Introduction to NGS and RNA-Seq

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(With some slides borrowed from Scott Hunicke-Smith and Jeff Barrick)

Some background

- Research scientist- bioinformatician at CBRS.
  - RNA-Seq
  - Genome Assembly
  - Exome data analysis
  - Benchmarking of tools

CCBB
Center for Compuational Biology
and Bioinformatics

FRI

BIG DATA IN BIOLOGY

- Training
  - Grad students, post-docs.
  - Undergraduate- FRI

Goals of the Class

- When considering an RNA-Seq experiment
  - What kind of options are available for library prep?
- When you have an RNA-Seq dataset
  - What kind of options are available for analysis?
- Hands-on experience running typical RNA-Seq workflows on TACC
  - Some unix, R, TACC skills
- Learn the terminology

Setting General Expectations

- Lots of background and basics to provide comfort with terminology and key concepts.

- Exposure to commands and typically used analysis tools using an example RNA-Seq dataset.
  - No one 'best' or 'standard' tool.

- A starting point for you to design your RNA-Seq study or analyze your dataset.

Resources

- BioIteam Wiki- Bookmark it!
  https://wikis.utexas.edu/display/bioiteam

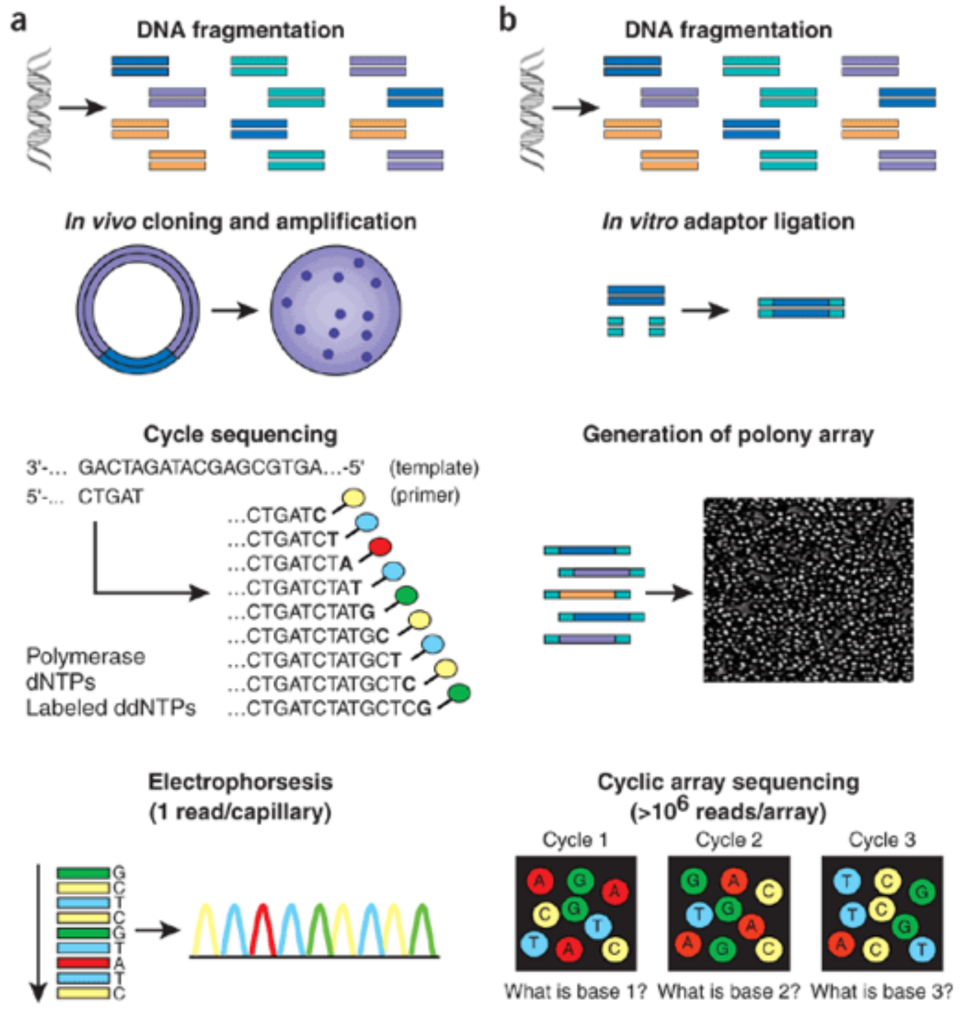
- Summer School course materials:
  https://wikis.utexas.edu/display/bioiteam/Introduction+to+RNA+Seq+Course

- Byte Club: Meets Third Wednesday of every month
- https://wikis.utexas.edu/display/bioiteam/Byte+Club

- CCBB Bioinformatics consultants

What is Next Generation(or) Second Generation Sequencing?

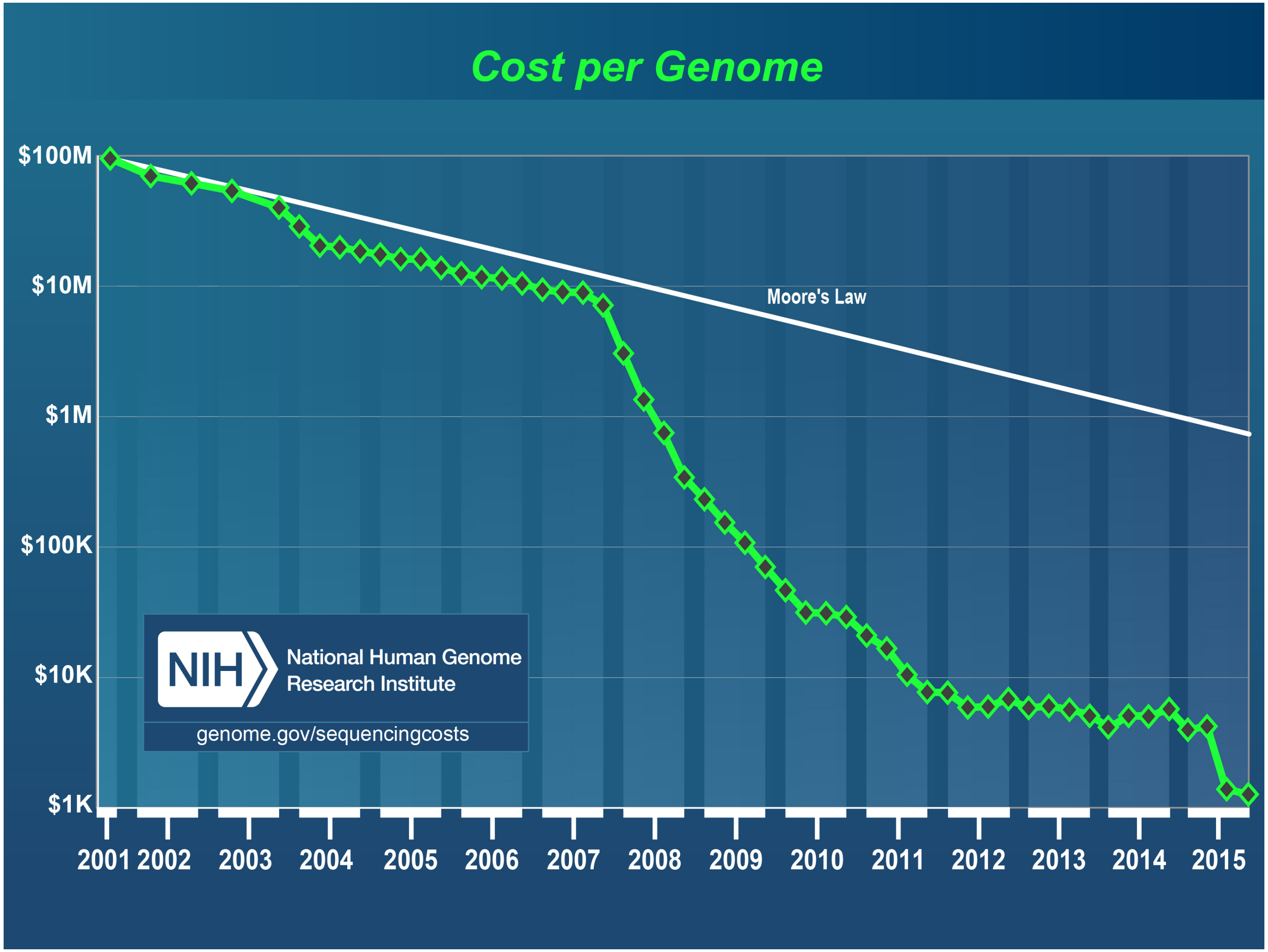
- Massively parallel sequencing
- The template DNA is attached to a cluster.
- Billions of clusters sequenced in parallel.
- 3-10 billion independent DNA fragments sequenced in one run.



