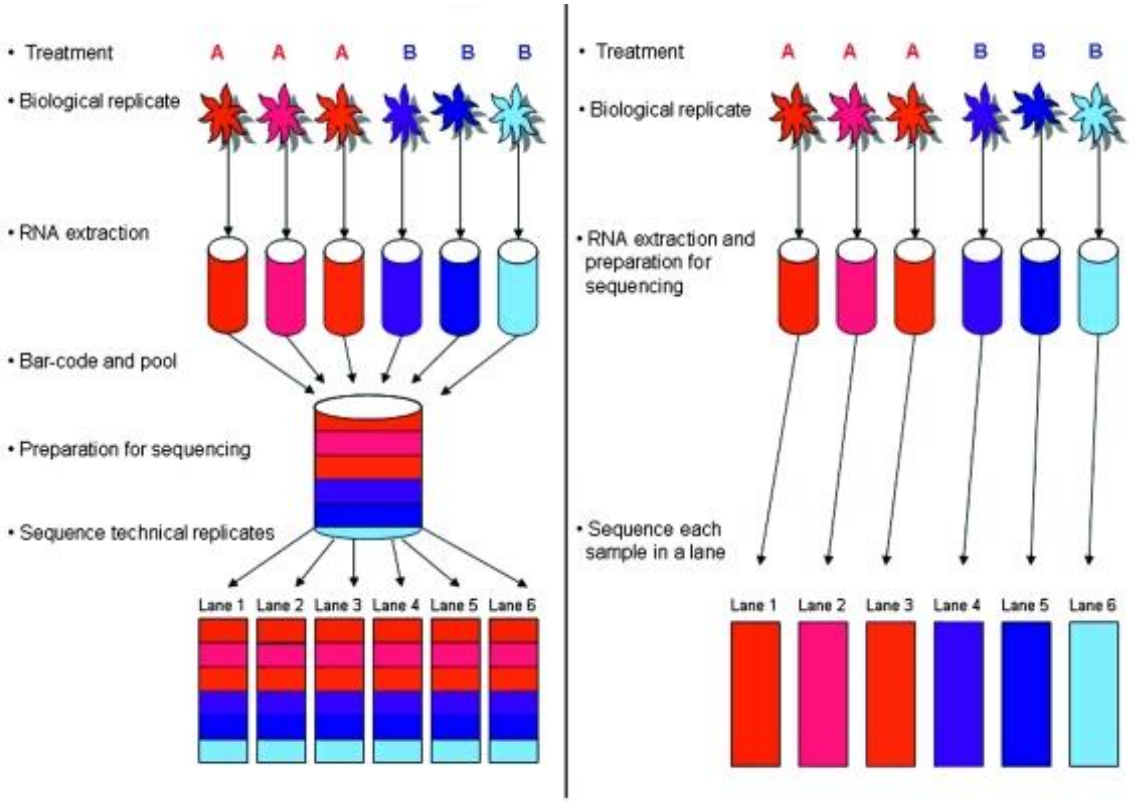
- It is impossible to partition biological variation from technical variation, when these two sources of variation are confounded.
- No amount of statistical sophistication can separate confounded factors after data have been collected.

Batch Effects

- Avoid batch effects -

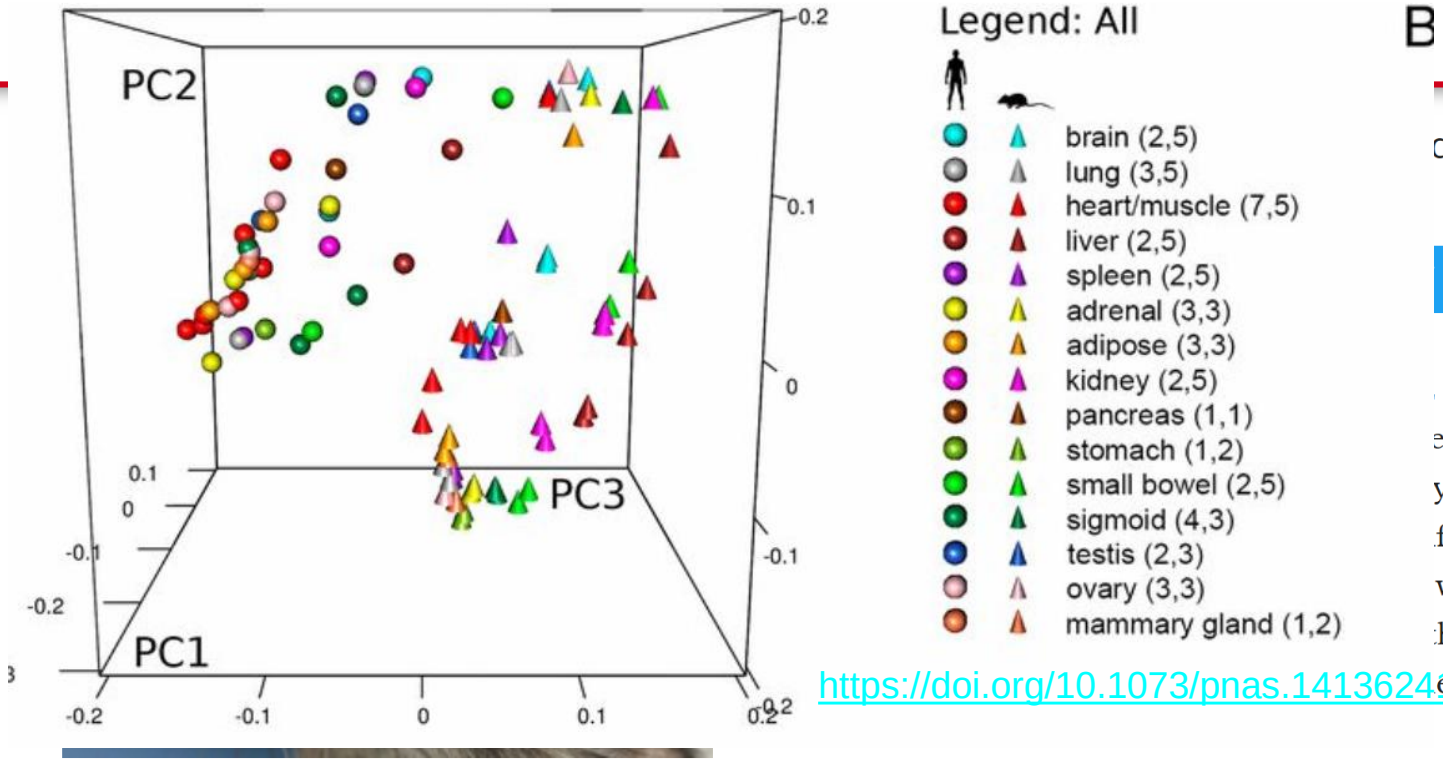
Technical sources of variation that have been added to samples during processing, such as extracting RNA with different kits or sequencing on different flowcells or lanes.

This design avoids the lane batch effect →



Auer and Doerge GENETICS *June 1, 2010 vol. 185 no. 2*

TheScientist



WIKIMEDIA, RAMA

https://doi.org/10.1073/pnas.1413624111

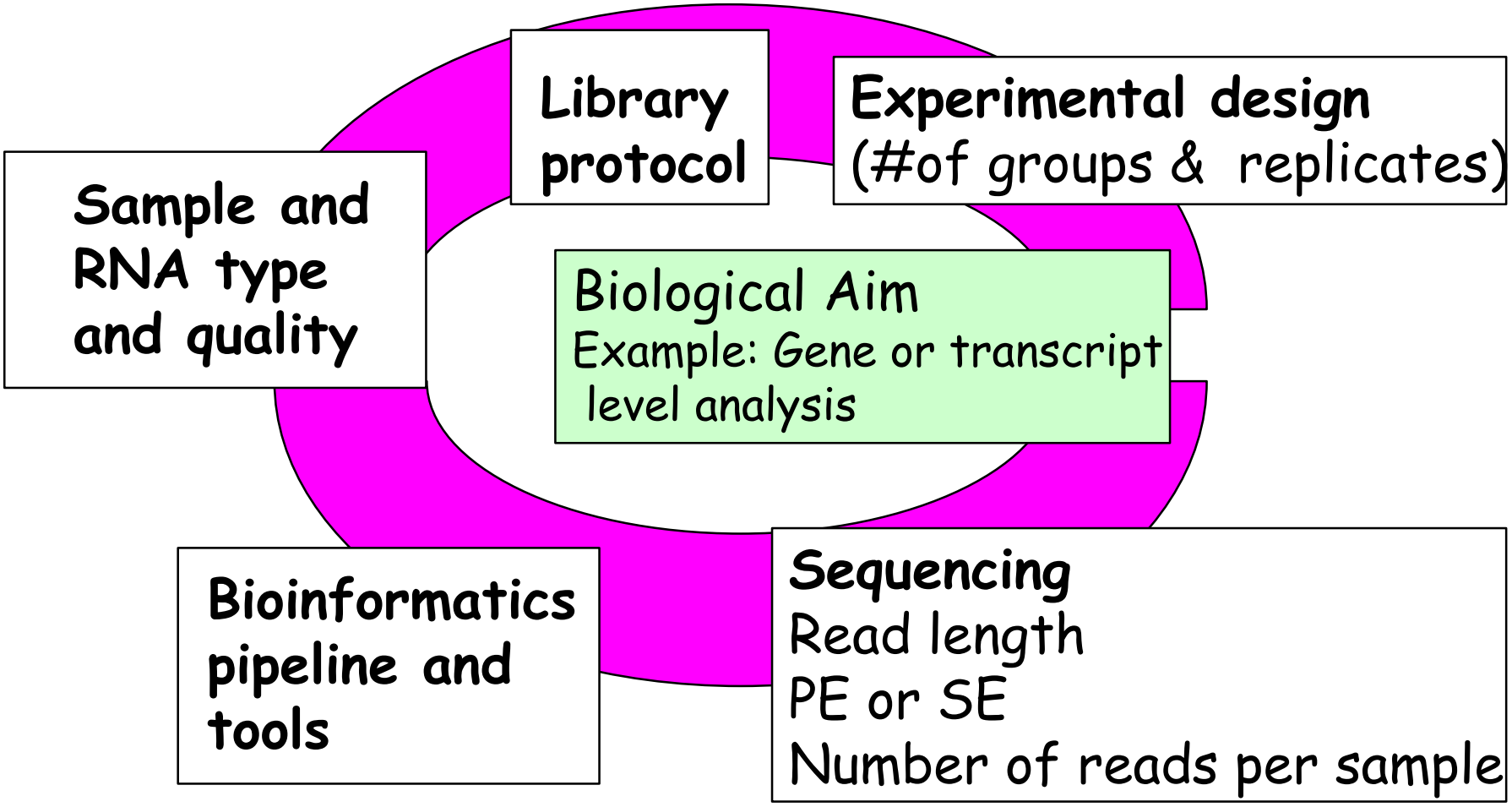
But earlier this month, Yoav Gilad of the University of Chicago called these results into question on Twitter. With a dozen or so 140-character dispatches (including three heat maps), Gilad suggested the results published in *PNAS* were an anomaly—a result of how the tissue samples were sequenced in different batches. If this "batch effect" was eliminated, he proposed, mouse and human tissues clustered in a tissue-specific manner, confirming previous results rather than supporting the conclusions reported by the Mouse ENCODE team.

Figure 1. Study design.

D87PMJN1 (run 253, flow cell D2GUAACXX, lane 7)	D87PMJN1 (run 253, flow cell D2GUAACXX , lane 8)	D4LHBFN1 (run 276, flow cell C2HKJACXX , lane 4)	MONK (run 312, flow cell C2GR3ACXX , lane 6)	HWI-ST373 (run 375, flow cell C3172ACXX , lane 7)
heart	adipose	adipose	heart	brain
kidney	adrenal	adrenal	kidney	pancreas
liver	sigmoid colon	sigmoid colon	liver	brain
small bowel	lung	lung	small bowel	spleen
spleen	ovary	ovary	testis	● Human
testis		pancreas		● Mouse

Gilad Y and Mizrahi-Man O. A reanalysis of mouse ENCODE comparative gene expression data [version 1]. F1000Research 2015, 4:121 (doi: 10.12688/f1000research.6536.1)

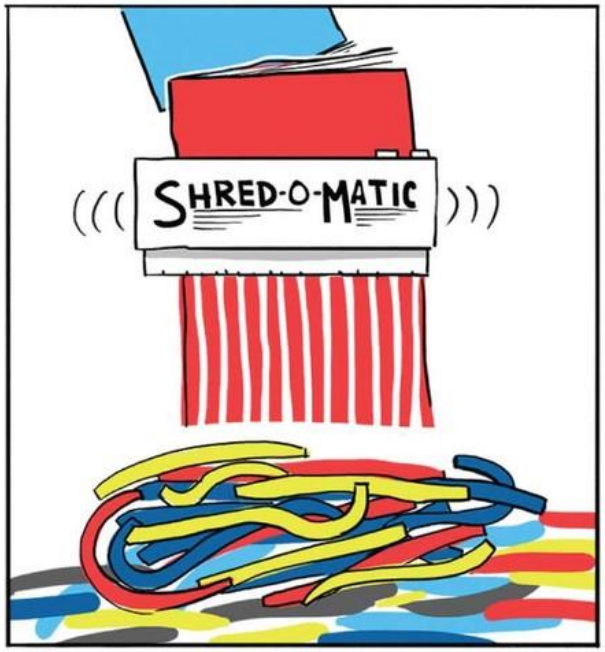
Summary RNA-Seq Experiment Planning



We recommend to come to a kick-off meeting with us, to help **plan** your experiment

