Outline

- Introduction to RNA-seq
- Sample preparation
- Quality control
- Transcript assembly
- Read alignment
- Differential gene expression
- Data visualization and plotting

Regulation of gene expression

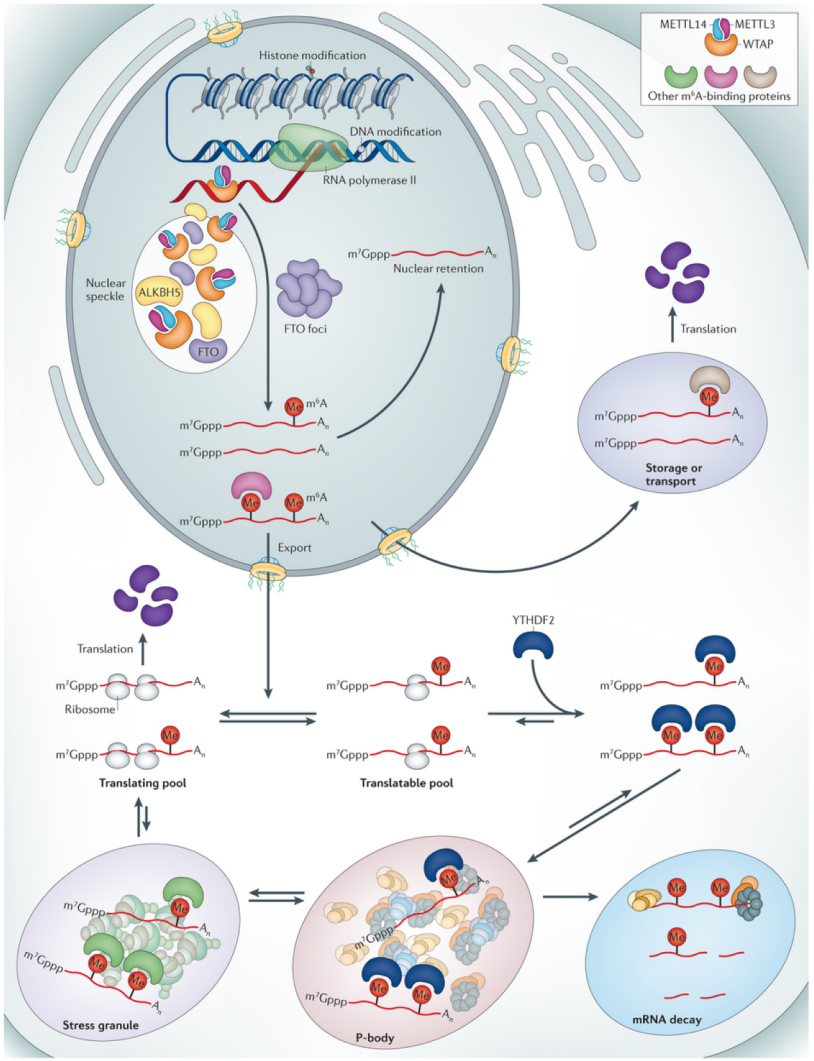
Regulation of transcription:
- Transcription factors
- Histone modifications
- DNA methylation

Regulation of RNA processing:
- Polyadenylation
- Splicing
- Capping
- RNA export

Regulation of translation:
- mRNA decay
- Translational repression
- Sequestration