So, what's so great about second generation sequencing?

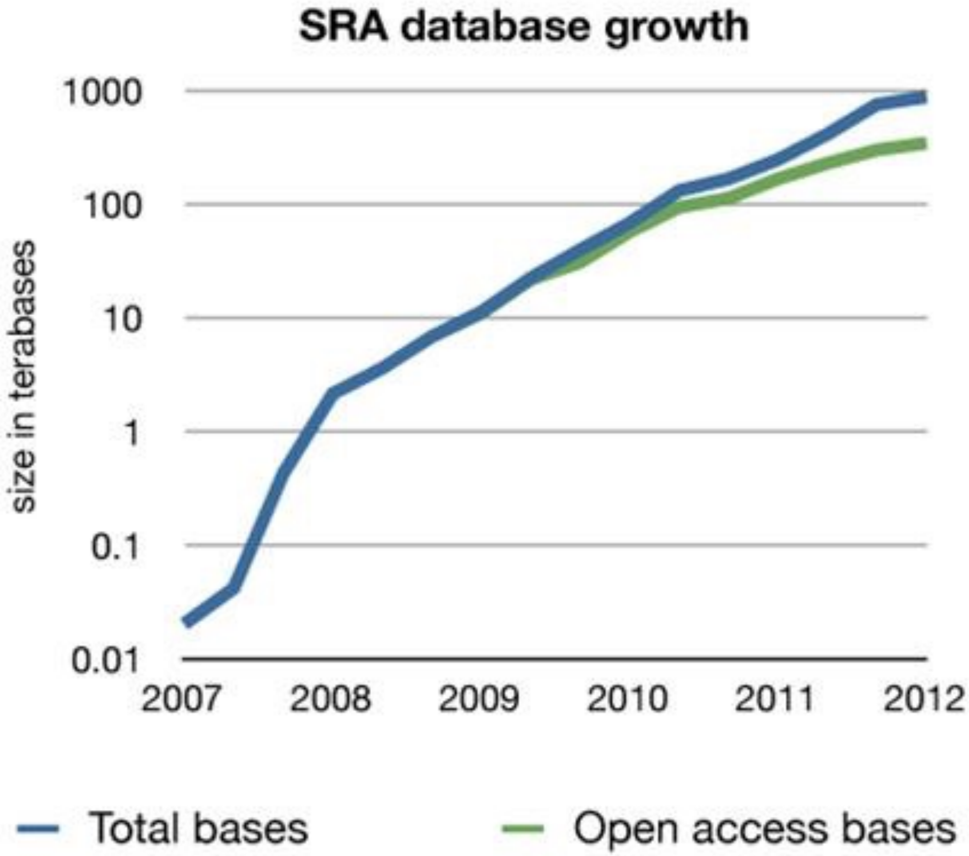
- **+** Sequence lots more, faster!

- **+** More cost effective.

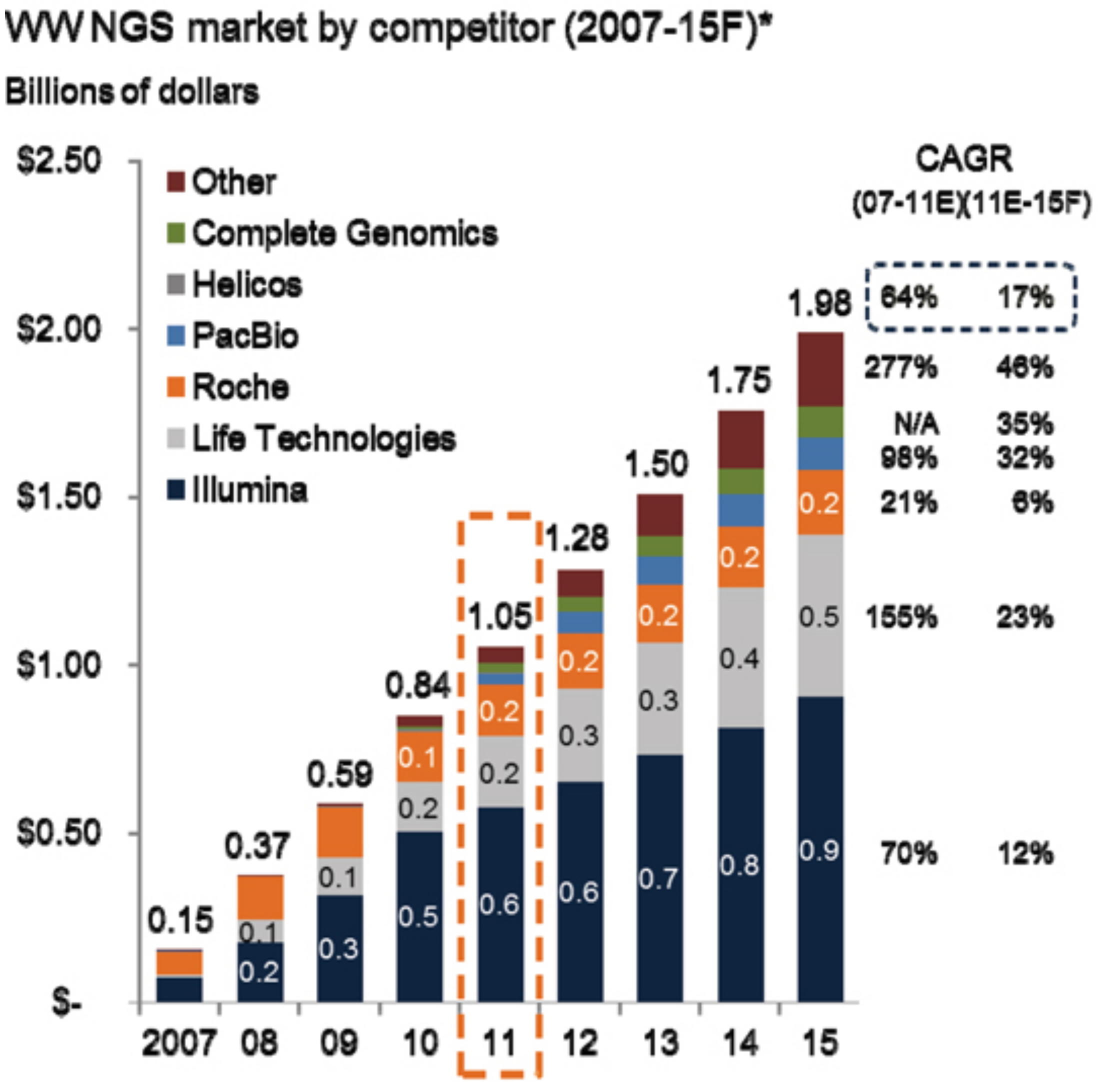


So, what's NOT so great about second generation sequencing?

- Data deluge
- Bioinformaticians and computational biologists to the rescue!