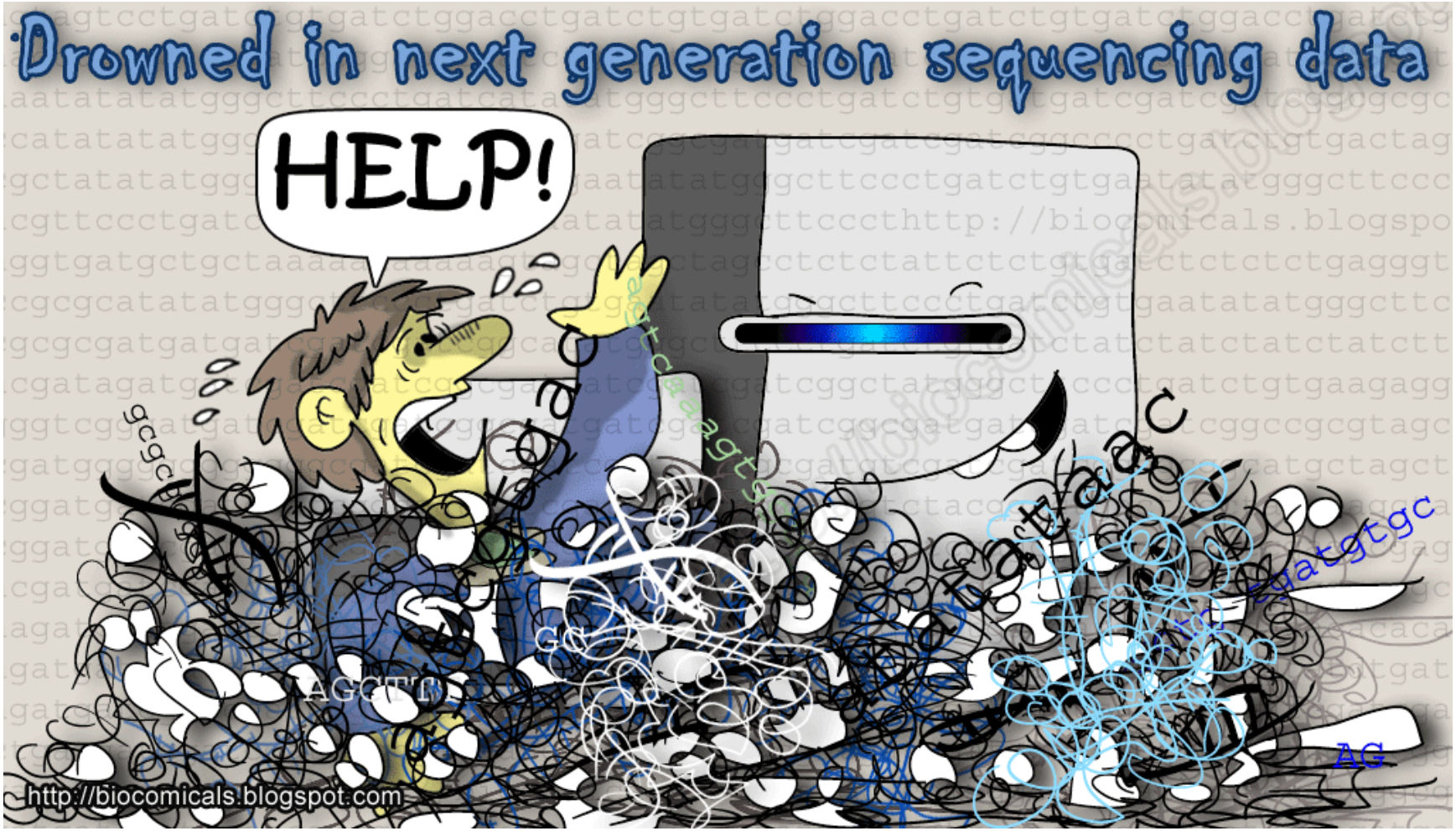
Agenda

- Introduction & Experimental design
- Analysing Gene expression from RNA-Seq data
- Analysing Gene expression from bulk MARS-Seq data



RNA-Seq is a straightforward process: you isolate RNA, sequence it with a high-throughput sequencer, and put it all back together. What is the problem?

Abigail Yu Nature Methods 10, 1165–1166 (2013)



HELP !!!!

I just got sequence data…

Sequence Output Format

- FASTQ

  Line 1: Unique ID for a sequencing read

  Line 2: Sequences

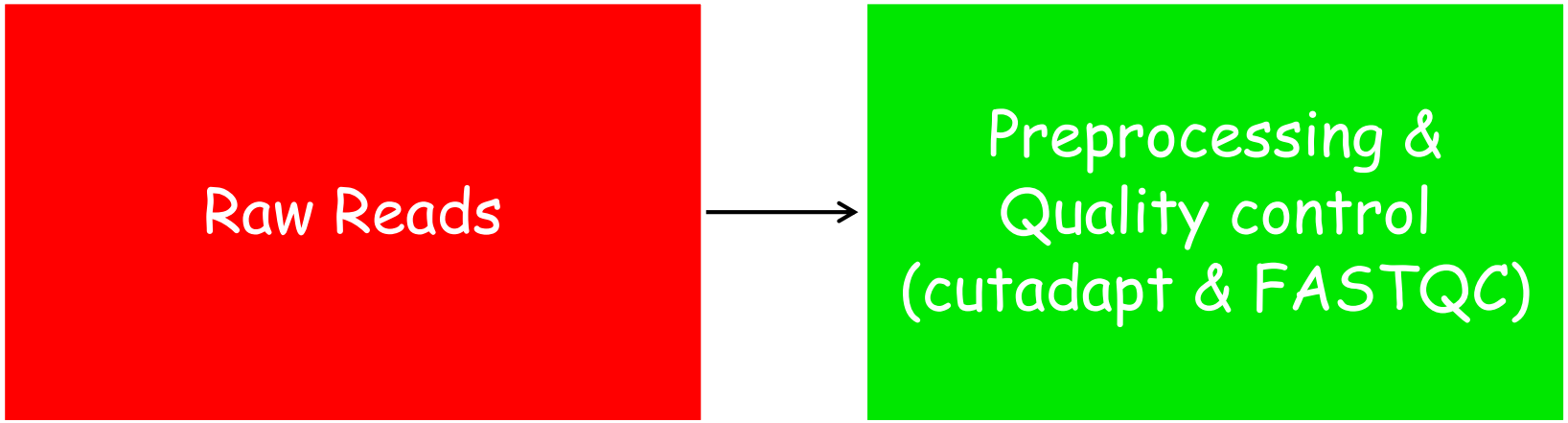
  Line 3:+

  Line 4: Base calling quality score (Analogous to Phred scores but in ASCII value)

  Example:

```
@HISEQ:126:H14YJADXX:1:1101:1118:2101 1:N:0:ATCACG
CTCCATAGTCAGAAACTTCAGCATGACAGTACCTCATGCTGCATCAGGTGATCATGAAAAGATTACAGGCTTTCTAAAATTATCAGCAAGATATGG
+
@@?ADDDD?ADHDIIIIIIIEIIIGEFHC<?FH4C9E9BGAFIGH<DG9BD?@DGGEGHHG<DCBBCC8C>FHCGEHIGEEE>EEHEEEEC>A>;;
```

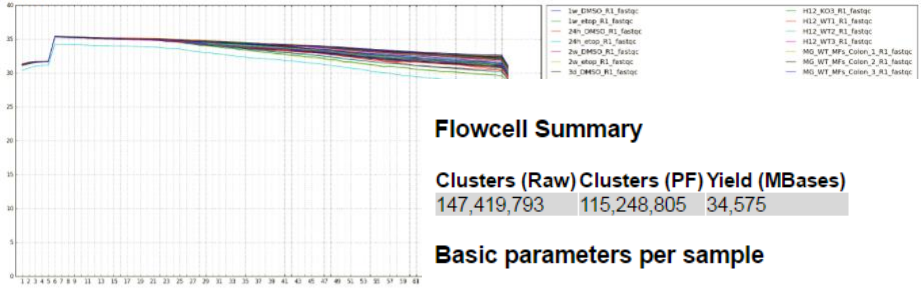
RNA-Seq Workflow



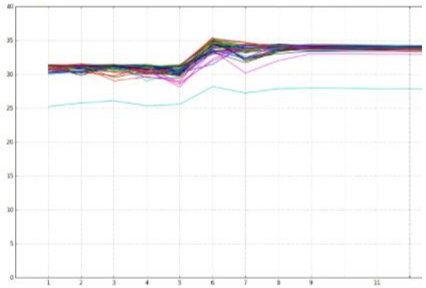
Example of QC report

Mean per base sequence quality

Read 1



Read 2



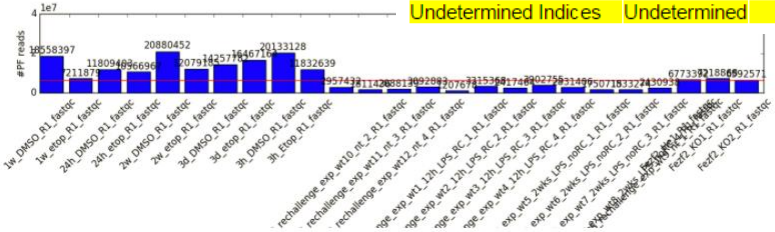
Flowcell Summary

Clusters (Raw)	Clusters (PF)	Yield (MBases)
147,419,793	115,248,805	34,575

Basic parameters per sample

Sample	Index	# PF Clusters	% Clusters per sample	Yield (MBases)	%≥Q30	FastQC Analysis	
A4_6bp_GAAGAA	GAAGAA	7,784,351	672.75	2,335	81.71 (R1) 72.38 (R2)	R1	R2
B4_6bp_AGGATC	AGGATC	5,678,462	490.50	1,703	82.04 (R1) 72.68 (R2)	R1	R2
C4_6bp_GACAGT	GACAGT	4,848,140	419.25	1,455	82.48 (R1) 71.31 (R2)	R1	R2
D4_6bp_CCTATG	CCTATG	11,618,248	1006.25	3,485	64.39 (R1) 54.91 (R2)	R1	R2
E4_6bp_TCGCCT	TCGCCT	4,487,566	387.50	1,346	80.13 (R1) 68.38 (R2)	R1	R2
H4_6bp_ATTCTA	ATTCTA	30,168,777	2621.00	9,050	61.09 (R1) 44.91 (R2)	R1	R2
P7-I1_ATCACG_Benny	ATCACG	130	0.00	0	23.08 (R1) 22.31 (R2)	R1	R2
P7-I2_CGATGT_Benny	CGATGT	98	0.00	0	57.14 (R1) 21.43 (R2)	R1	R2
P7-I3_TTAGGC_Benny	TTAGGC	6	0.00	0	(R1) (R2)	R1	R2
P7-I4_TGACCA_Benny	TGACCA	11	0.00	0	(R1) (R2)	R1	R2
P7-I7_CAGATC_Benny	CAGATC	29	0.00	0	20.69 (R1) 6.90 (R2)	R1	R2
P7-I8_ACTTGA_Benny	ACTTGA	103	0.00	0	50.49 (R1) 43.69 (R2)	R1	R2
P7-I8_ACTTGA_Benny	ACTTGA	103	0.00	0	50.49 (R1) 43.69 (R2)	R1	R2
Undetermined Indices	Undetermined	50,662,884	4402.25	15,200			

#PF reads per sample

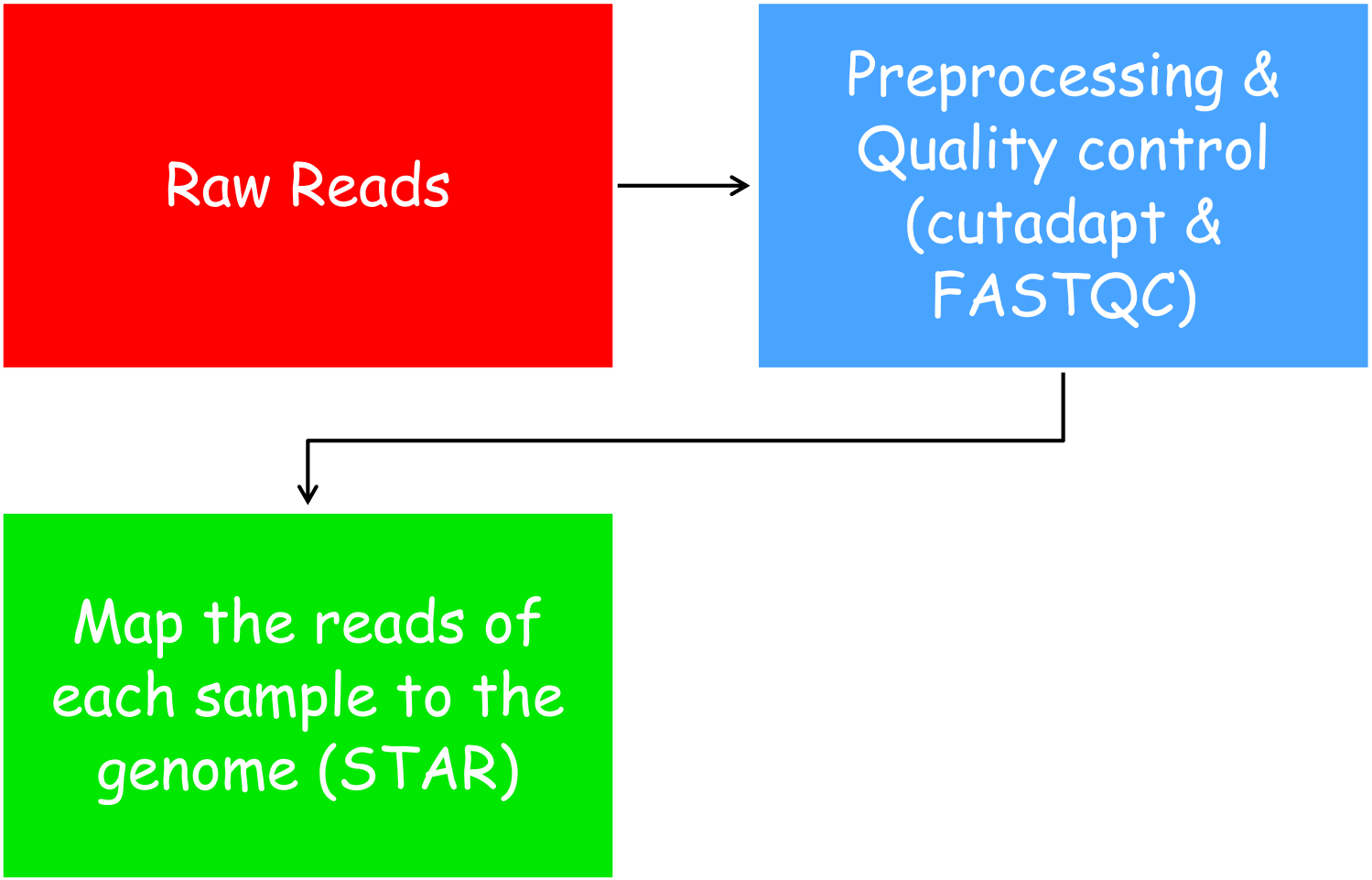


Pre-processing

- Recommendation is to use the high quality sequence data (is critical for de novo assembly), pre-processing includes:
  - Trim sequences if: the end is of low quality, contain adapter or polyA or polyT
  - Filter low quality reads
  - Avoid using samples with insufficient number of reads