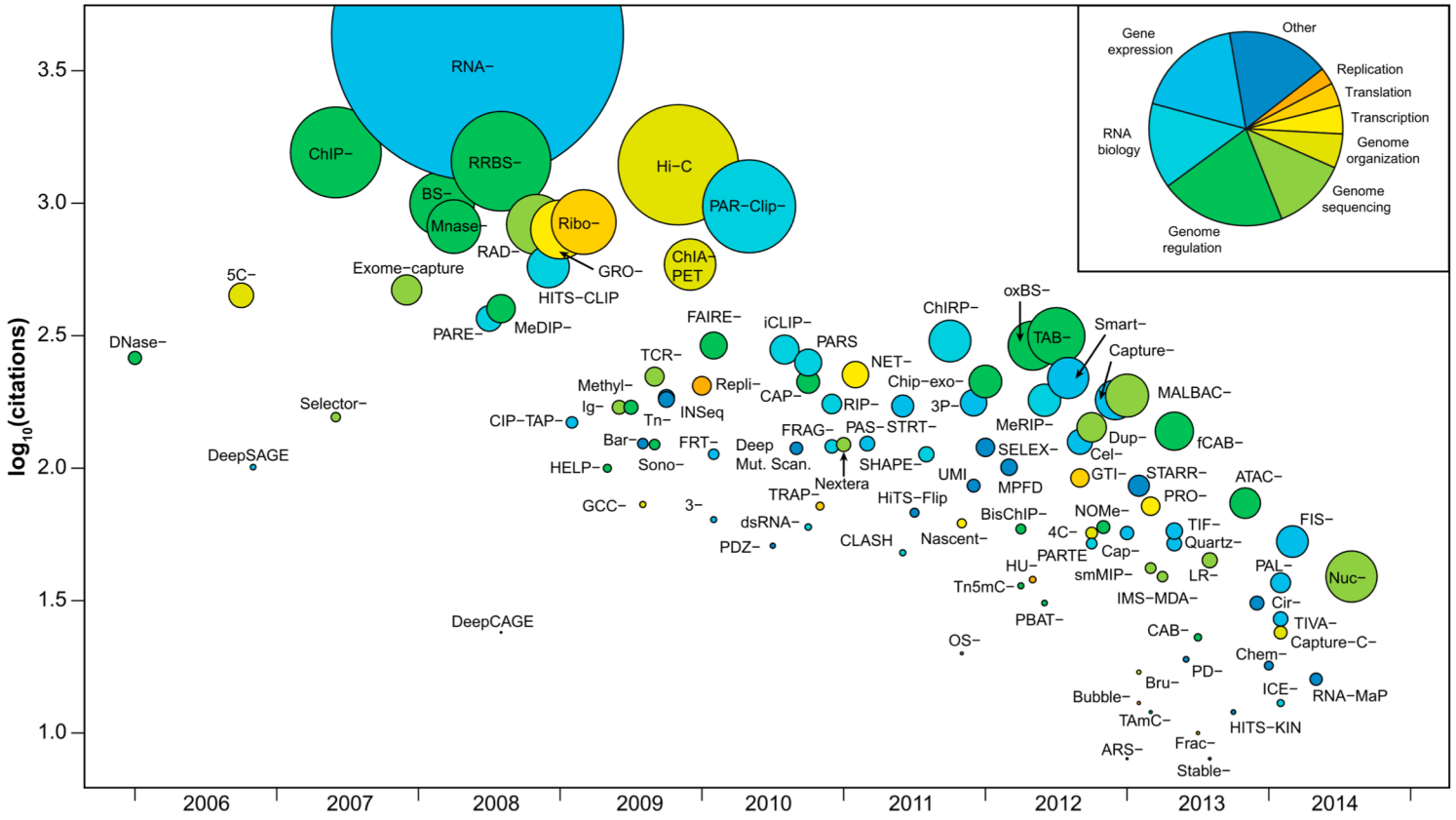
RNA-seq measures steady state mRNA levels and RNA sequence composition

Posttranslational regulation:
- Chemical modifications (e.g. phosphorylation)
- Protein turnover (proteolysis)



Fu et al. (2014)

RNA-seq is the most common HTS application



Reuter et al 2015

Sample preparation

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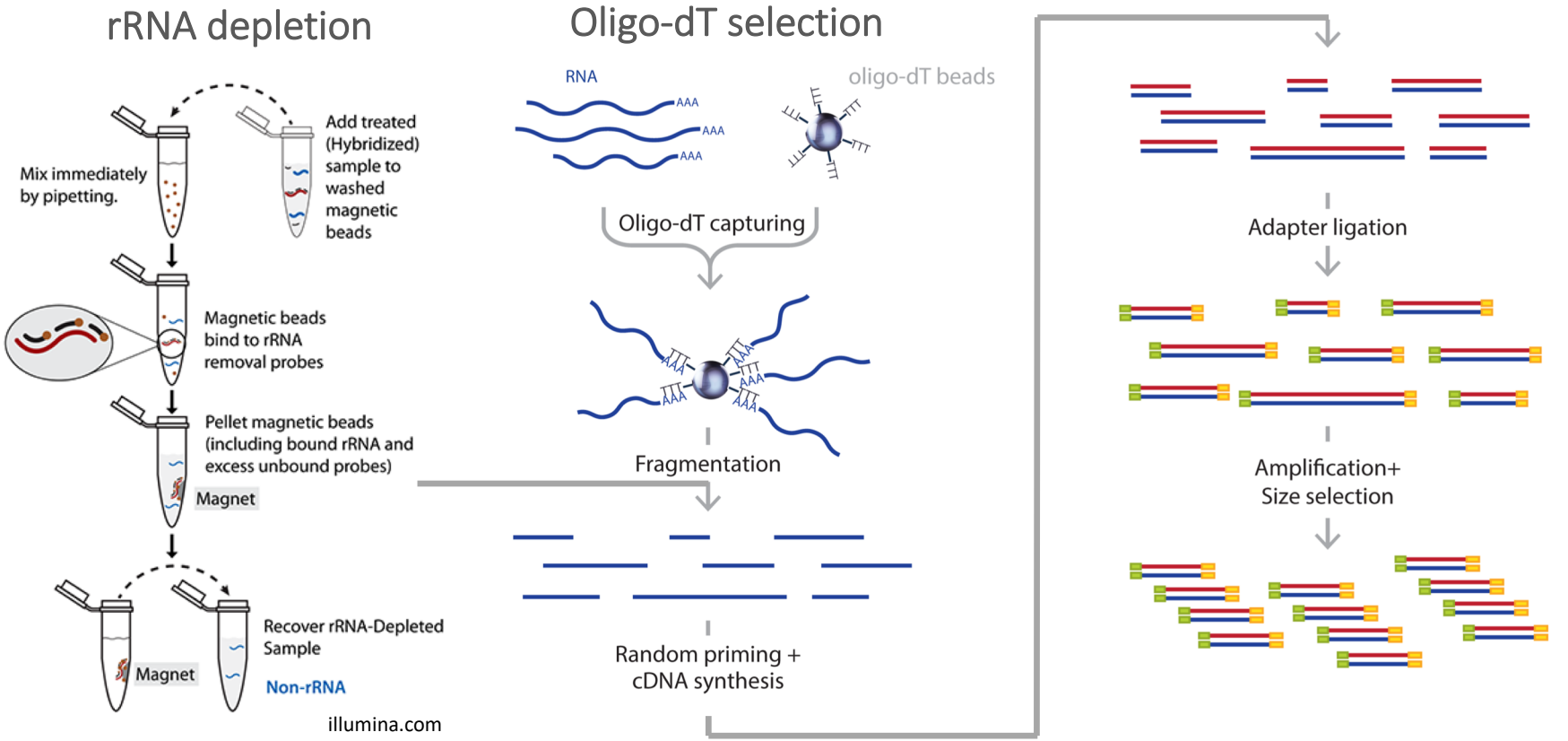
- Use high-quality RNA as starting material.
- Minor differences between samples can have a substantial impact on gene expression.
- Three biological replicates is the default but not ideal for every situation.
- Some recommended kits for standard RNA-seq:
  - NEBNext Ultra II Directional RNA Library Prep Ki
  - Illumina kits