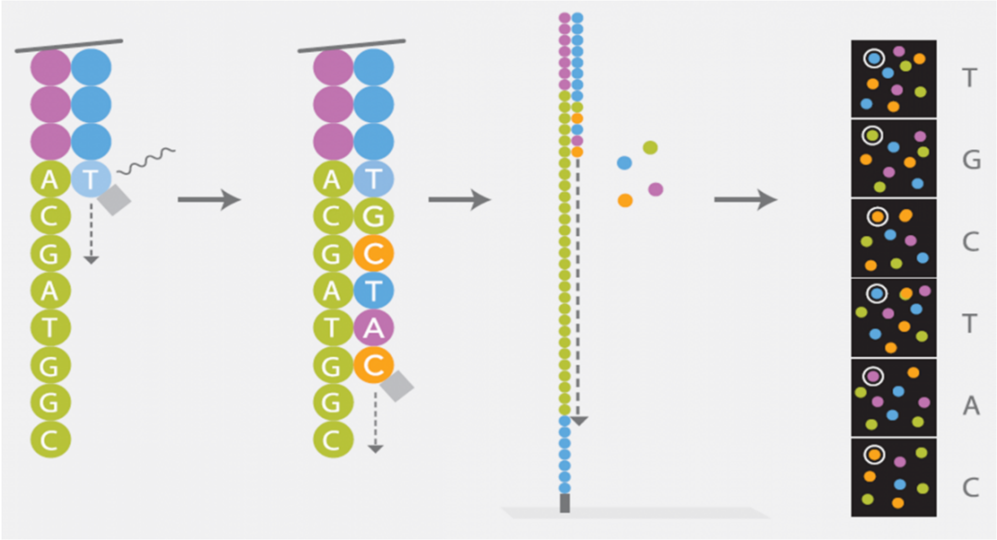
Who are the players?



How do next generation sequencers work?

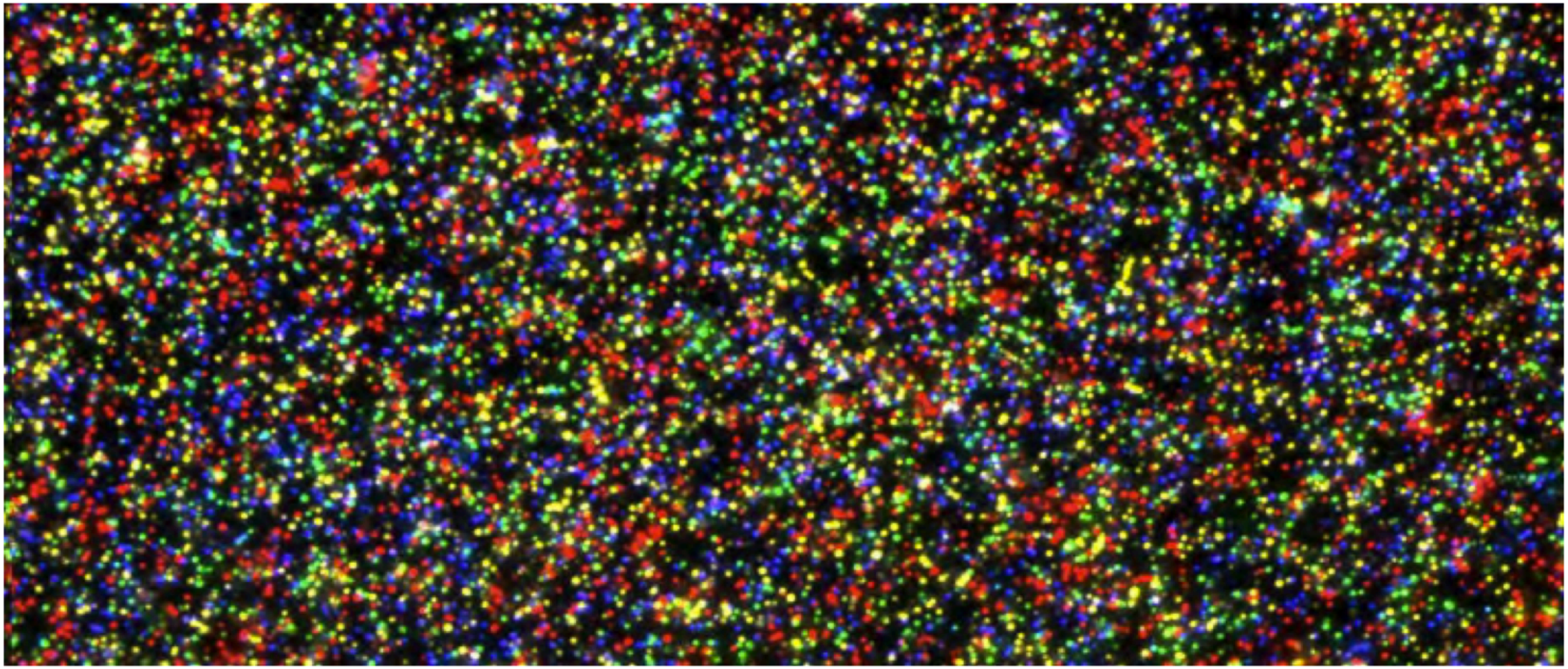
- Attach a short DNA template on a chip.
- Flood with polymerase, fluorescent labeled nucleobases.
- When a complementary base is generated, take a picture of the fluorescence.
- Do this for millions/billions of templates at the same time.

Sequencers simply observe DNA Replication



http://www.cegat.de/

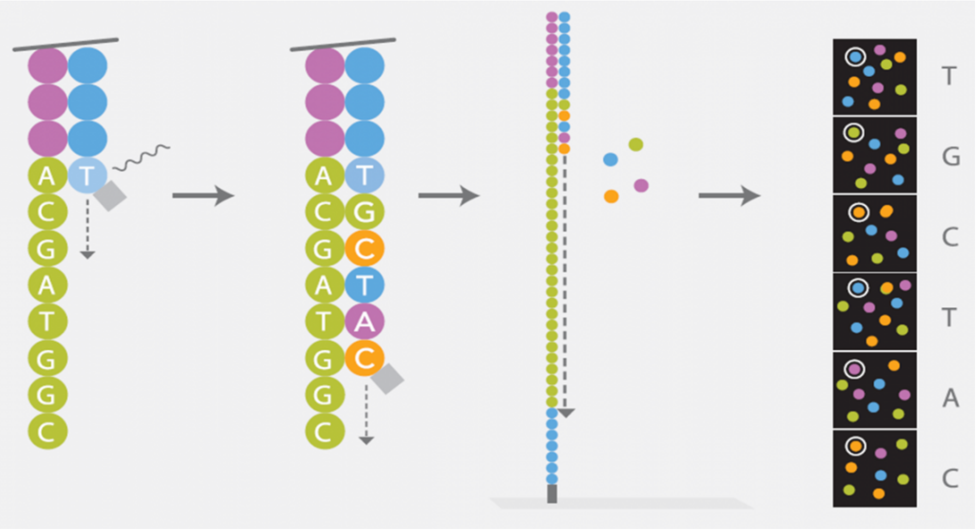
How do next generation sequencers work?



How does the sequencer work?