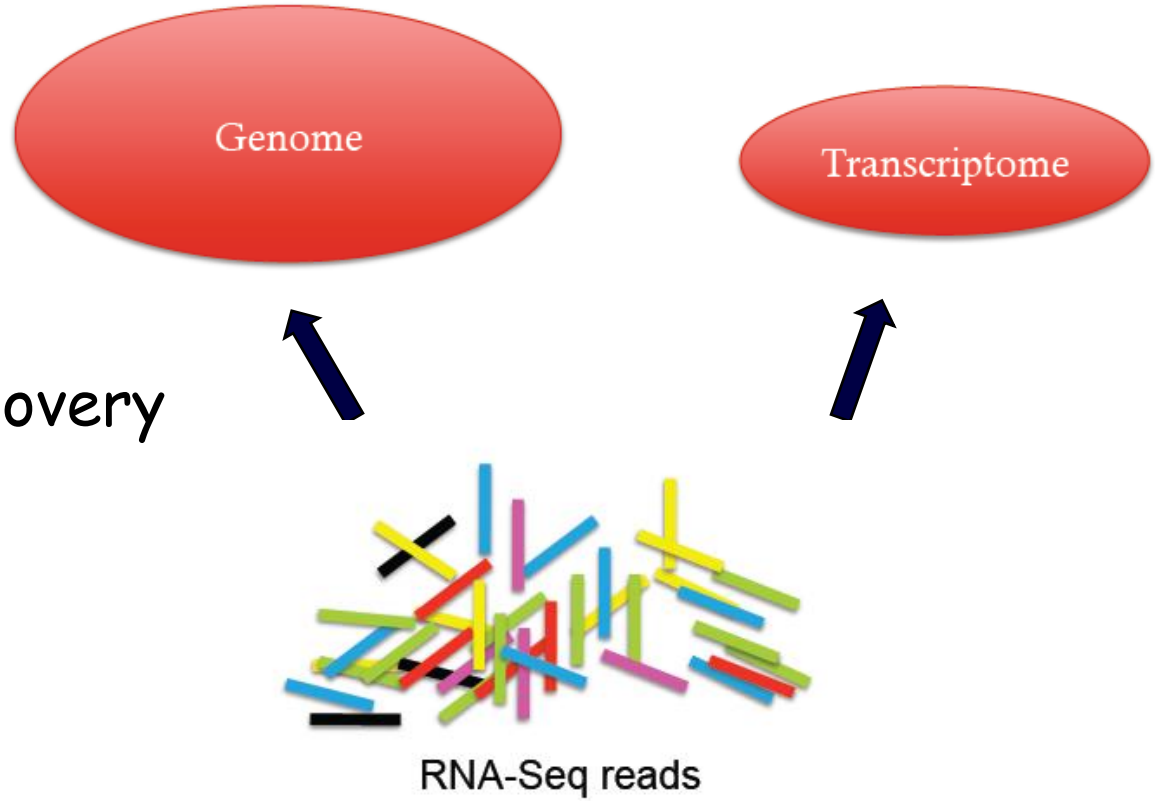
cutadapt

RNA-Seq Workflow

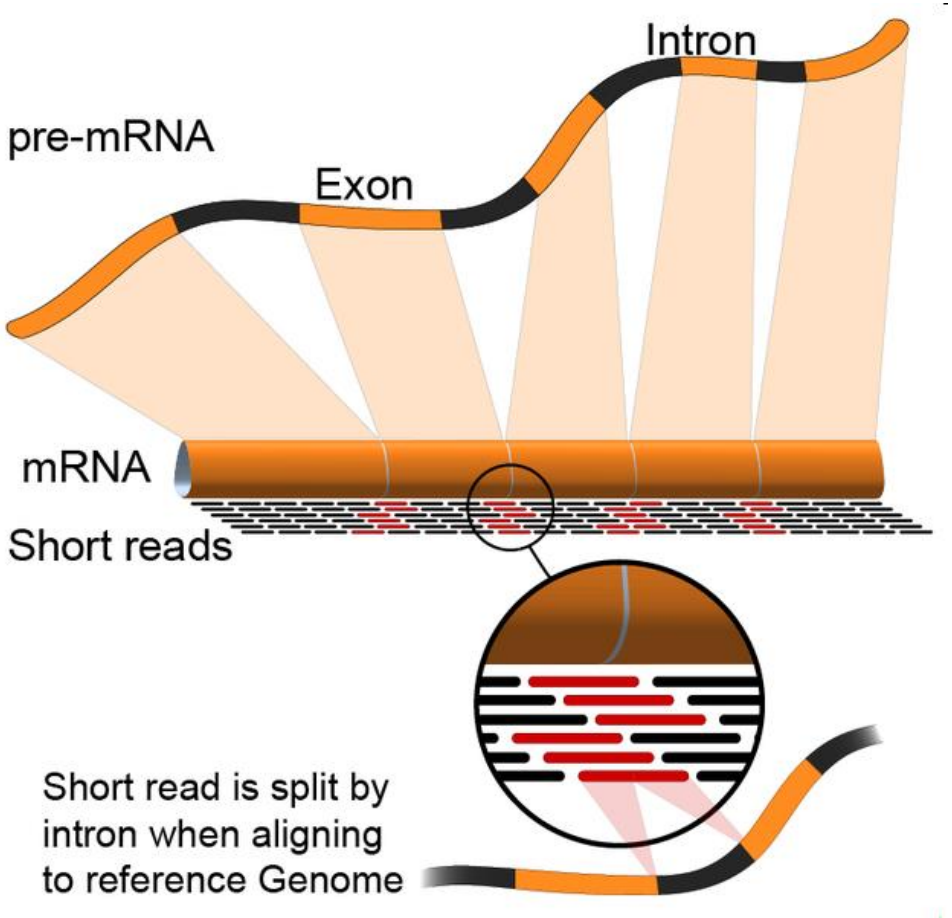


Mapping Short RNA-Seq Reads

Do I align the reads to the genome or to the transcriptome?



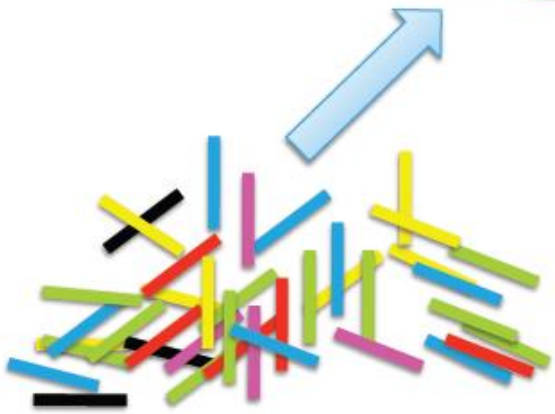
Mapping to Genome
How to align reads that span exons?



http://en.wikipedia.org/wiki/RNA-Seq

RNA-Seq mapping with TopHat

Goal: **identify** all transcripts and estimate relative amounts from RNA-Seq data



RNA-Seq reads

Bioinformatics. 2009 May 1; 25(9): 1105–1111. PMCID: PMC2672628
Published online 2009 March 16. doi: 10.1093/bioinformatics/btp120

TopHat: discovering splice junctions with RNA-Seq

Cole Trapnell,[1*] Lior Pachter,[2] and Steven L. Salzberg[1]

[1]Center for Bioinformatics and Computational Biology, University of Maryland, College Park, MD 20742 and [2]Department of Mathematics, University of California, Berkeley, CA 94720, USA
*To whom correspondence should be addressed.
Associate Editor: Ivo Hofacker

Genome Biology

METHOD Open Access

TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions

Daehwan Kim[1,2,3*], Geo Pertea[3], Cole Trapnell[5,6], Harold Pimentel[7], Ryan Kelley[8] and Steven L Salzberg[3,4]

Newer Aligners – Improving speed

Figure 2: Alignment speed of spliced alignment software for 20 million simulated 100-bp reads.

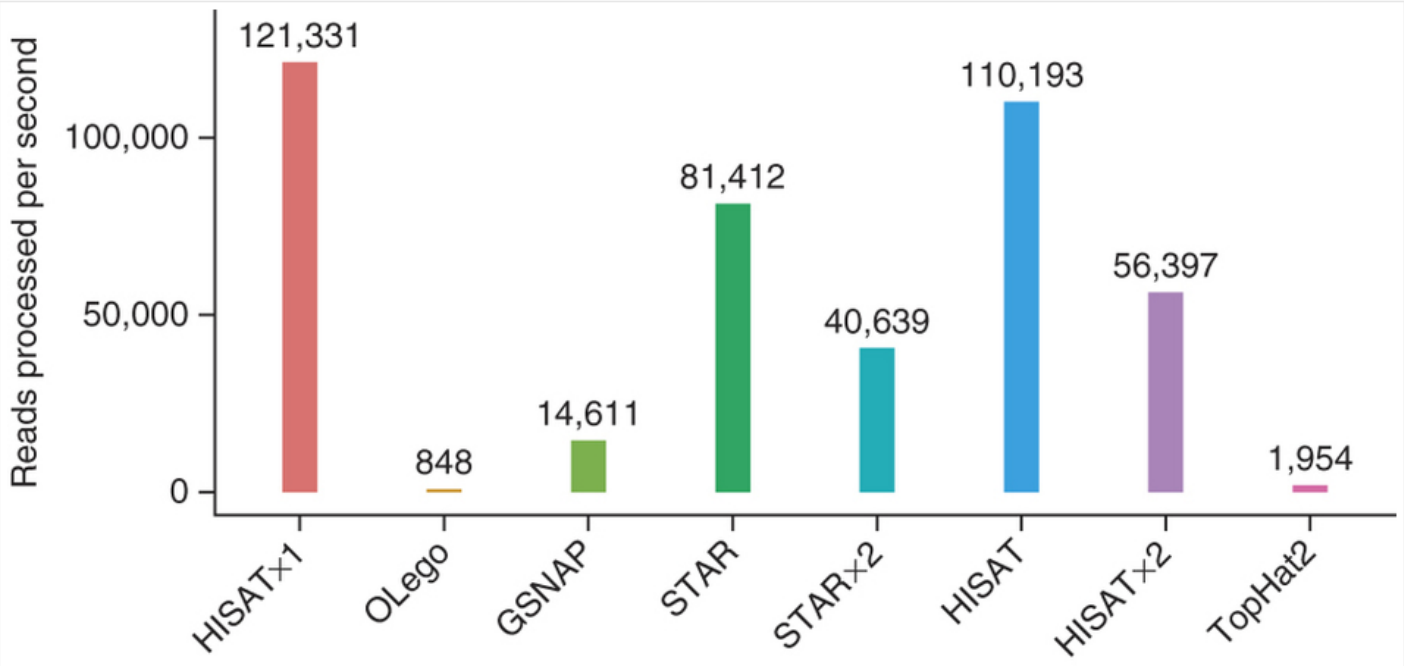
From

HISAT: a fast spliced aligner with low memory requirements

Daehwan Kim, Ben Langmead & Steven L Salzberg

Nature Methods **12**, 357–360 (2015) | doi:10.1038/nmeth.3317

Received 07 August 2014 | Accepted 16 January 2015 | Published online 09 March 2015



Alignment speed for all read types (defined in Fig. 1) combined, measured as the number of reads processed per second by the indicated tools. Supplementary Figure 2 provides the alignment speed for each type of read separately.

STAR-Spliced Transcripts Alignment to a Reference

(a)

In the first step, the algorithm finds the Maximal Exact Match (*MMP*) starting from the first base of the read

Next, the *MMP* search is repeated for the unmapped portion of the read

Speed of search is achieved since the suffix array is not compressed and therefore requires **increased memory usage (30-60Gb)**

Last stage is Clustering, stitching and scoring

(b)

If STAR does not find an exact matching sequence for each part of the read due to mismatches or indels, the previous MMPs will be extended.

(c)

If extension does not give a good alignment, then the poor quality or adapter sequence (or other contaminating sequence) will be soft clipped.