Sample preparation

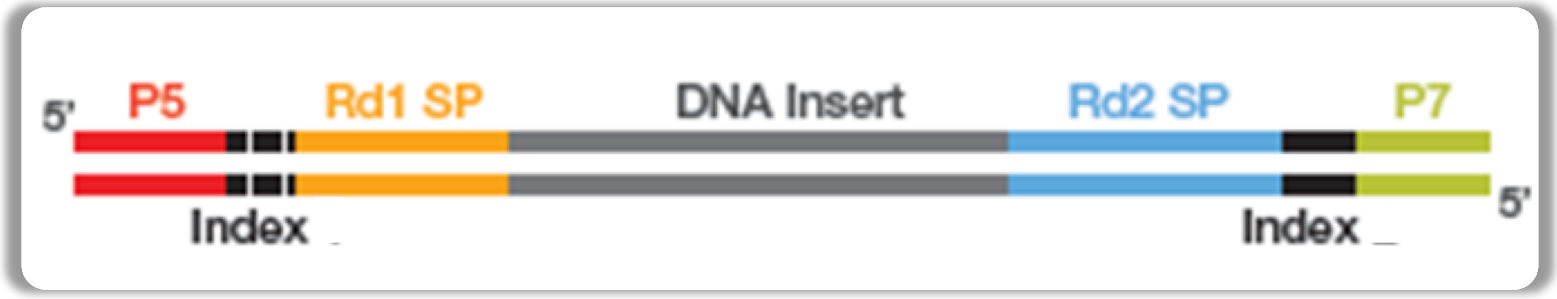
- Starting RNA
  - Typically 1-5 ug of high-quality total RNA is ideal.
- Sequencing depth
  - Typically you want about 20 million high quality reads/library.
- Considerations
  - Strand specific (default is yes)
  - Single-end or paired-end (single is sufficient for well annotated transcriptomes)
  - Long reads vs short reads (short Illumina reads, 50-150 nt, are usually sufficient)
  - rRNA depletion or oligo-dT
  - Low quantity/single cell

RNA-seq library preparation

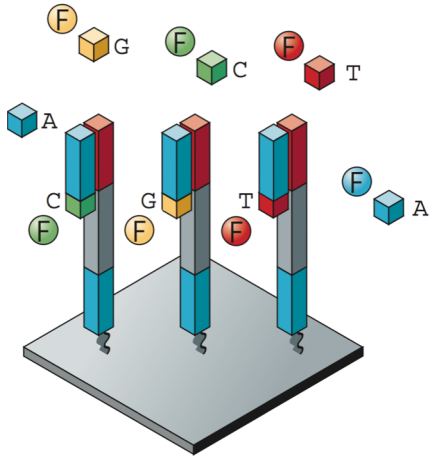


Zhernakova et al. (2009)

Library composition



Dual Index Library shown



Metzker, M.L. (2010) NRG

HiSeq 2500

Slide content courtesy of Illumina

FASTQ format

Index sequence

Read 1

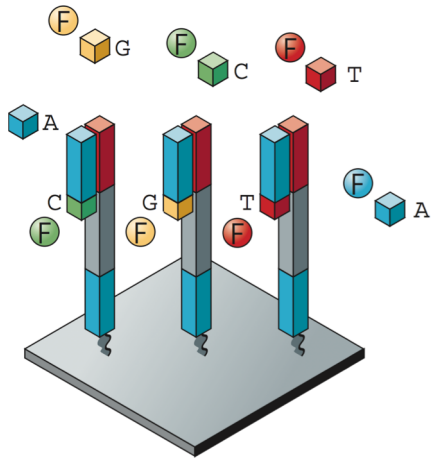
```
1 @D64TDFP1:248:C50DMACXX:5:1101:1241:2095 1:N:0:ATCACG
2 CACCGCCCGTCGCTATCCGGGACTGGAATTCTCGGGTGCCAAGGAACTCCA
3 +
4 CCCFFFFFHHHHHJIJGHJJJJIJJJJJGGGFFFFEABDHHHFHFF@@DD>
```