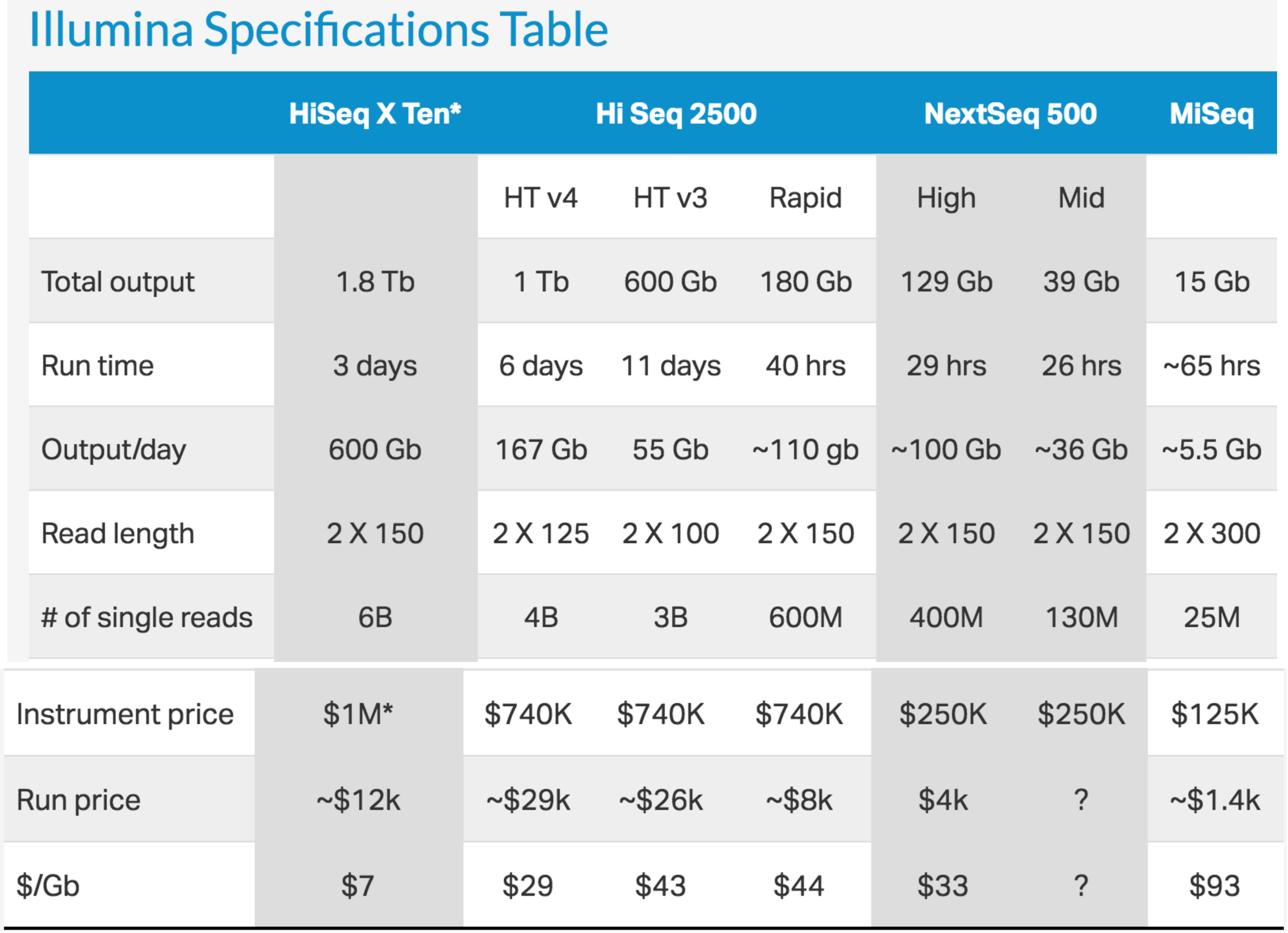
- Library prep
- Cluster generation/ amplification
- Sequencing by synthesis
- Done in parallel for billions clusters at once.

- Let's watch the official Illumina video.



http://www.cegat.de/

Different Types of Illumina Sequencers



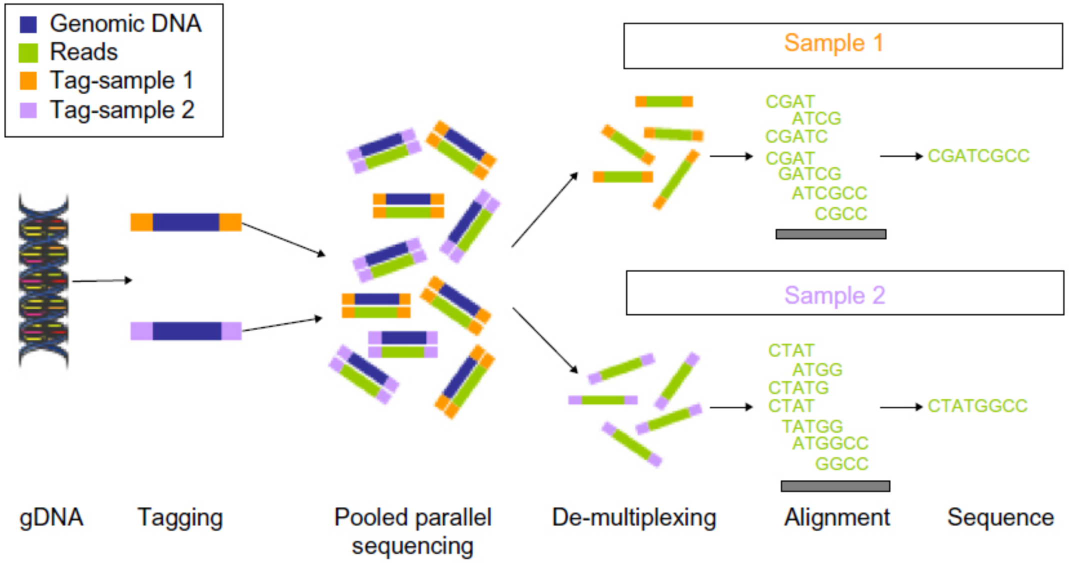
Illumina Specifications Table

	HiSeq X Ten*	Hi Seq 2500			NextSeq 500		MiSeq
		HT v4	HT v3	Rapid	High	Mid	
Total output	1.8 Tb	1 Tb	600 Gb	180 Gb	129 Gb	39 Gb	15 Gb
Run time	3 days	6 days	11 days	40 hrs	29 hrs	26 hrs	~65 hrs
Output/day	600 Gb	167 Gb	55 Gb	~110 gb	~100 Gb	~36 Gb	~5.5 Gb
Read length	2 X 150	2 X 125	2 X 100	2 X 150	2 X 150	2 X 150	2 X 300
# of single reads	6B	4B	3B	600M	400M	130M	25M
Instrument price	$1M*	$740K	$740K	$740K	$250K	$250K	$125K
Run price	~$12k	~$29k	~$26k	~$8k	$4k	?	~$1.4k
$/Gb	$7	$29	$43	$44	$33	?	$93

allseq.com/knowledgebank/sequencing-platforms/illumina

Multiplexing

- Sample specific Indexes/ Barcodes are attached to the DNA template.
- 6-8bp indexes/barcodes
- Data off the sequencer must first be demultiplexed to identify which reads belong to which sample.



https://doi.org/10.2147/BLCTT.S51503

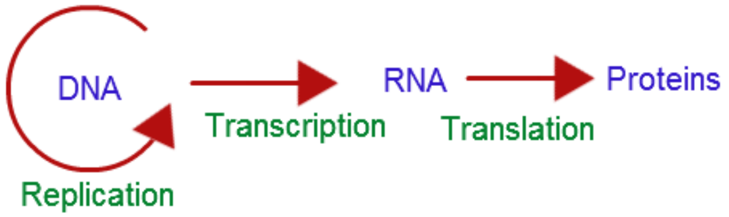
What are the Limations/Challenges?

- Amplification can cause problems.
  - Clusters are made by using PCR amplification.

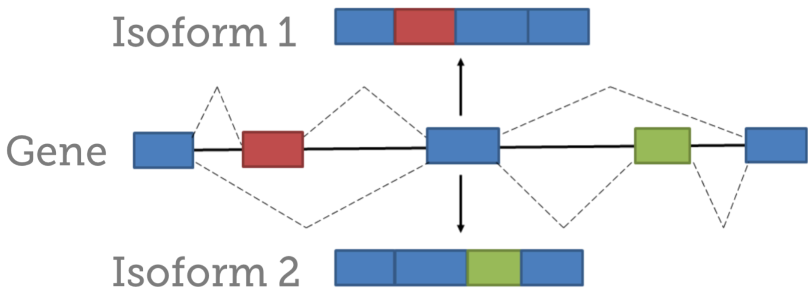
- Reads are short
  - difficult to align, assemble.
  - too short to span long repeat regions.
  - Difficult to detect large structural variations like inversions.

What is RNA-Seq?

- Examine the state of the transcriptome.