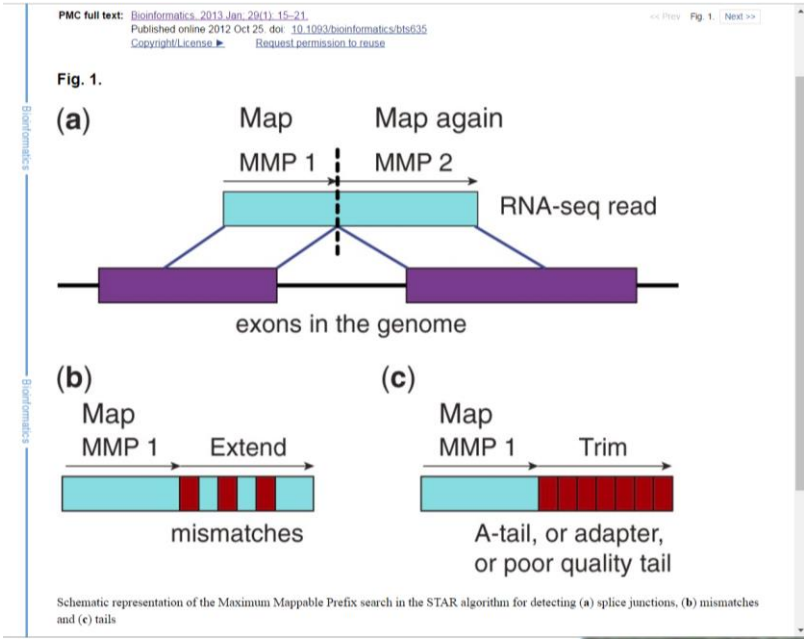
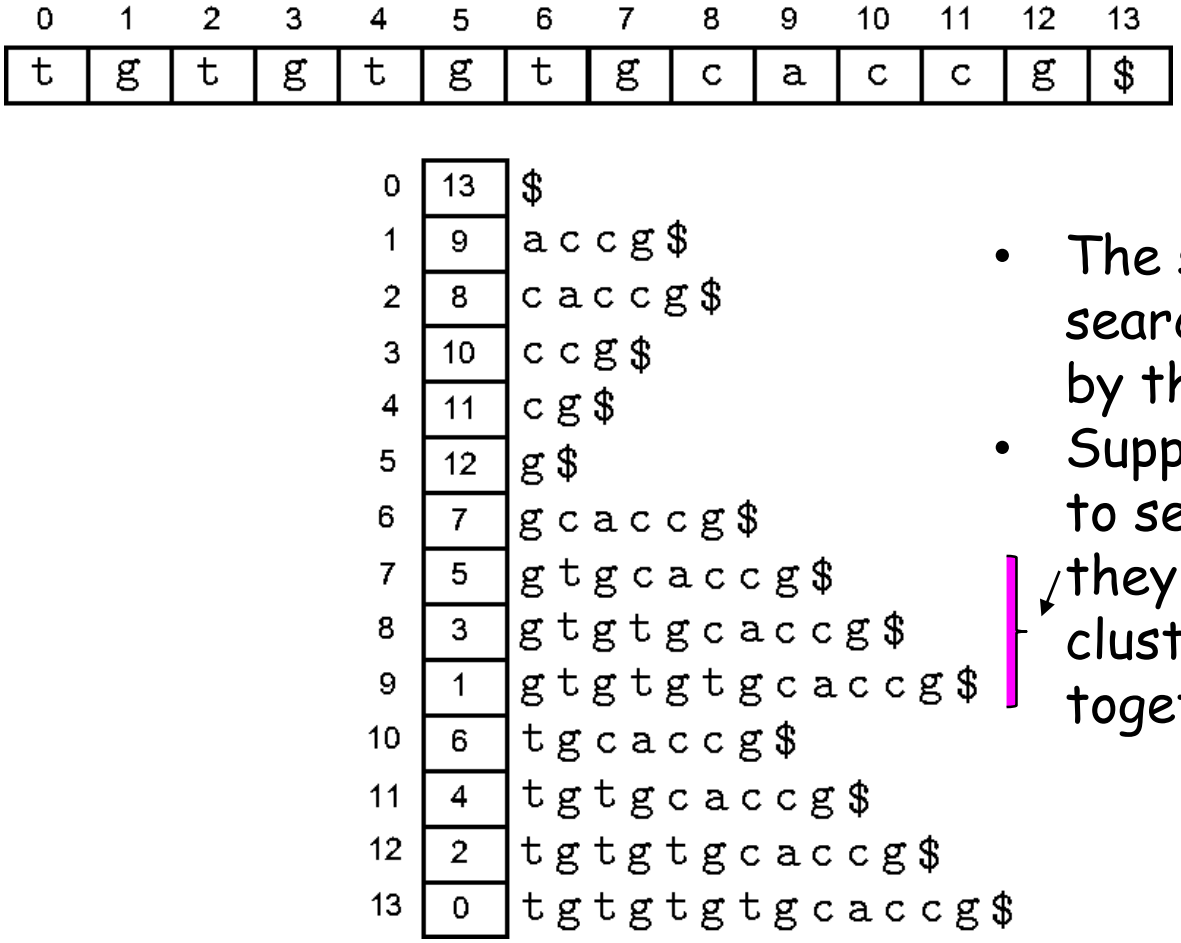


Figure 1: A string (above) and its suffix array (shown vertically) along with the position index on the left and the ...



Briefings in Bioinformatics, Volume 15, Issue 2, March 2014, Pages 138–154, https://doi.org/10.1093/bib/bbt081

Examples of Input and Output

Sequences – fastq

```
@HISEQ:226:C95PJANXX:2:1106:7378:57379 1:N:0:CGCTATGT
CCCGTCTCAAAAGGGAAACAGGTTGATATTCCTGTGCCAATAGTATTATGAGTTTCTTAGA
+
CCCCCGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGEECGGGGDDGGGGGGGGGGGGG
@HISEQ:226:C95PJANXX:2:1106:7467:57387 1:N:0:CGCTATGT
CTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
+
CCCCCGGGGGGGGBGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
@HISEQ:226:C95PJANXX:2:1106:7460:57432 1:N:0:CGCTATGT
CTTGCATTGTTATTTCTTGTCACTACCTCTCTTCTTTAGAATTGGGTAATTTACGCGCCTG
+
CCCCCGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
@HISEQ:226:C95PJANXX:2:1106:7269:57438 1:N:0:CGCTATGT
GTGGGGAGTTTGACTGGGGCGGTACATCTGTTAAATATTAACGCAGATGTCCAAAGACAAG
+
CCCCCGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
```

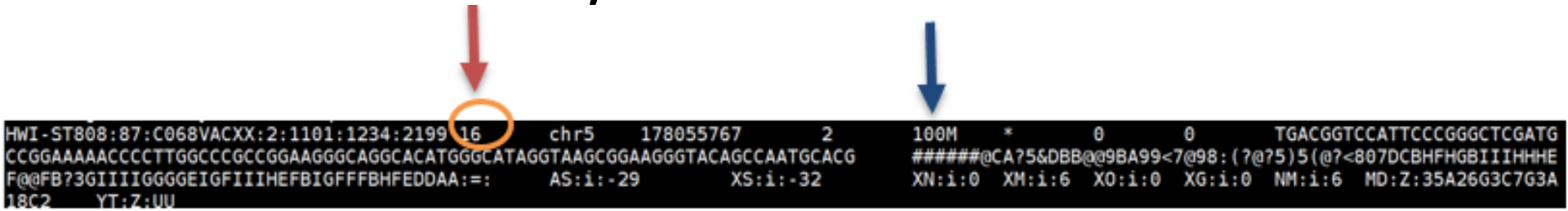
Mapping to genome

```
HISEQ:153:H8PNPADXX:1:1106:16824:79365  83      chr1    4839877 255     101M    =       4839864 -114
GGTAACTTTCTGTAGTTGGTAAGCCTACCAAGAATTCATGGTTACATCAGGCATCTTATTTTTAAGATATTTCTTTGAC DDEEDDDEDDDDBFFFFHHHHH
IGEJJJJJJJJJJJJJJIIGJIJJJIJJJJJJJJJJJJJJHHHHHFFFFFCCC NH:i:1  HI:i:1  AS:i:200        nM:i:0  MD:Z:1
```

Mapped Reads – bam format

Mapping Output - Alignment file

- SAM or binary BAM file



1.4 The alignment section: mandat

In the SAM format, each alignment line typicall
has 11 mandatory fields. These fields always appe
can be '0' or '*' (depending on the field) if the co
gives an overview of the mandatory fields in the

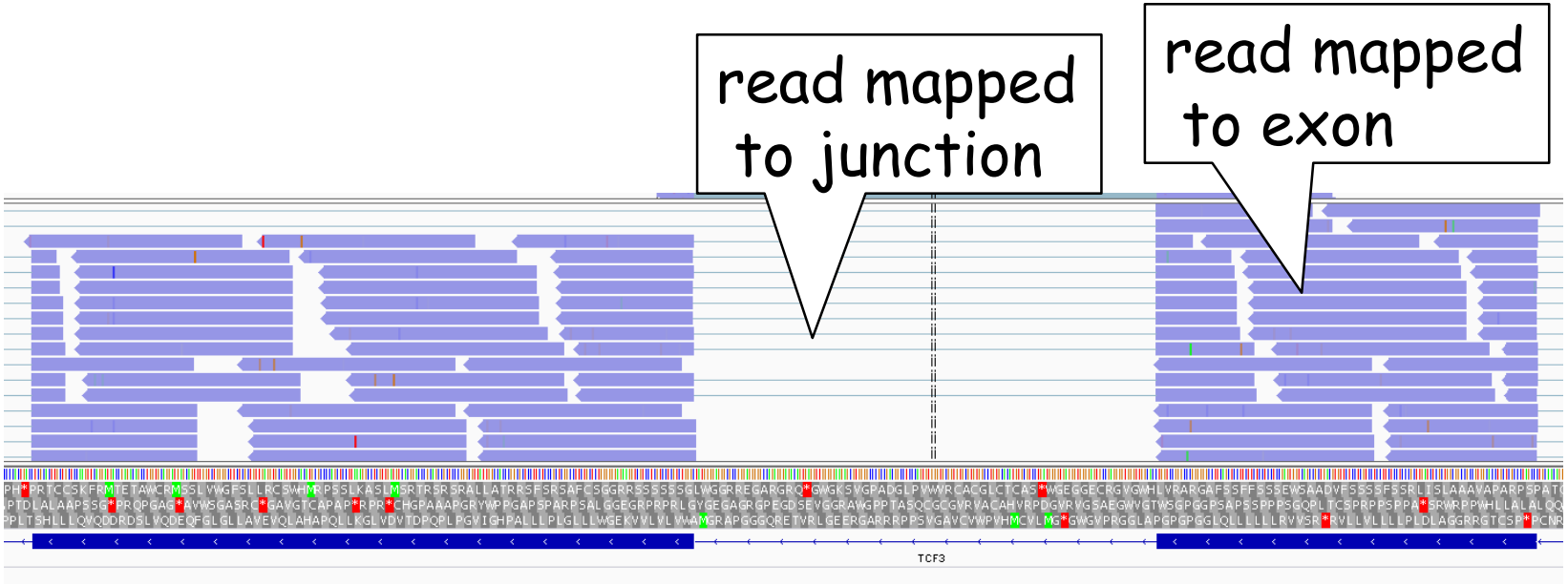
Col	Field	Type	Regexp/Range
1	QNAME	String	[!-?A-~]{1,254}
2	FLAG	Int	[0,2^{16}-1]
3	RNAME	String	*|[!-()+-<>-~]
4	POS	Int	[0,2^{31}-1]
5	MAPQ	Int	[0,2^{8}-1]
6	CIGAR	String	*|([0-9]+[MIDN
7	RNEXT	String	*|=|[!-()+-<>-
8	PNEXT	Int	[0,2^{31}-1]
9	TLEN	Int	[-2^{31}+1,2^{31}-1]
10	SEQ	String	*|[A-Za-z=.]+
11	QUAL	String	[!-~]+

understand the FLAG code of SAM format

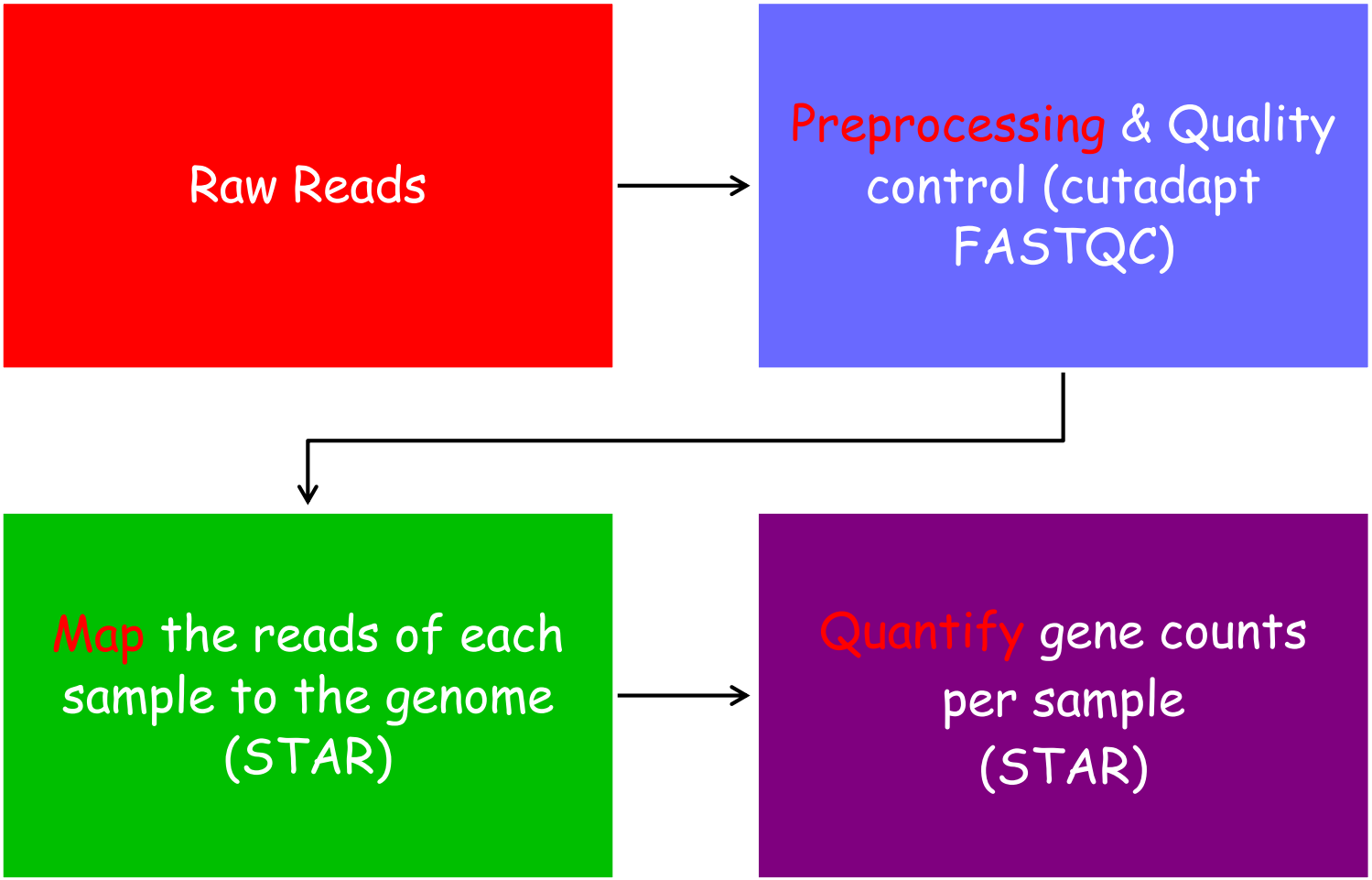
Here is what SAM specification stated for the FLAG column:

```
FLAG: bitwise FLAG. Each bit is explained in the following table:
Bit Description
0x1 template having multiple segments in sequencing (1)
0x2 each segment properly aligned according to the aligner (2)
0x4 segment unmapped (4)
0x8 next segment in the template unmapped (8)
0x10 SEQ being reverse complemented (16)
0x20 SEQ of the next segment in the template being reversed (32)
0x40 the rst segment in the template (64)
0x80 the last segment in the template (128)
0x100 secondary alignment (256)
0x200 not passing quality controls (512)
0x400 PCR or optical duplicate (1024)
```