Read 2

```
1 @D64TDFP1:248:C50DMACXX:5:1101:1371:2154 1:N:0:ATCACG
2 TCAATATTTGCATAGGGTATCTGGAATTCTCGGGTGCCAAGGAACTCCAGT
3 +
4 CCCFFFFFHHHHHJJJJGFHIJJJJJJJJJJJJJFHHIIJJHGHJFGHJJI
```

Read 3

```
1 @D64TDFP1:248:C50DMACXX:5:1101:1461:2205 1:N:0:ATCACG
2 GAAAGACGTCTTCCTAGATTATGGAATTCTCGGGTGCCAAGGAACTCCAGT
3 +
4 CCCFFFFFHHHHHJJJJJJJJJJJIJJJJJJJJJHIJJJJJGIIJFGIJJJ
```



Metzker, M.L. (2010) NRG

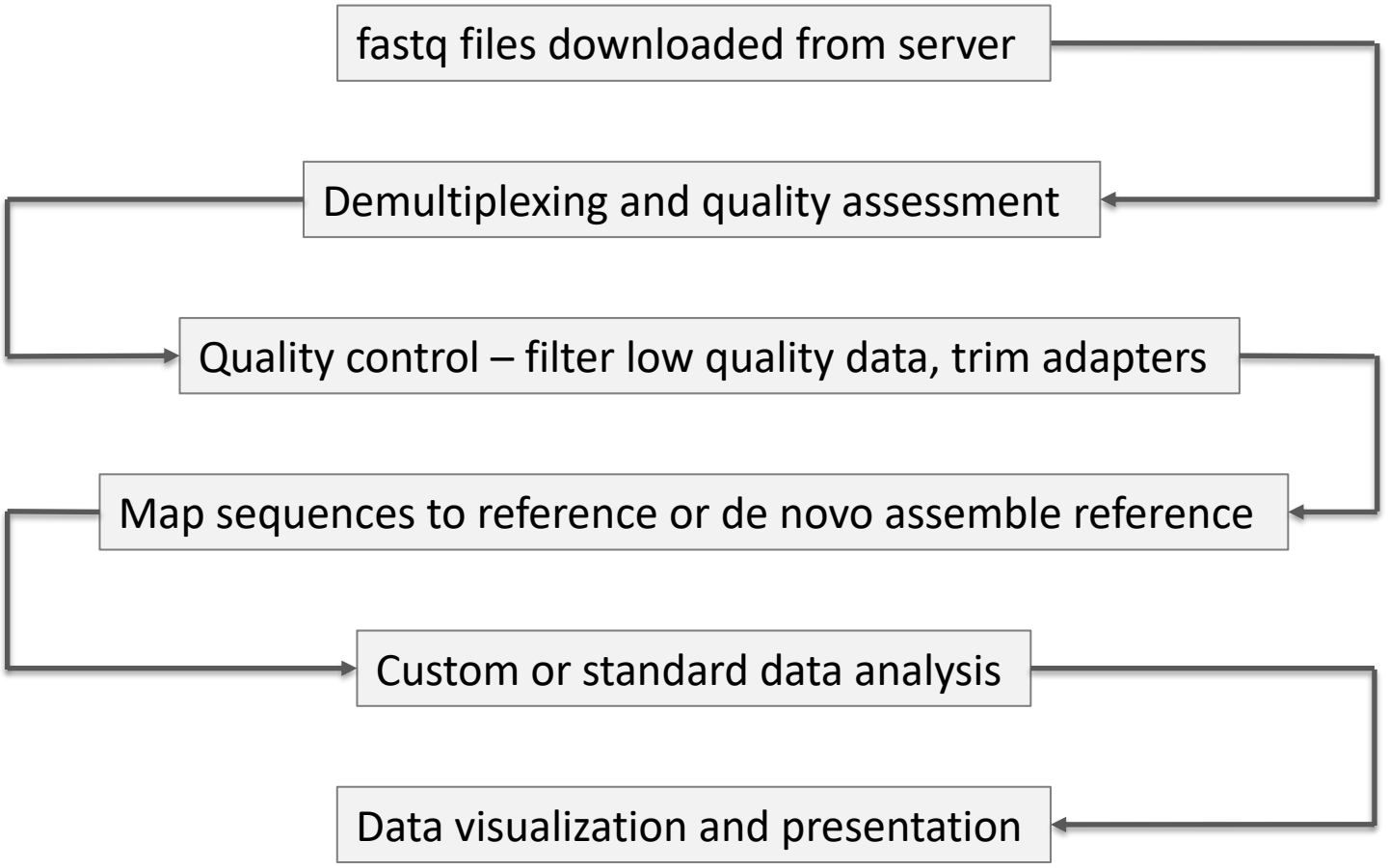
Line 1: sequence ID, description, and index; begins with @