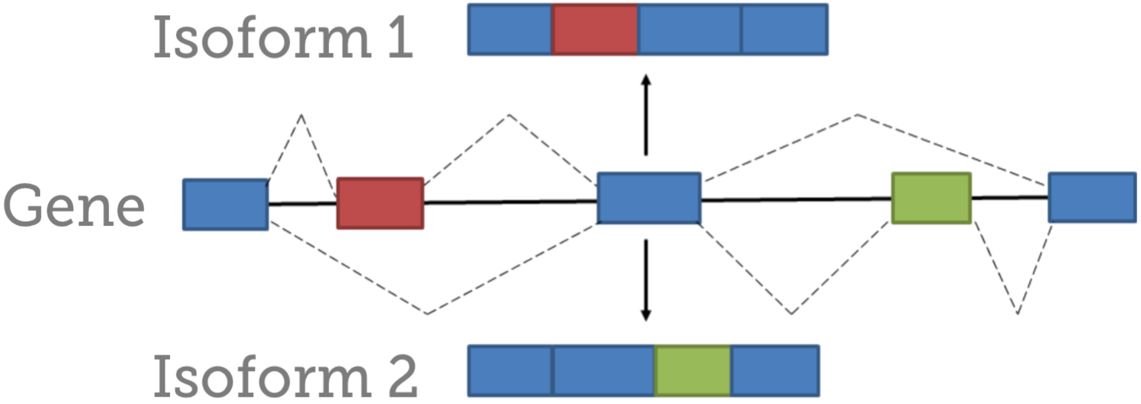
- Genes expression patterns vary in:
  - Tissue types
  - Cell types
  - Development stages
  - Disease conditions
  - Time points



- RNA-Seq measures these expression variations using high-throughput sequencing technologies.

What is RNA-Seq?

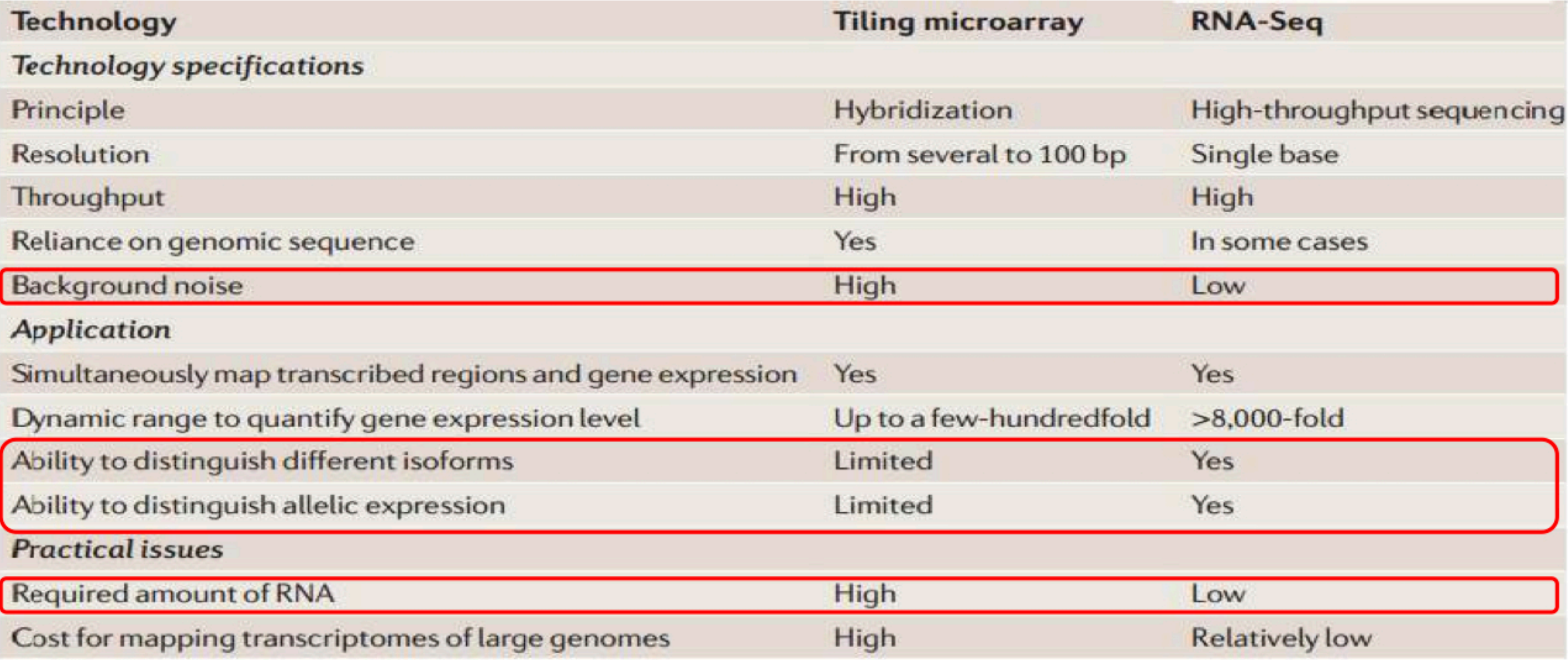
- RNA-Seq measures these expression variations
  - At gene level
  - At isoform level



Other Uses of RNA-Seq

- Assembling and annotating a transcriptome
- Characterization of alternative splicing patterns
- Gene fusion detection
- Small RNA profiling
- Targeted approaches using RNA-Seq
- Direct RNA sequencing

Advantages of RNA-Seq



Technology	Tiling microarray	RNA-Seq
Technology specifications		
Principle	Hybridization	High-throughput sequencing
Resolution	From several to 100 bp	Single base
Throughput	High	High
Reliance on genomic sequence	Yes	In some cases
Background noise	High	Low
Application		
Simultaneously map transcribed regions and gene expression	Yes	Yes
Dynamic range to quantify gene expression level	Up to a few-hundredfold	>8,000-fold
Ability to distinguish different isoforms	Limited	Yes
Ability to distinguish allelic expression	Limited	Yes
Practical issues		
Required amount of RNA	High	Low
Cost for mapping transcriptomes of large genomes	High	Relatively low

RNA-Seq: a revolutionary tool for transcriptomics

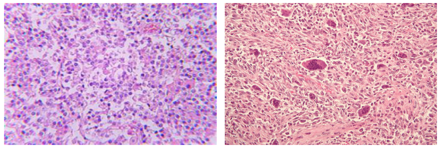
Zhong Wang, **Mark Gerstein**, and **Michael Snyder**

Nat Rev Genet. 2009 January ; 10(1): 57–63. doi:10.1038/nrg2484.

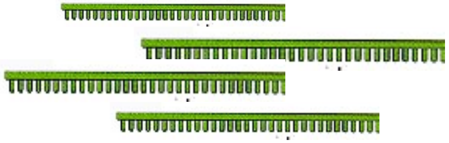
What are your questions ?

- This determines how you set up your experiment and how you analyze the data.
- What are you looking for?
  - Annotating a transcriptome?
  - Differential expression?
    - Novel transcripts/isoforms, junctions?
    - Differential gene expression?
    - Differential exon level counts?
    - Differential regulation?
  - Small RNA?

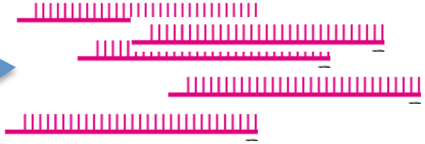
RNA-Seq… at it’s Most Basic Form



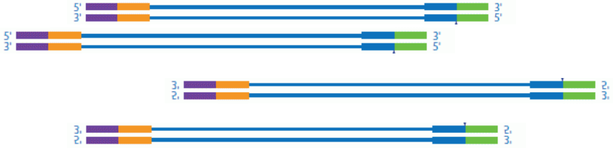
Samples from two conditions



Isolate RNA

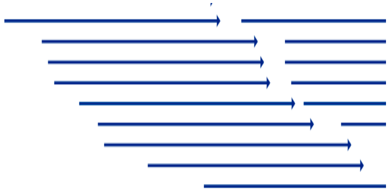


Generate cDNA



Create sequencing library by fragmenting, size selection and adding adaptors

Run sequencer



Generate short reads

defense response
negative regulation of programmed cell death
negative regulation of cell death
chromosome organization
regulation of cell proliferation
response to DNA damage stimulus
cell cycle process
programmed cell death
response to organic substance
cellular response to stress
regulation of cell death