Visualization of Bam outputs in a Genome Browser (IGV)

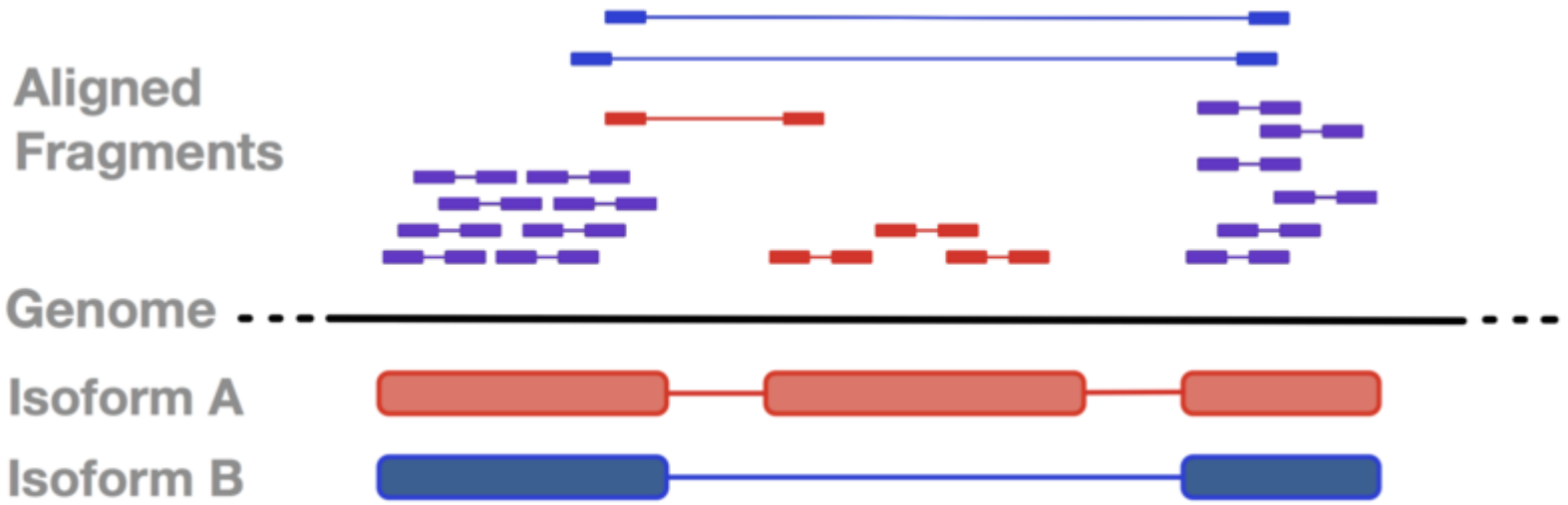


RNA-Seq Workflow



Gene Quantification

- A gene is quantified by counting the number of fragments/reads which align uniquely to all its exons.
- The gene exons are given to the program as a GTF file
- We do not need to determine from which transcript the read was derived



Trapnell et al. Nat Biotechnol. 2010 May;28(5):511-5.

Gene Transfer Format (GTF)

- GTF file is used to capture gene structure information.
- It is a tab-delimited text format

```
chr1    unknown exon    3214482 3216968 .       -       .       gene_id "Xkr4"; gene_name "Xkr4"; p_id "P15391"; transcript_id "NM_001011874"; tss_id "TSS27105";
chr1    unknown stop_codon      3216022 3216024 .       -       .       gene_id "Xkr4"; gene_name "Xkr4"; p_id "P15391"; transcript_id "NM_001011874"; tss_id "TSS27105";
chr1    unknown CDS     3216025 3216968 .       -       2       gene_id "Xkr4"; gene_name "Xkr4"; p_id "P15391"; transcript_id "NM_001011874"; tss_id "TSS27105";
chr1    unknown CDS     3421702 3421901 .       -       1       gene_id "Xkr4"; gene_name "Xkr4"; p_id "P15391"; transcript_id "NM_001011874"; tss_id "TSS27105";
chr1    unknown exon    3421702 3421901 .       -       .       gene_id "Xkr4"; gene_name "Xkr4"; p_id "P15391"; transcript_id "NM_001011874"; tss_id "TSS27105";
chr1    unknown CDS     3670552 3671348 .       -       0       gene_id "Xkr4"; gene_name "Xkr4"; p_id "P15391"; transcript_id "NM_001011874"; tss_id "TSS27105";
chr1    unknown exon    3670552 3671498 .       -       .       gene_id "Xkr4"; gene_name "Xkr4"; p_id "P15391"; transcript_id "NM_001011874"; tss_id "TSS27105";
chr1    unknown start_codon     3671346 3671348 .       -       .       gene_id "Xkr4"; gene_name "Xkr4"; p_id "P15391"; transcript_id "NM_001011874"; tss_id "TSS27105";
chr1    unknown exon    4290846 4293012 .       -       .       gene_id "Rp1"; gene_name "Rp1"; p_id "P17361"; transcript_id "NM_001195662"; tss_id "TSS6138";
chr1    unknown stop_codon      4292981 4292983 .       -       .       gene_id "Rp1"; gene_name "Rp1"; p_id "P17361"; transcript_id "NM_001195662"; tss_id "TSS6138";
```

GTF format

GTF (Gene Transfer Format) is a refinement to GFF that tightens the specification. The first eight GTF fields are the same as GFF. The *group* field has been expanded into a list of *attribute* Each attribute consists of a type/value pair. Attributes must end in a semi-colon, and be separated from any following attribute by exactly one space.

The attribute list must begin with the two mandatory attributes:

- **gene_id *value*** - A globally unique identifier for the genomic source of the sequence.
- **transcript_id *value*** - A globally unique identifier for the predicted transcript.

Example:
Here is an example of the ninth field in a GTF data line:

```
gene_id "Em:U62317.C22.6.mRNA"; transcript_id "Em:U62317.C22.6.mRNA"; exon_number 1
```

The Genome Browser groups together GTF lines that have the same *transcript_id* value. It only looks at features of type *exon* and *CDS*.

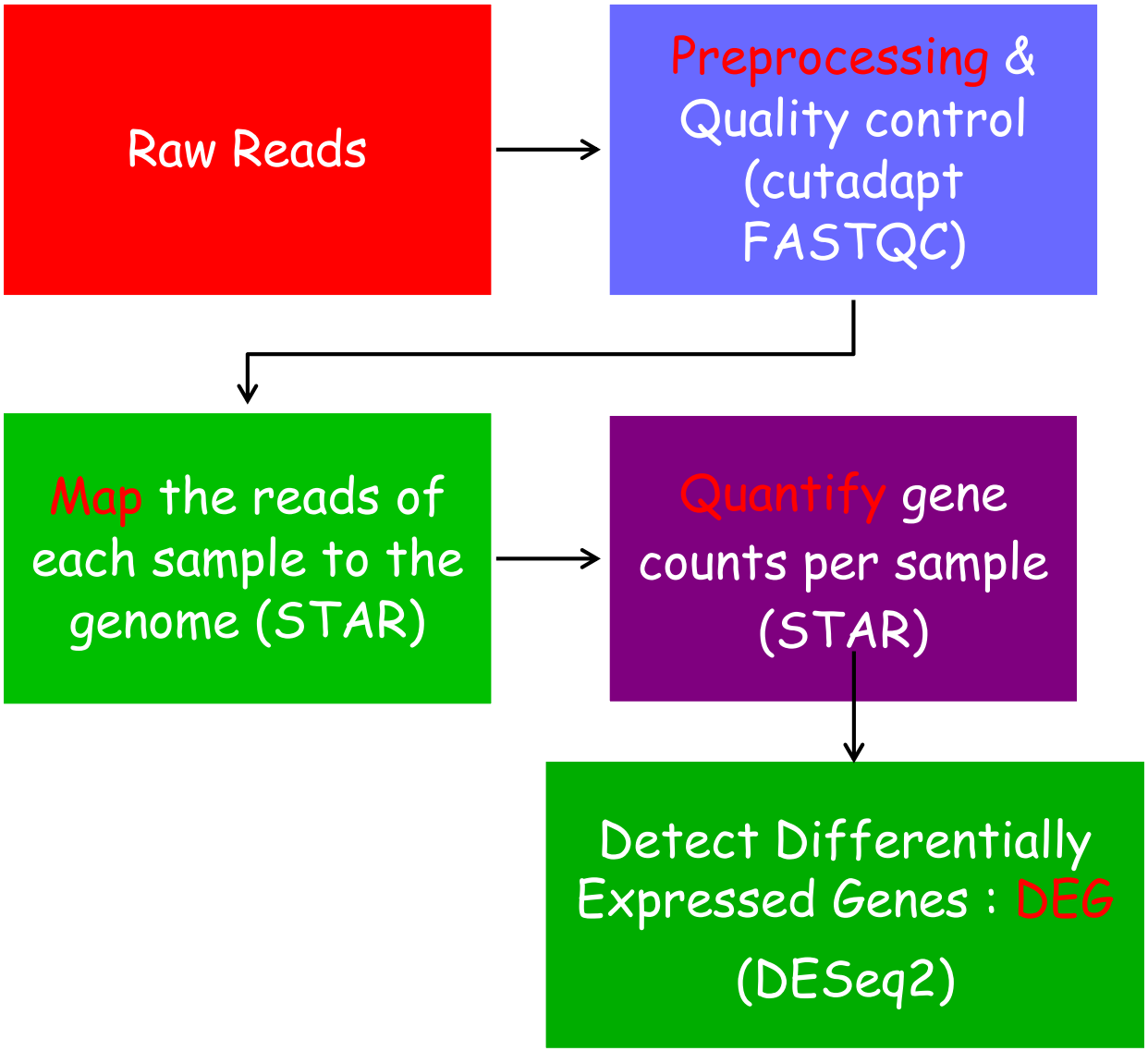
For more information on this format, see http://mblab.wustl.edu/GTF2.html. If you would like to obtain browser data in GTF format, please refer to Genes in gtf or gff format on the wiki.

STAR/HTSeq Result
A count matrix

	sample_1	sample_2	sample_3	sample_4
gene_1	15	9	11	18
gene_2	19	21	21	40
gene_3	106	114	153	207
gene_4	569	565	756	992
gene_5	1029	1260	1559	1968
gene_6	5049	10029	7537	200
SUM	**10 M**	**30 M**	**20 M**	**10 M**

Need to account for the differences in sequence amount between the samples

RNA-Seq Workflow



DESeq2 Normalization
median-of-ratios method

- Create a "virtual reference sample" by taking, for each gene, the geometric mean of counts over all samples
- Normalize each sample to this reference, to get one scaling factor ("size factor") per sample.

Example-DESeq Normalization

	sample_1	sample_2	sample_3	sample_4	geometric mean		ratio sample_1
gene_1	15	9	11	18	12.79		1.17
gene_2	19	21	21	40	24.06		0.79
gene_3	106	114	153	207	139.87		0.76
gene_4	569	565	756	992	700.73		0.81
gene_5	1029	1260	1559	1968	1412.26		0.73
gene_6	5049	5897	7537	10029	6887.68		0.73
						Median	0.77

The scaling factor for sample 1 is 0.77

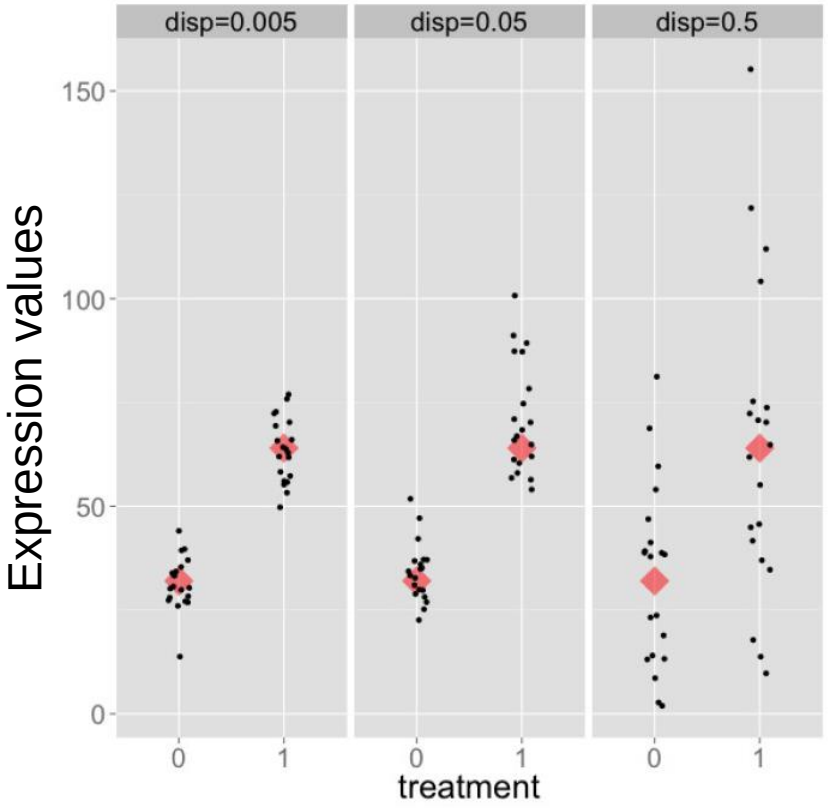
DESeq2 Normalization

Need to normalize the amount of sequence data between the samples

1. Geometric mean is calculated for each gene across all samples.
2. The counts for a gene in each sample is then divided by this mean.
3. The median of these ratios in a sample is the size factor for that sample.
4. The counts are divided by the sample-specific size factors

This procedure corrects for library size and RNA composition bias, which can arise for example when only a small number of genes are very highly expressed in one experiment condition but not in the other.