Line 2: sequence; contains only A, C, T, G, and N

Line 3: optional sequence ID; begins with +

Line 4: signal quality of each base, cryptic code, phred 33 or 64

Data analysis workflow



Quality control

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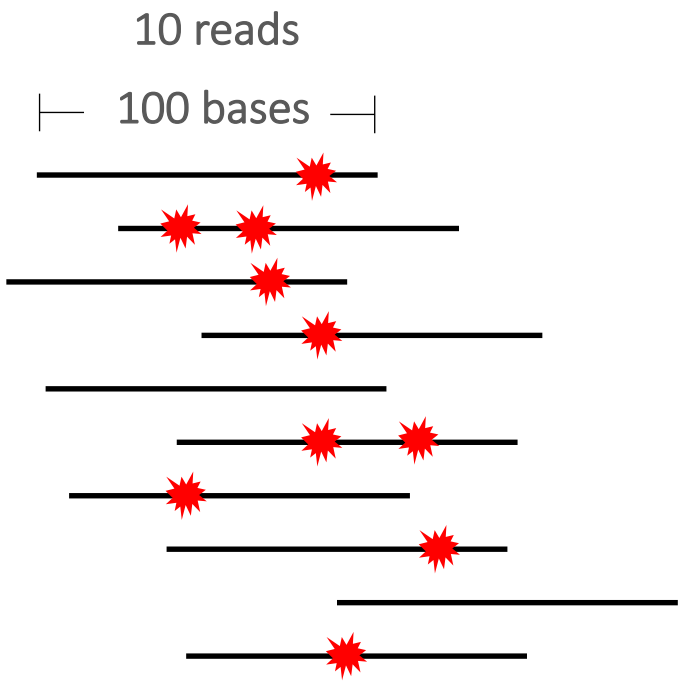
Assessing Read Quality

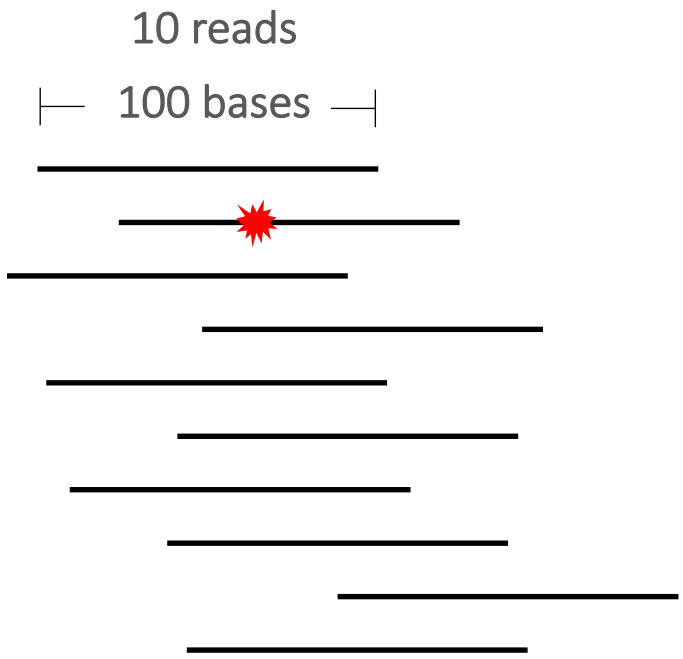
Phred quality score: a measure of the quality of base calling:

$Q = -10 \log(P)$ where P is the error probability

Phred Quality Score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%
60	1 in 1,000,000	99.9999%

Quality control





P = 0.01
Q = 20 (Q20)

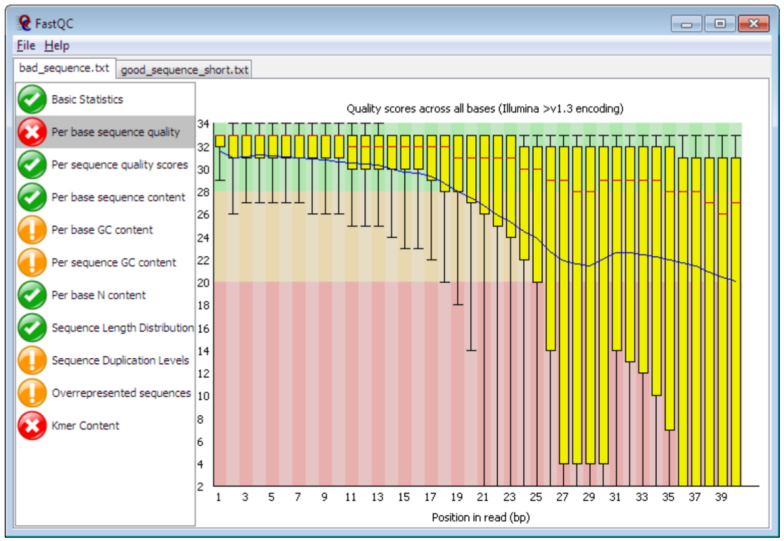
Q = -10 log(P)

P = ?
Q = ?