Identify differentially expressed genes

RNA Illumina Tru-Seq library prep

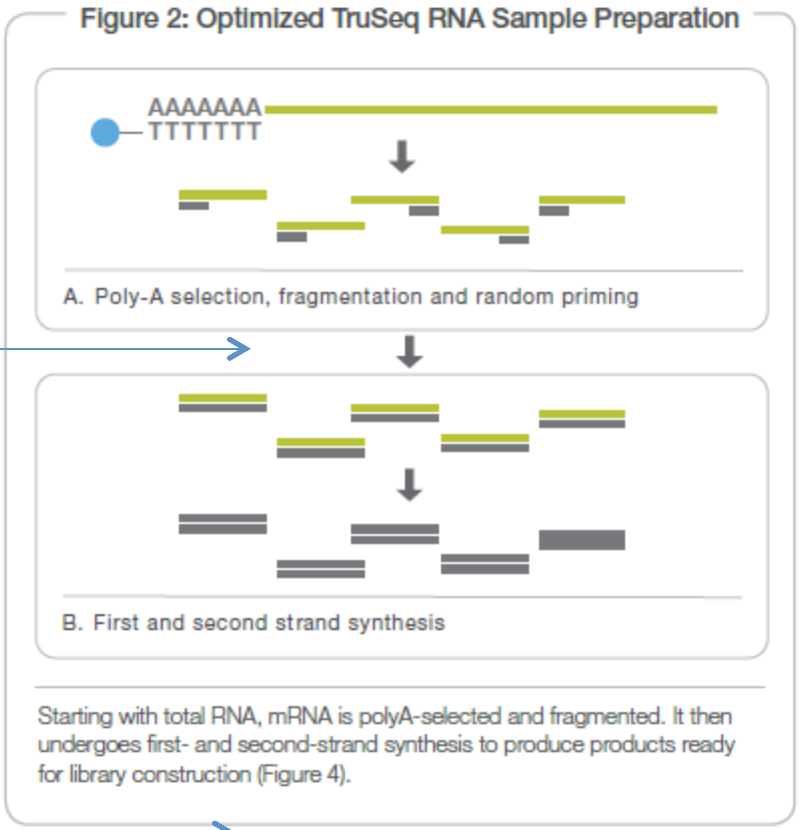


Figure 2: Optimized TruSeq RNA Sample Preparation

A. Poly-A selection, fragmentation and random priming

B. First and second strand synthesis

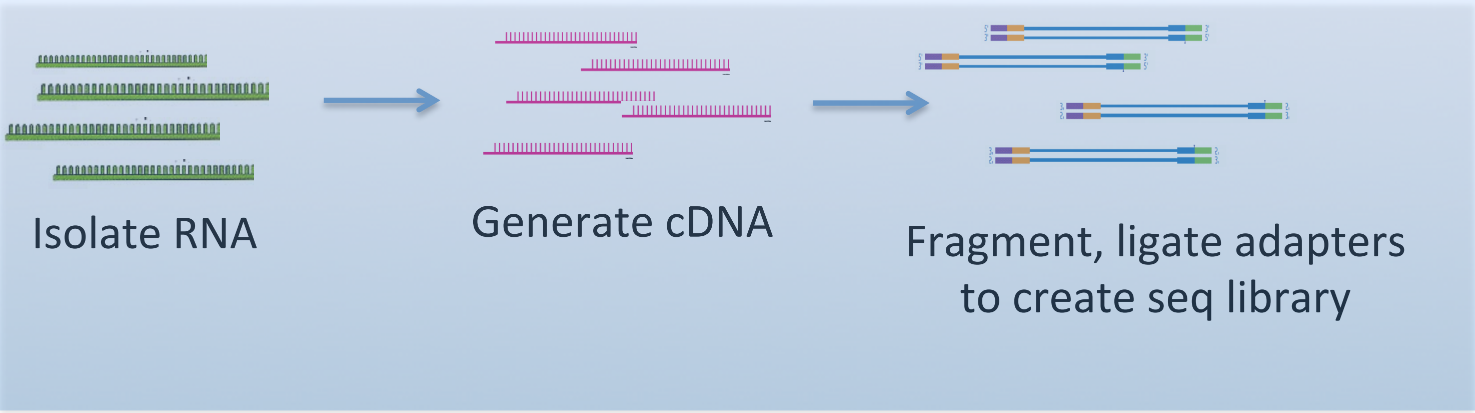
Starting with total RNA, mRNA is polyA-selected and fragmented. It then undergoes first- and second-strand synthesis to produce products ready for library construction (Figure 4).