Determining Differentially Expressed Genes



http://www.molgen.mpg.de/1242892/rnaseq.pdf

- Aim: finding genes which have a significant difference between the groups which is larger than the "noise" – variation within the groups
- The advantage of having many replicates allows us to learn about the biological variation within the groups tested

Determining Differentially Expressed Genes (DEG)

- Our input are genes counts, i.e. discrete values
- In order to determine the DEG genes we need to module the data i.e. make assumptions on the statistical properties
- Incorrect assumptions can lead to poor false discovery rate (FDR) control and inaccurate true positive identification in the DEG calls.

RNA-Seq Noise

Suppose we sequence the same library twice to the same depth. For instance sequence it on two different lanes?

Which kind of replicates are these ?

Will we get the same gene counts?

RNA-Seq a Sampling Experiment

- A typical RNA library is estimated to have 2.408×10^{12} different molecules.
- If we sequence 30 million reads –this means 30M molecules are sampled.
- Our sample represents approximately 0.0013% of the total number of available molecules.
- It is therefore clear that when we sample twice we will observe a variance in the gene counts

RNA-Seq Noise

- In the case of sequencing the same library twice, since we have a large total number of reads and only a small fraction of reads mapping to each gene, then the observed read counts for an individual gene can be well approximated by a Poisson distribution.
- Poisson distribution is sometimes called the law of small numbers because it is the probability distribution of the number of occurrences of an event that happens rarely but has very many opportunities to happen.

Poisson Distribution

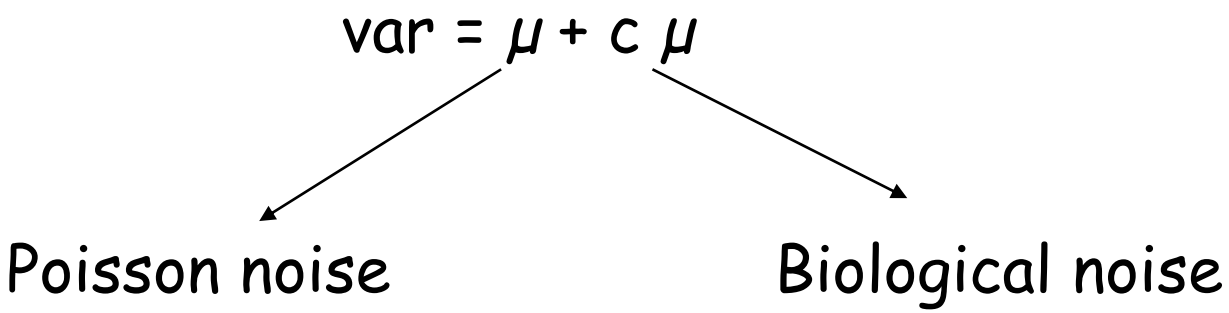
- Assuming the gene counts in a RNA-Seq experiment follow a Poisson distribution we would expect that the average gene count and the variance of the counts are equal.

  var = μ

- https://youtu.be/fxtB8c3u6l8
- https://youtu.be/HK7WKsL3c2w

Biological Variation

- When we sequence biological replicate samples the concentration of a given gene will vary around a mean value with a certain standard deviation
- This standard deviation needs to be to be estimated from the data, in the case of RNA-Seq we need to estimate it with a limited number of replicates



Negative Binomial

- In RNA-Seq analysis the negative binomial distribution is used as an alternative to the Poisson since it takes into account variance that exceeds the gene mean
- The count data is used to estimate the variance

  Orange line: the fitted observed curve for the variance

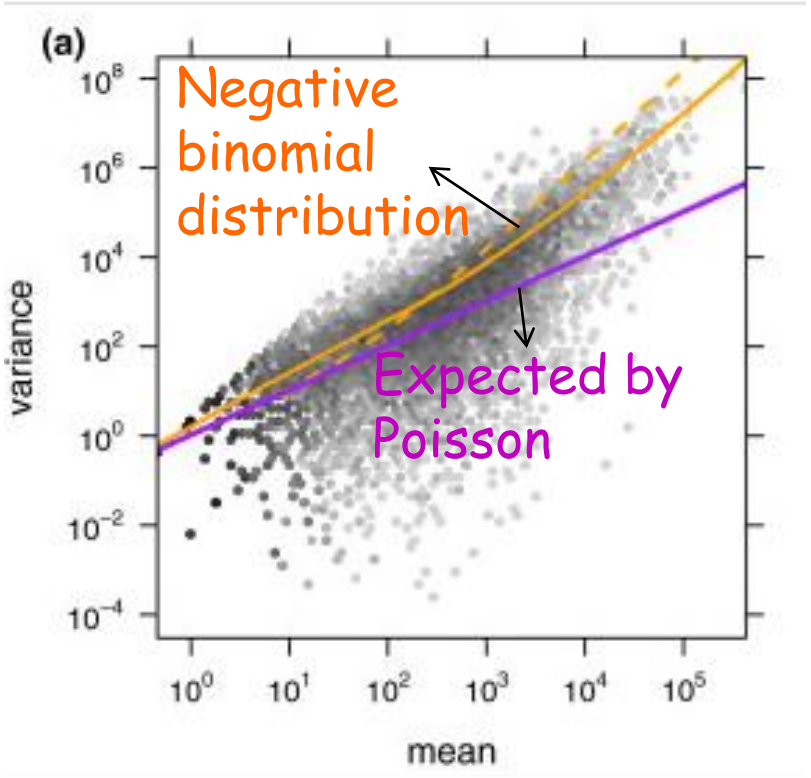
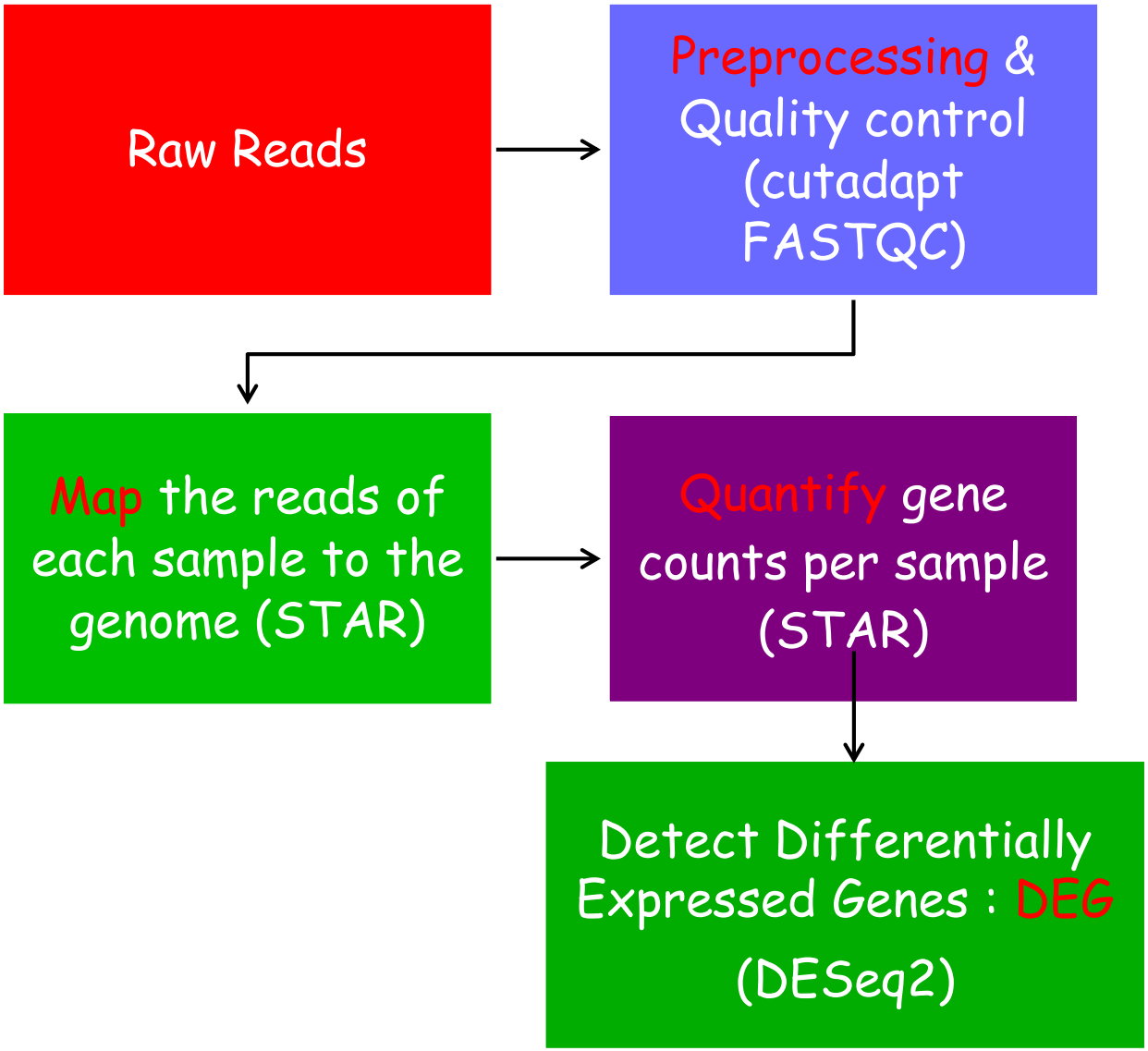


Fig. 1 from Anders & Huber, 2010: Dependence

Detecting Differentially Expressed Genes

- DESeq2 tests for differential expression by the use of negative binomial generalized linear models
- The output consists of:
  - Log fold change (treatment/control)
  - p-value - indicates the probability that the observed difference between treatment and control will be observed even though the there is no true treatment effect
  - Adjusted p value – multiple test correction
    - In the RNA-Seq study we simultaneously tested all genes

RNA-Seq Workflow



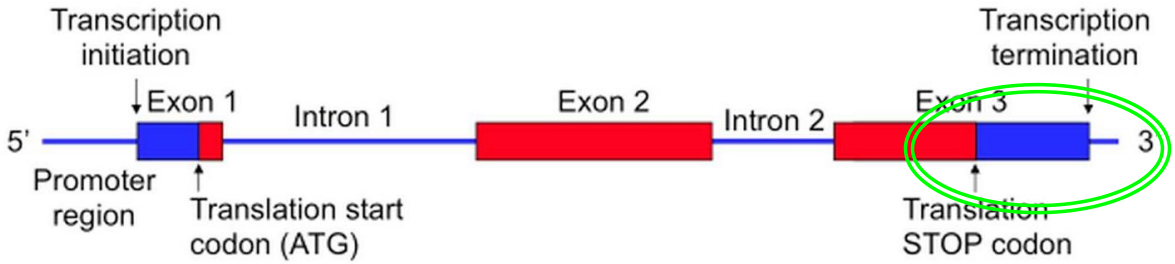
Agenda

- Introduction & Experimental design
- Analysing Gene expression from RNA-Seq data
- Analysing Gene expression from bulk MARS-Seq data

MARS-Seq

Differences between RNA-Seq and bulk MARS-Seq:

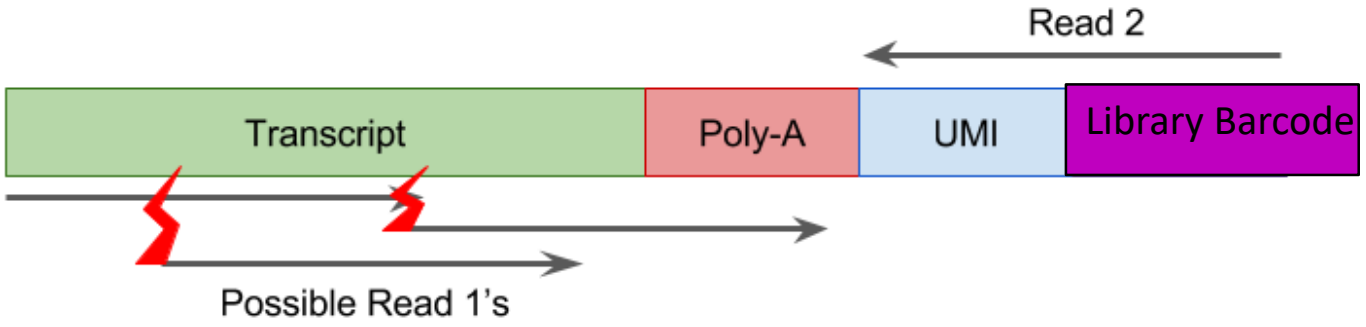
- Library generated contains only 3' end of the transcripts



- Low input material - UMI (Unique Molecular Identifier)