Q30 is a common quality threshold or quality criterion

Quality control

FastQC: a GUI tool for assessing the quality of high-throughput sequencing data.



Trimmomatic: software for trimming adapter sequences and low-quality bases from sequencing reads.

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Sequence mapping/alignment

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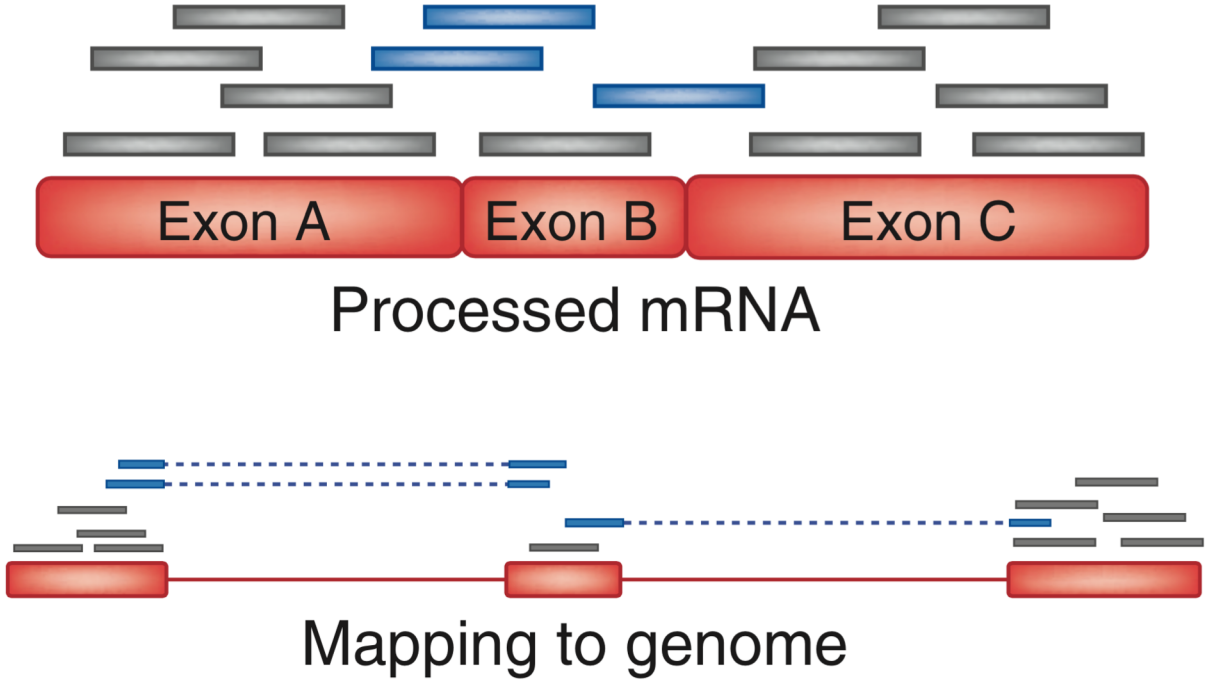
Table 1 A selection of short-read analysis software

Program	Website	Open source?	Handles ABI color space?	Maximum read length
Bowtie	http://bowtie.cbcb.umd.edu	Yes	No	None
BWA	http://maq.sourceforge.net/bwa-man.shtml	Yes	Yes	None
Maq	http://maq.sourceforge.net	Yes	Yes	127
Mosaik	http://bioinformatics.bc.edu/marthlab/Mosaik	No	Yes	None
Novoalign	http://www.novocraft.com	No	No	None
SOAP2	http://soap.genomics.org.cn	No	No	60
ZOOM	http://www.bioinfor.com	No	Yes	240

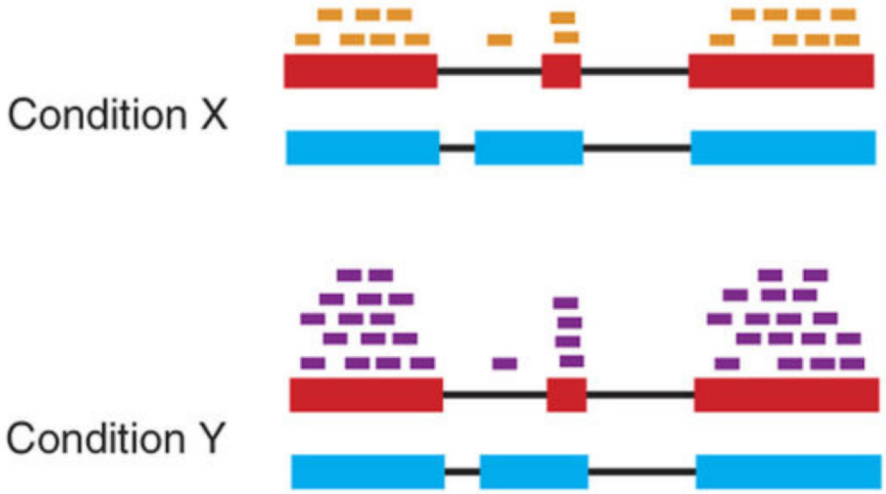
Trapnell and Salzberg (2009)