Size selection step

Adaptor ligation and standard library preparation

2 days for 8 samples

RNA-Seq Libraries… with More Details

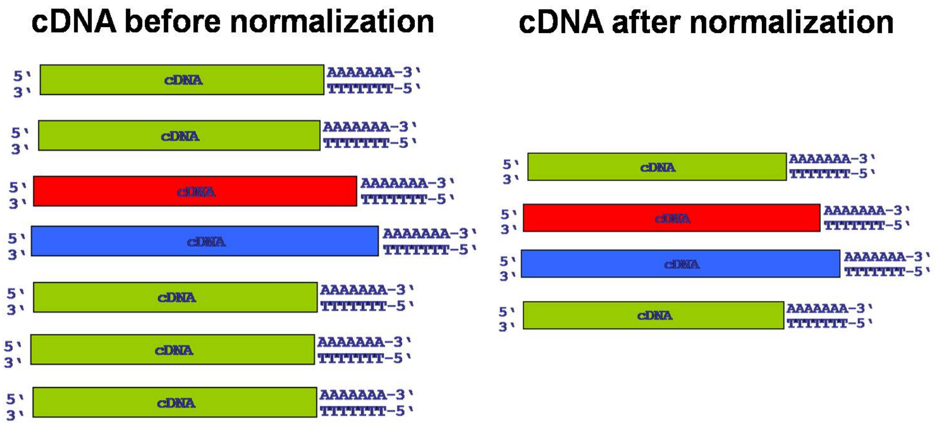


Isolate RNA → Generate cDNA → Fragment, ligate adapters to create seq library

A. rRNA Depletion

Ribominus kit

B. Normalized library



cDNA before normalization

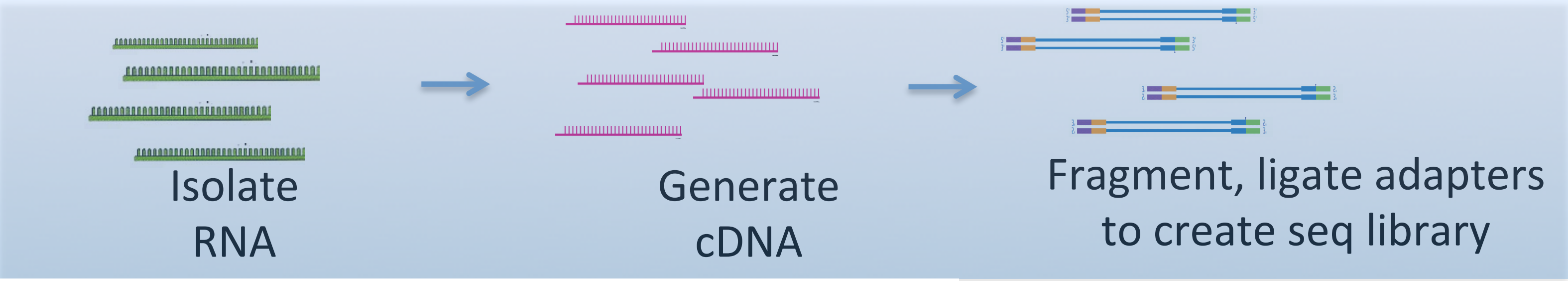
cDNA after normalization

Image from :www.genxpro.info

C. Size selection

Reserved for miRNA, siRNA profiling

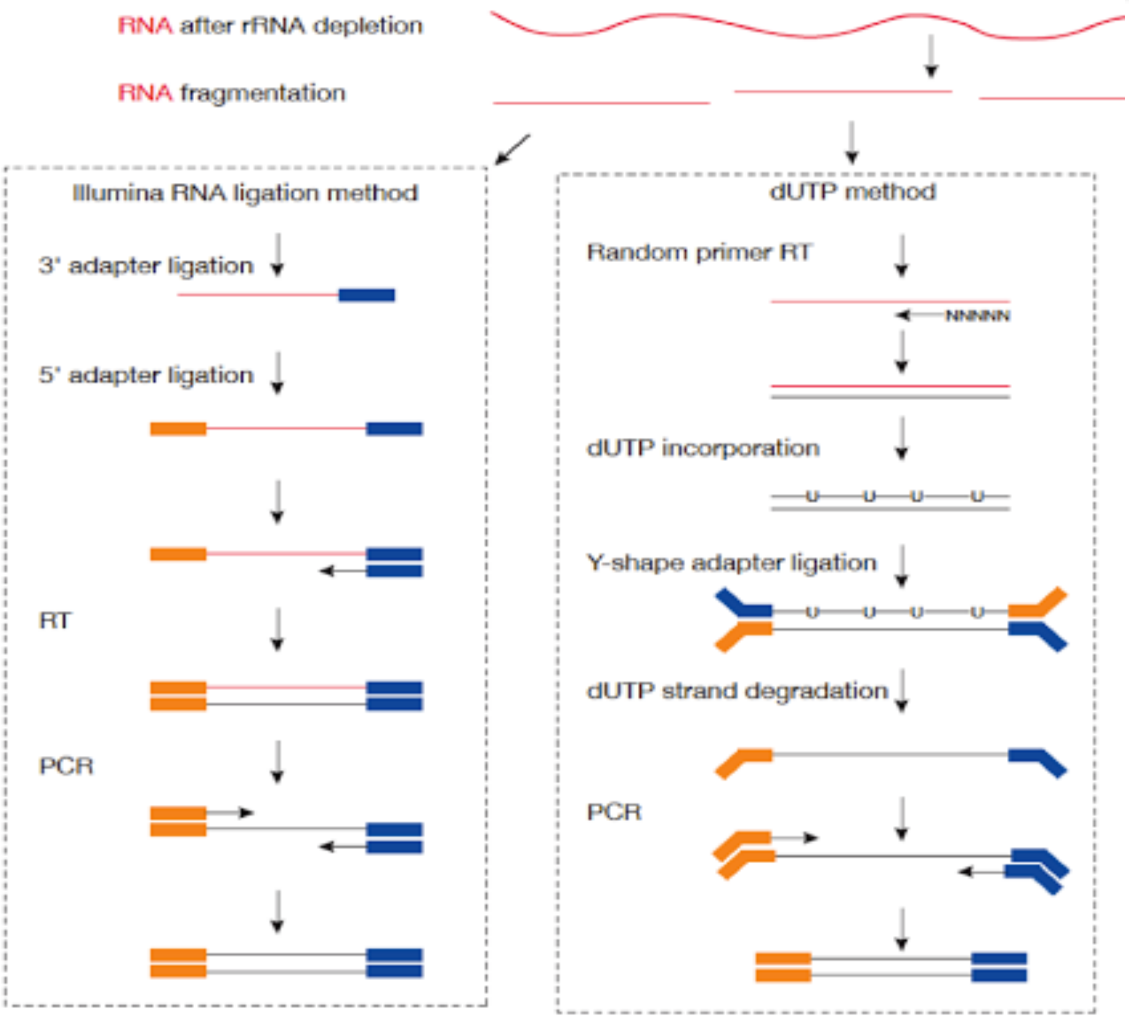
RNA-Seq Libraries… with More Details



Isolate RNA

Generate cDNA

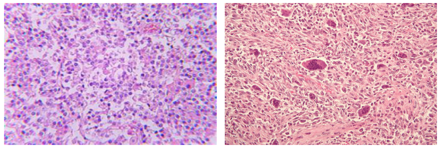
Fragment, ligate adapters to create seq library



Second Strand Synthesis- Many Strand Specific Methods.

Strand-specific libraries for high throughput RNA sequencing prepared without poly(A) selection, Zhang et al.

RNA-Seq… at it's Most Basic Form



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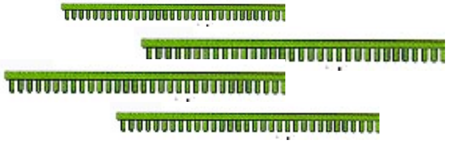
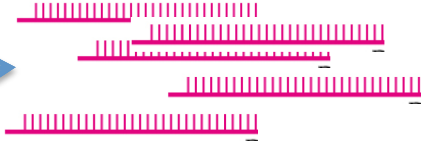
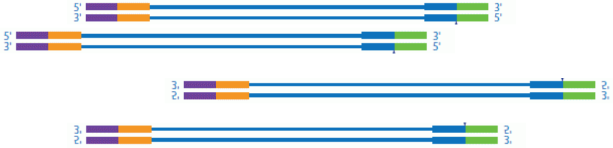