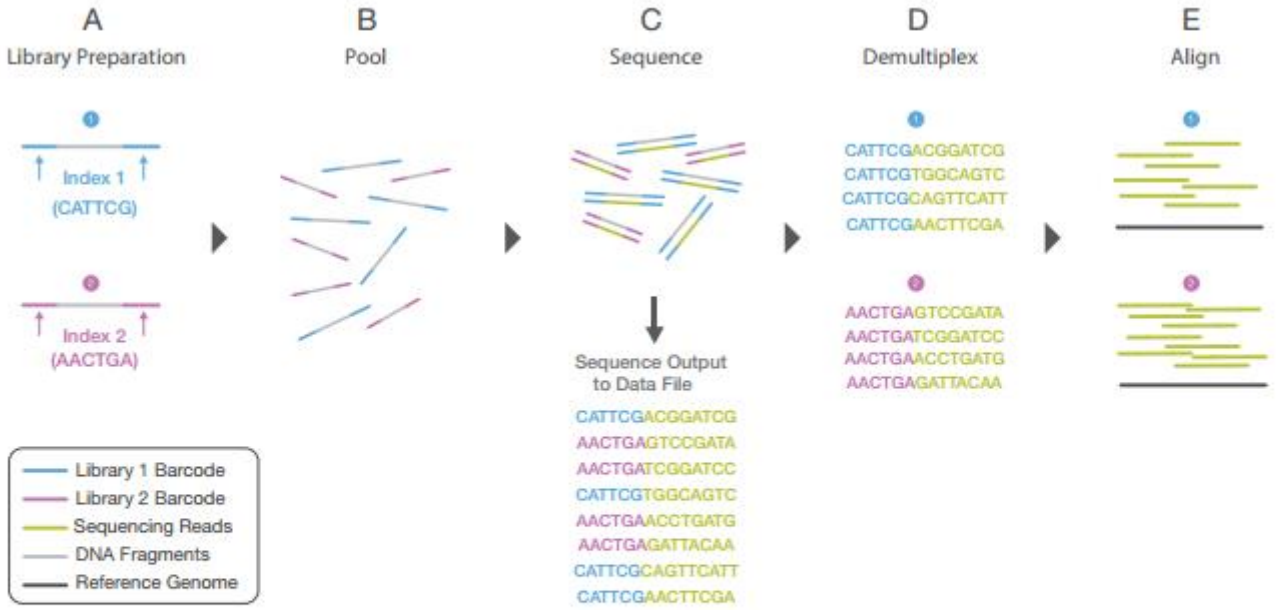
MARS-Seq – Paired end sequencing

- Read 1 (R1) contains 3′ cDNA insert sequence
- Read2 (R2) contains the library barcode and the UMI sequence



Library Barcode

- Multiplexing: the process of pooling samples together and sequencing them simultaneously
- Demultiplexing: separating reads using the library barcode to identify the origin sample



http://www.illumina.com/content/dam/illumina-marketing/documents/products/illumina_sequencing_introduction.pdf

Figure 5: Library Multiplexing Overview.

a. Two distinct libraries are attached to unique index sequences. Index sequences are attached during library preparation.

b. Libraries are pooled together and loaded into the same flow cell lane.

c. Libraries are sequenced together during a single instrument run. All sequences are exported to a single output file.

d. A demultiplexing algorithm sorts the reads into different files according to their indexes.

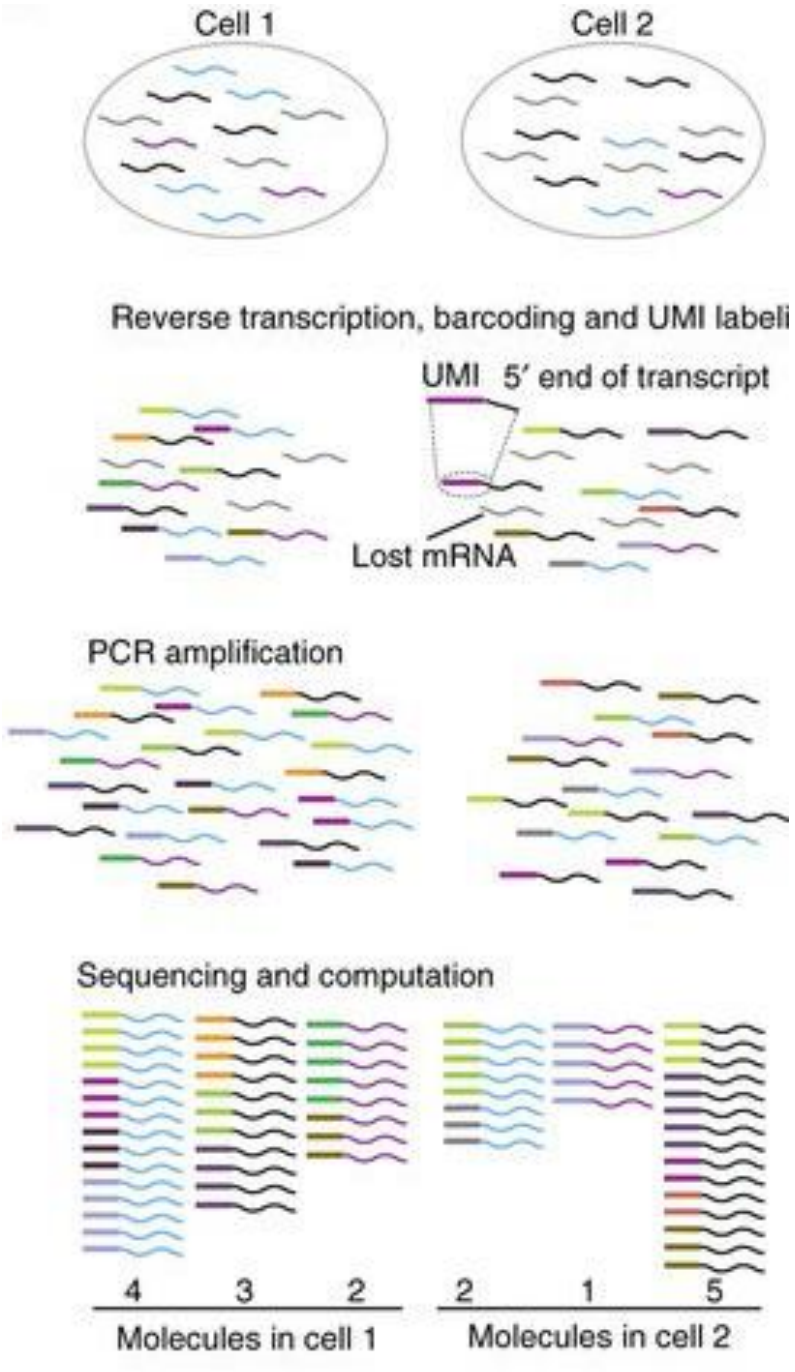
e. Each set of reads is aligned to the appropriate reference sequence.

Illumina Sample Index (barcodes)

Since the library barcode in the Illumina protocols and MARS-Seq protocol is not compatible, these libraries should not be pooled together

UMI

- The 8 base UMI is used as an identifier of a specific transcript molecule
- 4^8 = 65536 theoretical possibilities
- Reads are considered PCR duplicated, if they map to the same gene and have the same UMI
- Instead of counting reads we will count number of unique UMIs per gene

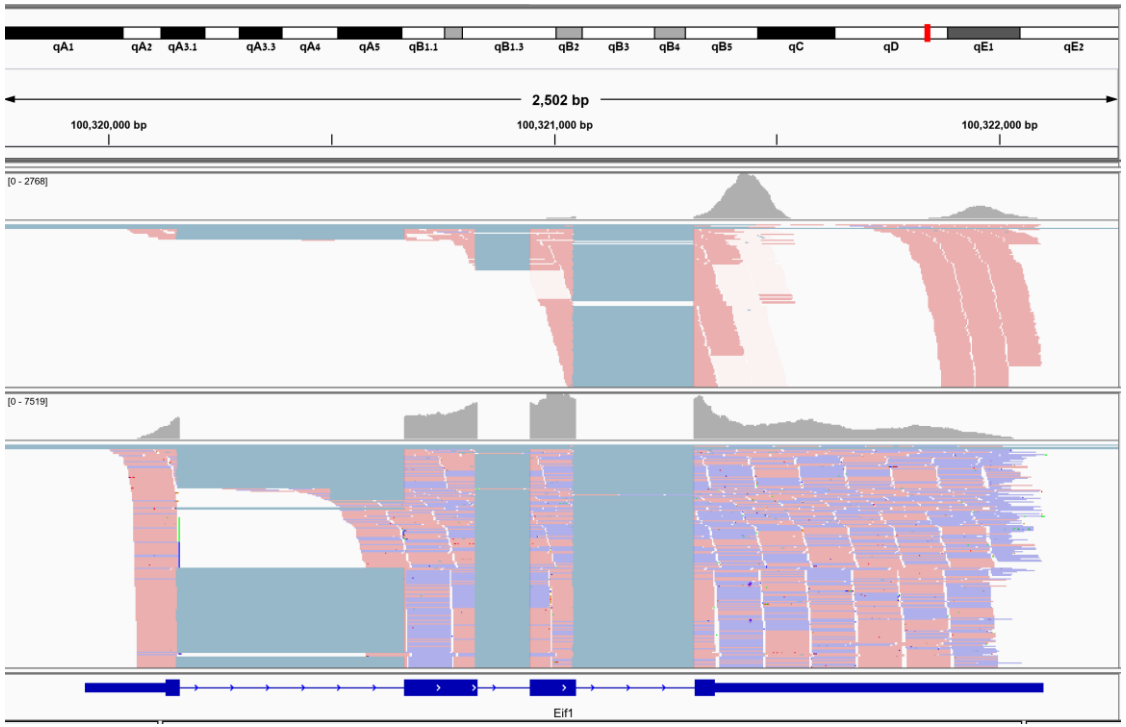


This figure is adapted from Islam et al (2014)

Differences MARS-Seq vs RNA-Seq

	MARS-Seq	RNA-Seq
Gene coverage	3'	The whole transcript
# READS per sample	5M	20M
Sequencing protocol	PE	SE or PE
Location of library index	R2	Illumina index (i5 & i7)
Location of UMI barcode	R2	NO UMI