Aligning reads to mRNAs



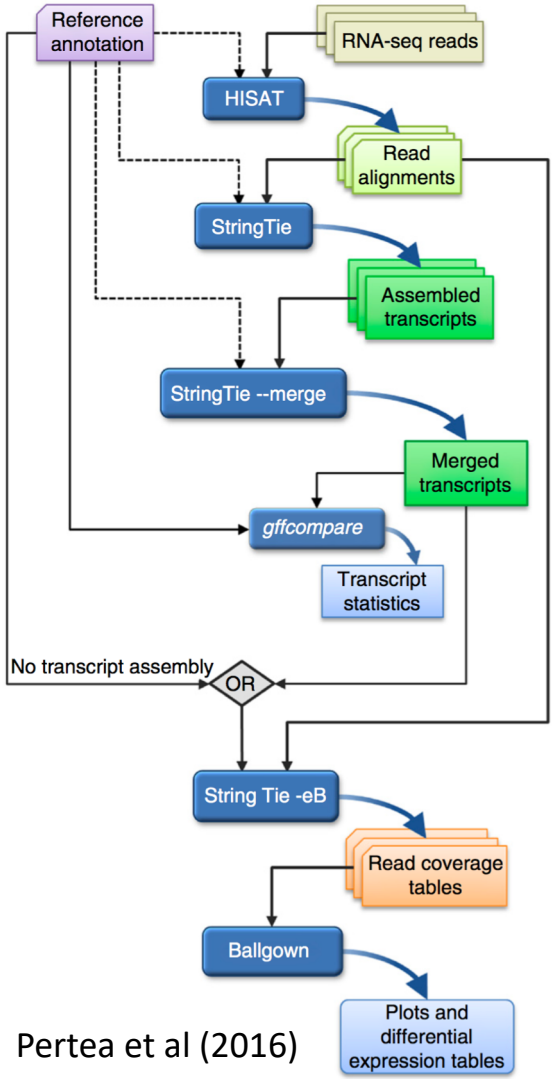
Trapnell et al (2009)

Differential gene expression



Trapnell et al (2010)

RNA-seq pipelines



Pertea et al (2016)

No reference genome? Use Trinity to assemble transcripts

Other mRNA aligners: Star, GNSAP, Tophat2

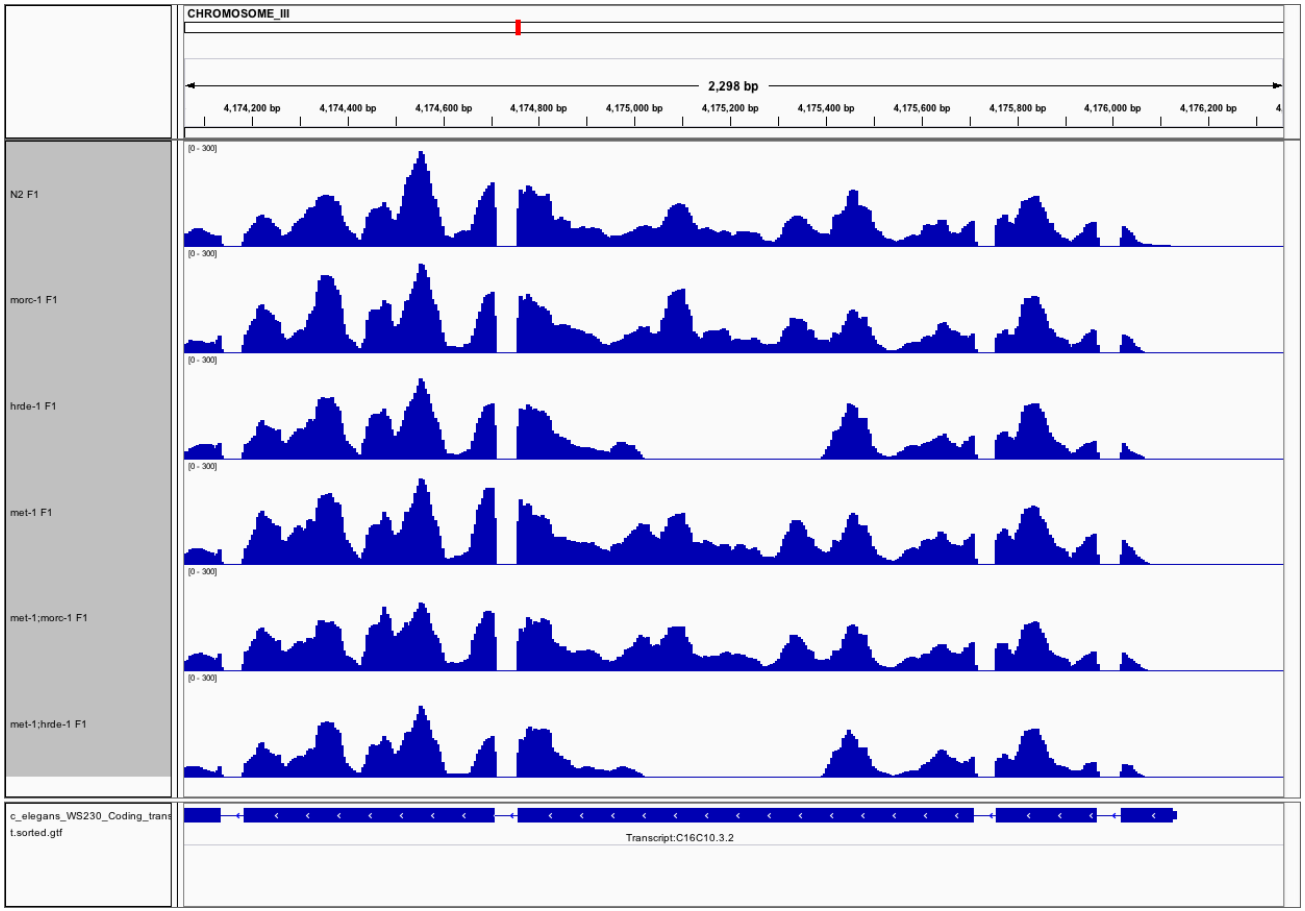
Other abundance estimators: RSEM, htseq-count