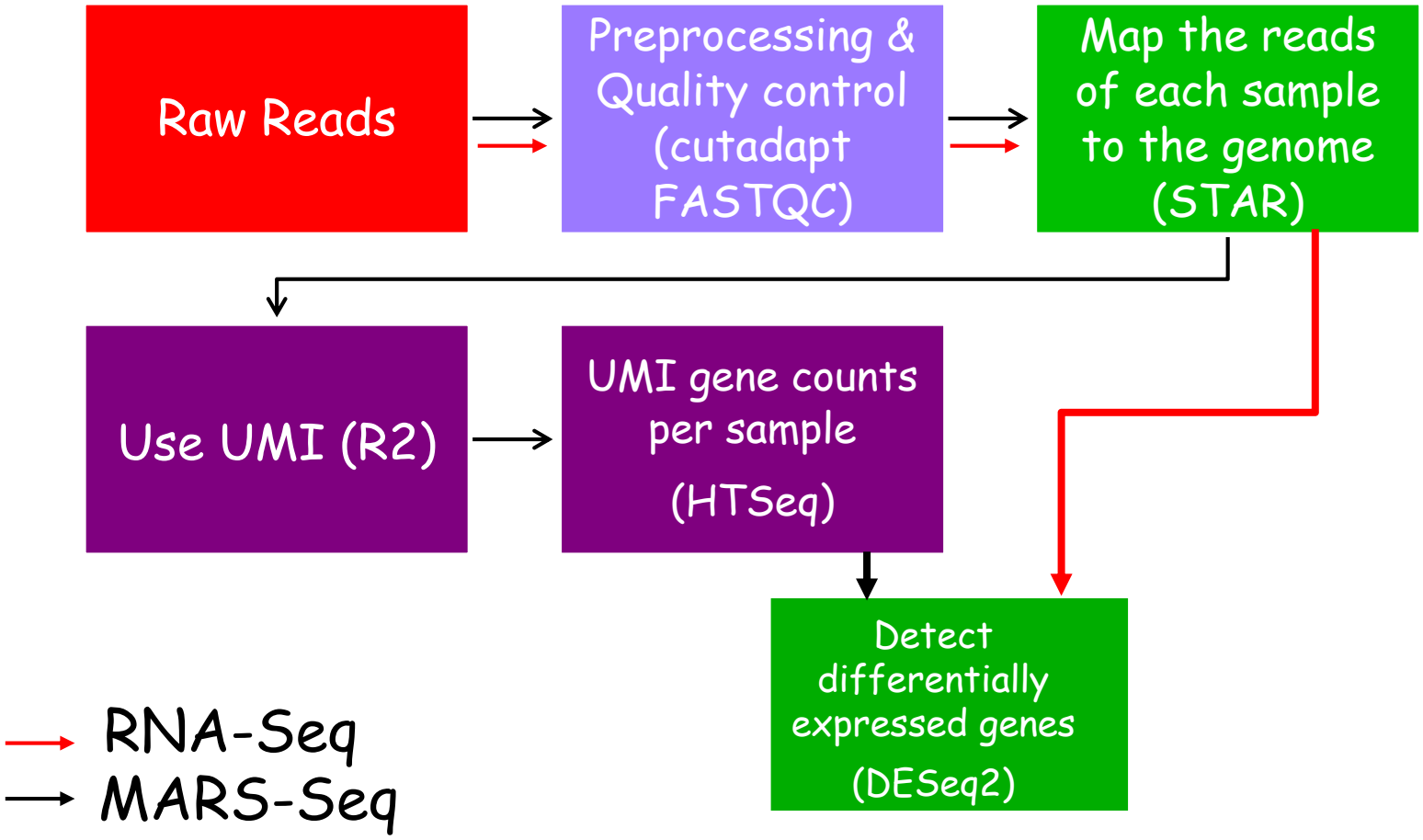
Genome Browser View



MAR-Seq

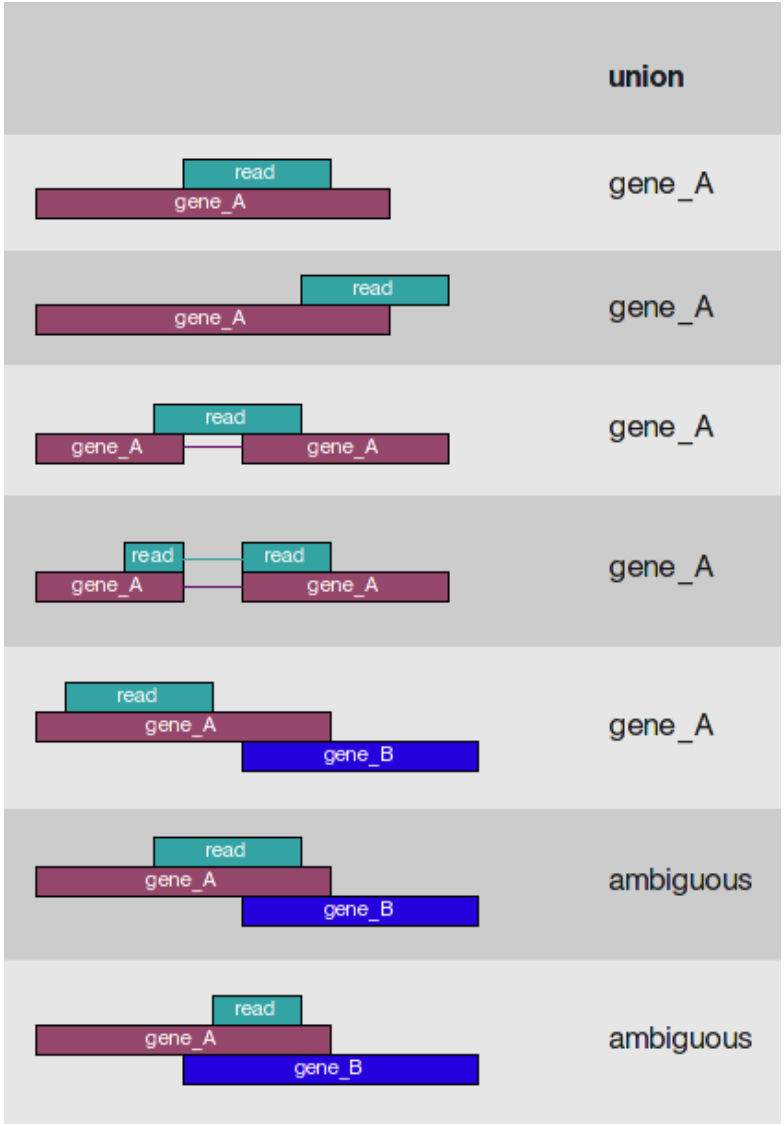
RNA-Seq

Bioinformatics Workflow



MARS-Seq Gene Quantification

- HTSeq criteria to count reads: uniquely mapped to the 3' of gene (using a modified annotation file)



http://www-huber.embl.de/users/anders/HTSeq/doc/count.html

In BAM File Add Feature Tag and Mark Duplicates

UMI barcode

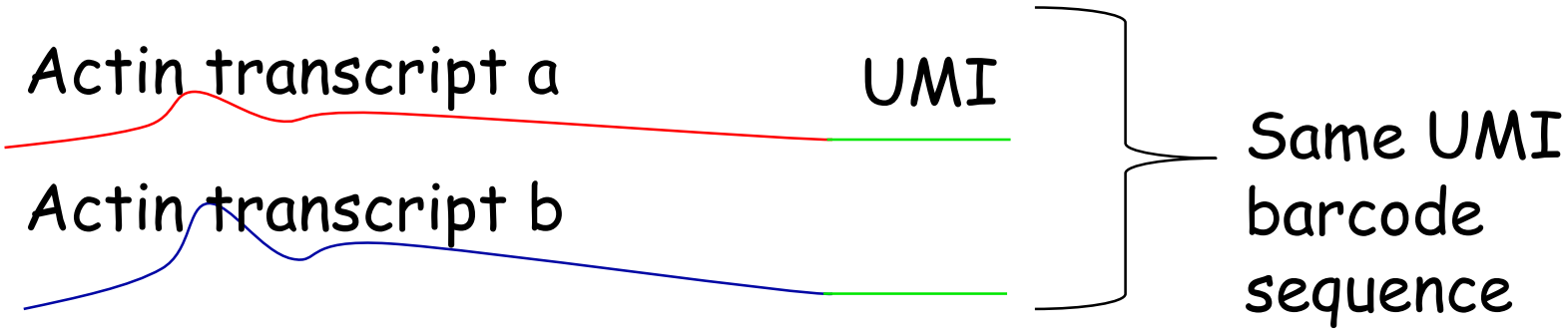
Mark OK

```
K00279:102:HJK2GBBXX:1:1104:3995:7222  0     chr1    164437130     255     101M    *       0       0
GTCCGTTGTGTTAACCTGACTGACACAAGATCAACAAGAGCACTGTACTCCTGGCAATTATTACATATGCTAGAACATGGATTTGCACTGTAGACAACACT
-AFFFJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJFFJJFJJFJJFFJ   NH:i:1  HI:i:1  AS:i:99 nM:i:0  RX:Z:ATTTTTTT
QX:Z:JJJJJJJJ   XF:Z:Nme7       rp:Z:K00279:102:HJK2GBBXX:1:1104:3995:7222      nd:i:8
```

Mark AS DUPLICATE

```
K00279:102:HJK2GBBXX:1:1102:23399:31734 1024     chr1    164437180     255     100M    *       0       0
CTGGCAATTATTACATATGCTAGAACATGGATTTGCACTGTAGACAACACTTAACACCAGTCTATGGGGTACTGCATTGCTTTTTATAAAGTTCAAAATA
<A-FFJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJFJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJ   NH:i:1  HI:i:1  AS:i:98 nM:i:0  RX:Z:ATTTTTTT
QX:Z:JJJJJJJJ   XF:Z:Nme7       rp:Z:K00279:102:HJK2GBBXX:1:1104:3995:7222      nd:i:8
K00279:102:HJK2GBBXX:1:1102:30919:42724 1024     chr1    164437198     255     101M    *       0       0
GCTAGAACATGGATTTGCACTGTAGACAACACTTAACACCAGTCTATGGGGTACTGCATTGCTTTTTATAAAGTTCAAAATAAAGATTTTTATTTTCAAAC
AAAFFJJJJJJJJJJJJJJJJJJJFJJJJJFJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJFJFJJAJJJFJFJJJJJF<JFJJJJFJJJJJJJ   NH:i:1  HI:i:1  AS:i:99 nM:i:0  RX:Z:ATTTTTTT
QX:Z:JJJJJJJJ   XF:Z:Nme7       rp:Z:K00279:102:HJK2GBBXX:1:1104:3995:7222      nd:i:8
K00279:102:HJK2GBBXX:1:1103:31994:17438 1024     chr1    164437199     255     100M    *       0       0
CTAGAACATGGATTTGCACTGTAGACAACACTTAACACCAGTCTATGGGGTACTGCATTGCTTTTTATAAAGTTCAAAATAAAGATTTTTATTTTCAAAC
AAFAFJJJJJJJJJJFJJJJJJJJJJJJJJJJJJJJJJJJJJFFJJJJJJJJJJJFJJJF<JJFJJJAF7<-AFFF77A<FJJF-7FJJJJJJJJ<FFJF   NH:i:1  HI:i:1  AS:i:98 nM:i:0  RX:Z:ATTTTTTT
QX:Z:JJJJJJJJ   XF:Z:Nme7       rp:Z:K00279:102:HJK2GBBXX:1:1104:3995:7222      nd:i:8
K00279:102:HJK2GBBXX:1:1105:22343:7468  1024     chr1    164437210     255     98M     *       0       0
ATTTGCACTGTAGACAACACTTAACACCAGTCTATGGGGTACTGCATTGCTTTTTATAAAGTTCAAAATACAGATTTTTATTTTCAAACAAGTGACTT
AA<A-<A7A-FJ<FFJA-FJFAFAJJJ-FA-7---F--A--77F-77<FFFJ<7--JF7FF<-<FJA7<--<A<-<7AF-FA-7FJJFAAF7-7F7FA   NH:i:1  HI:i:1  AS:i:94 nM:i:1  RX:Z:ATTTTTTT
QX:Z:JJFJFJJJ   XF:Z:Nme7       rp:Z:K00279:102:HJK2GBBXX:1:1104:3995:7222      nd:i:8
K00279:102:HJK2GBBXX:1:1102:4564:36200  1024     chr1    164437211     255     101M    *       0       0
TTTGCACTGTAGACAACACTTAACACCAGTCTATGGGGTACTGCATTGCTTTTTATAAAGTTCAAAATAAAGATTTTTATTTTCAAACAAGTGACTTTGGT
AAFFFJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJ   NH:i:1  HI:i:1  AS:i:99 nM:i:0  RX:Z:ATTTTTTT
QX:Z:JJJJJJJJ   XF:Z:Nme7       rp:Z:K00279:102:HJK2GBBXX:1:1104:3995:7222      nd:i:8
K00279:102:HJK2GBBXX:1:1103:13869:49089 1024     chr1    164437214     255     101M    *       0       0
GCACTGTAGACAACACTTAACACCAGTCTATGGGGTACTGCATTGCTTTTTATAAAGTTCAAAATAAAGATTTTTATTTTCAAACAAGTGACTTTGGTTTT
AAFFFJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJFJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJFJFJFJFF   NH:i:1  HI:i:1  AS:i:99 nM:i:0  RX:Z:ATTTTTTT
QX:Z:JJJJJJJJ   XF:Z:Nme7       rp:Z:K00279:102:HJK2GBBXX:1:1104:3995:7222      nd:i:8
K00279:102:HJK2GBBXX:1:1104:13362:2563  1024     chr1    164437214     255     101M    *       0       0
GCACTGTAGACAACACTTAACACCAGTCTATGGGGTACTGCATTGCTTTTTATAAAGTTCAAAATAAAGATTTTTATTTTCAAACAAGTGACTTTGGTTTT
AAFFFJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJFJJJJJJJJJJAJJJJJJFJJJJJJJJJJJFFAJFAFFJFAFF   NH:i:1  HI:i:1  AS:i:99 nM:i:0  RX:Z:ATTTTTTT
QX:Z:JJJJJJJJ   XF:Z:Nme7       rp:Z:K00279:102:HJK2GBBXX:1:1104:3995:7222      nd:i:8
```

UMI Count Correction

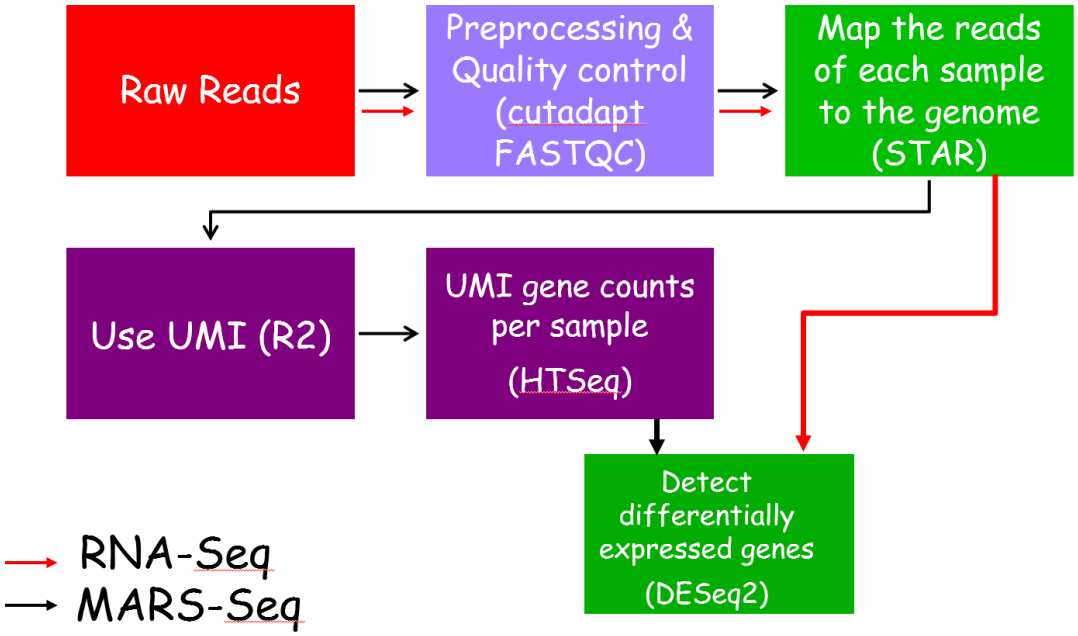


- UMI barcodes are connected to cDNA randomly, so it might happen that two independent transcripts derived from the same gene get assigned the same UMI barcode -> clash
- Genes that are highly gene expressed, have a higher chance of clashing
- Correction is applied to UMI counts taking into account the chance of clashing

UTAP: User-friendly Transcriptome Analysis Pipeline

Refael Kohen, Jonathan Barlev, Gil Hornung, Gil Stelzer, Ester Feldmesser, Kiril Kogan, Marilyn Safran and Dena Leshkowitz

BMC Bioinformatics 2019 **20**:154
https://doi.org/10.1186/s12859-019-2728-2
Received: 19 August 2018 | **Accepted:** 13 March 2019 | **Published:** 25 March 2019



Refael Kohen

Tools References

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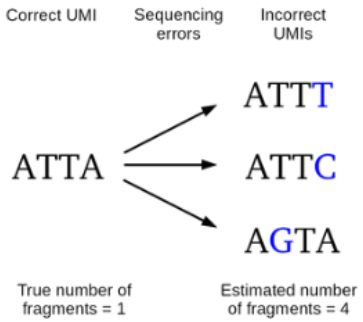
The END

THANKS FOR LISTENING

QUESTIONS?

Correction For Barcode Error Currently Not Implemented in UTAP



Sequencing errors inflate the apparent numbers of unique fragments sequenced

https://cgatoxford.wordpress.com/2015/08/14/unique-molecular-identifiers-the-problem-the-solution-and-the-proof/