Other common DE software: DESeq2, edgeR, cuffdiff

Various GUIs and R-based tools for drawing plots

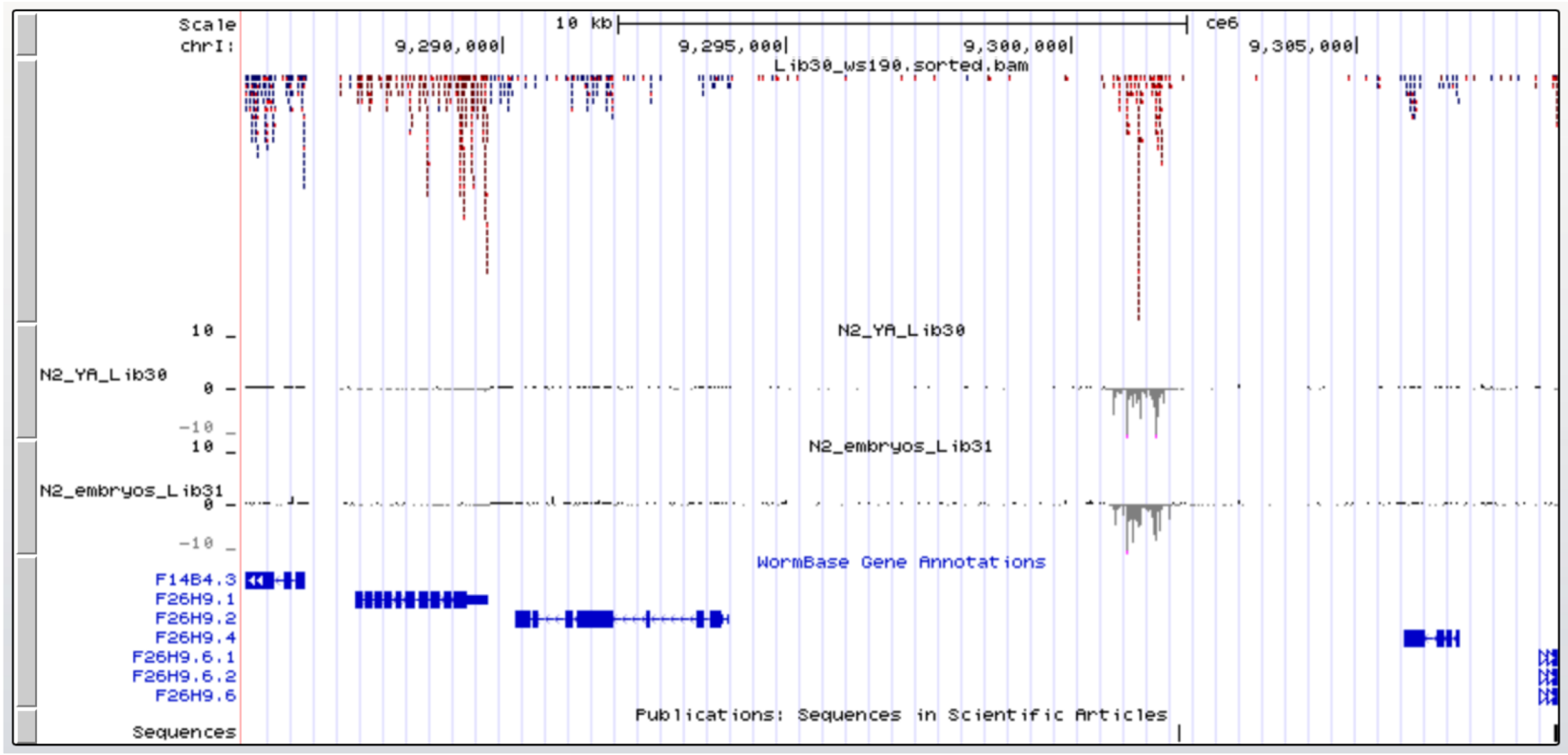
Genome browsers

Integrative Genomics Viewer (IGV)



Genome browsers

UCSC Genome Browser



Trinity workflow