Isolate RNA

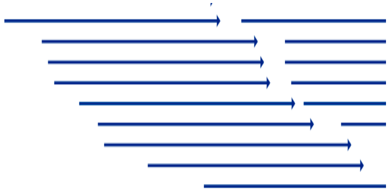


Generate cDNA

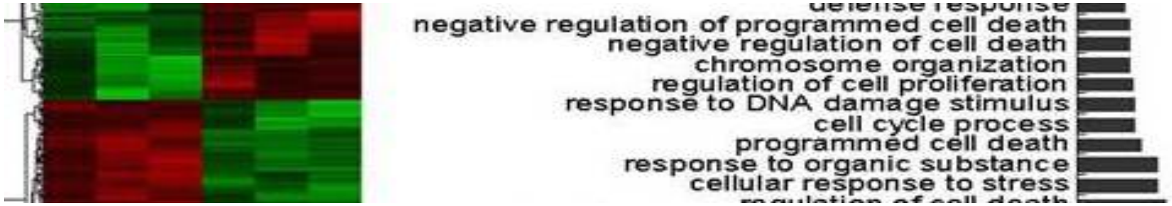


Create sequencing library by size selection and adding adaptors

Run sequencer



Generate short reads

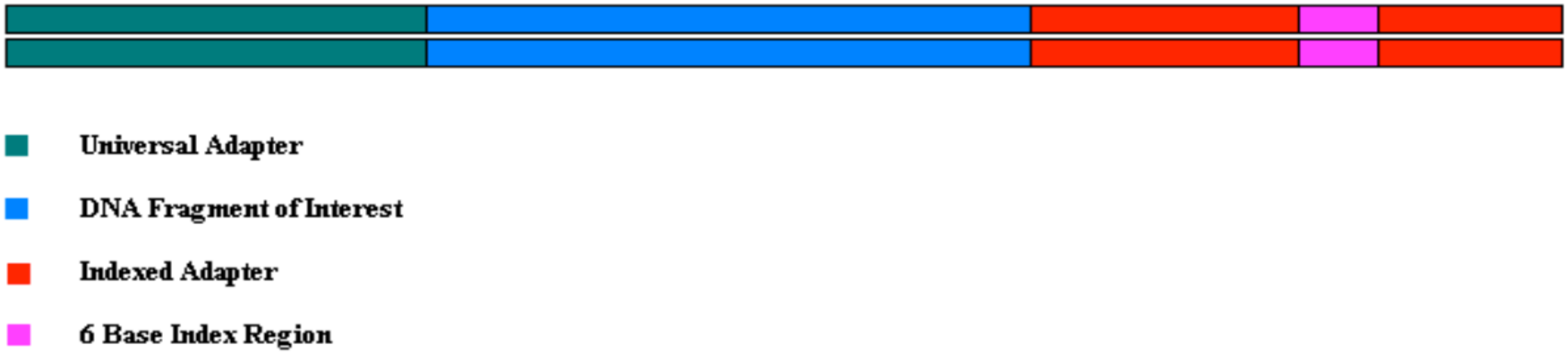


Identify differentially expressed genes

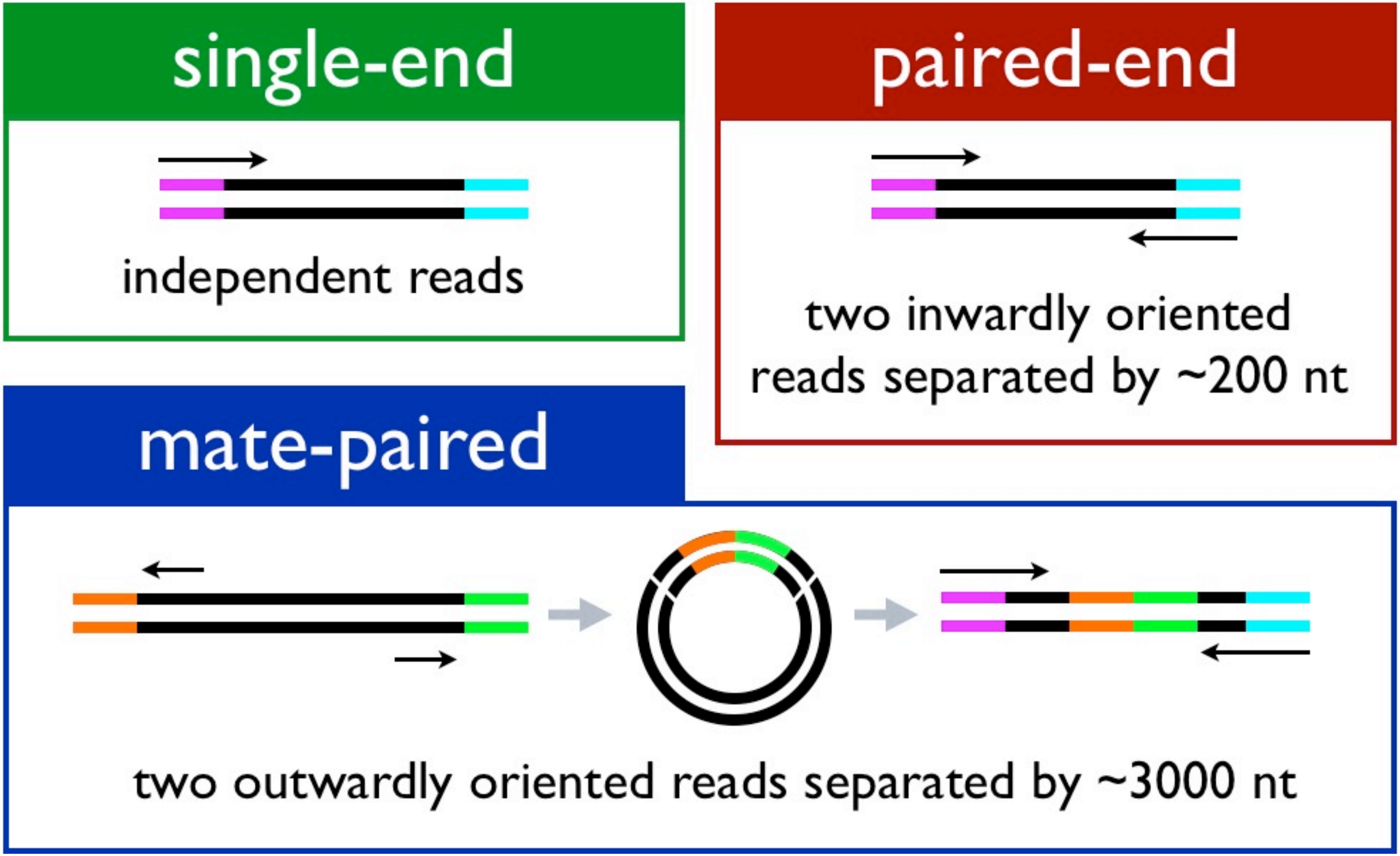
What is an adaptor?

Adaptor:

- Allows the template DNA to attach to the flowcell/cluster
- Has primer sequences to start synthesis off of.
- Has barcodes/indexes for multiplexing



Types of Illumina Fragment Libraries



single-end

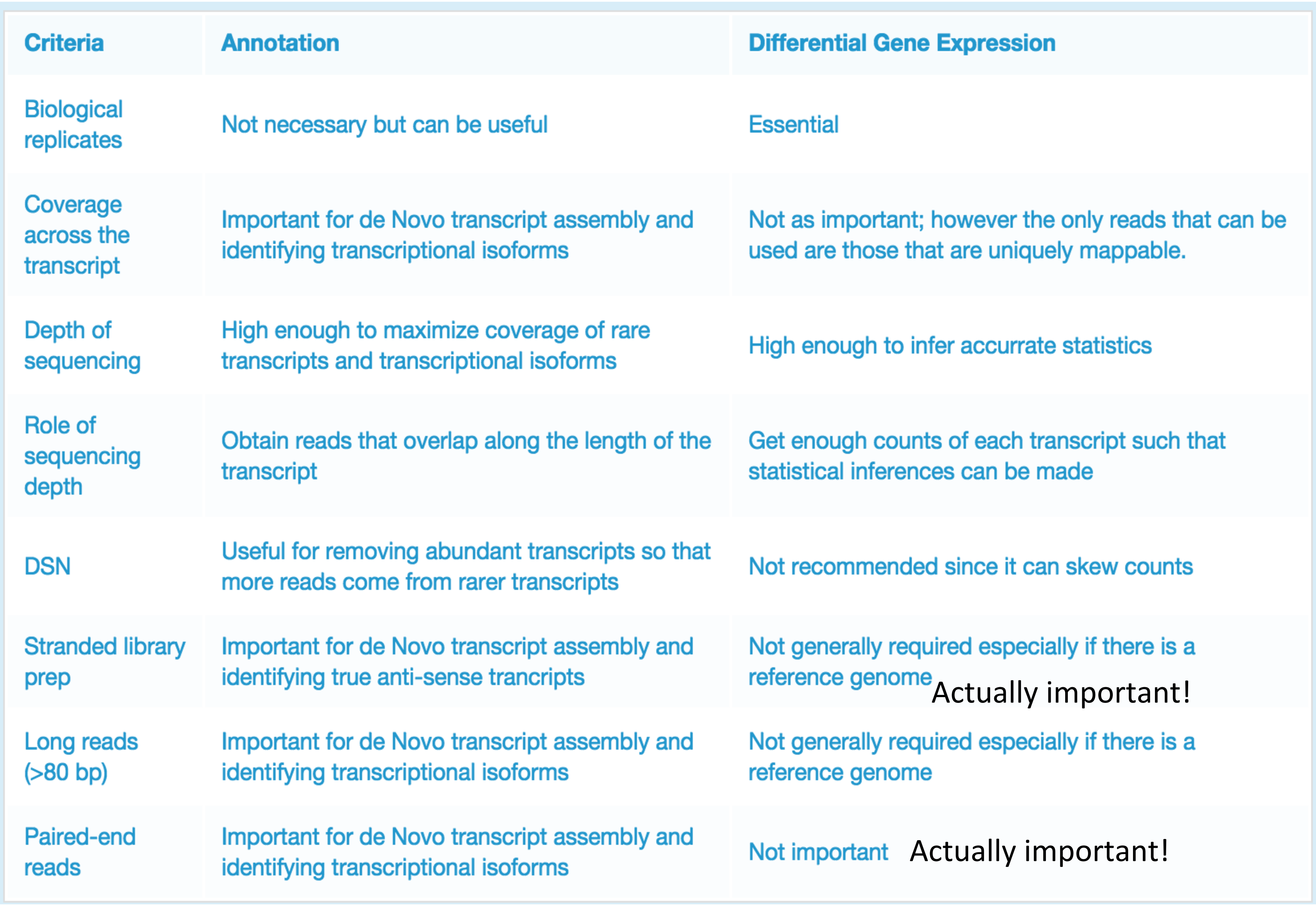
independent reads

paired-end

two inwardly oriented reads separated by ~200 nt

mate-paired

two outwardly oriented reads separated by ~3000 nt



Criteria	Annotation	Differential Gene Expression
Biological replicates	Not necessary but can be useful	Essential
Coverage across the transcript	Important for de Novo transcript assembly and identifying transcriptional isoforms	Not as important; however the only reads that can be used are those that are uniquely mappable.
Depth of sequencing	High enough to maximize coverage of rare transcripts and transcriptional isoforms	High enough to infer accurrate statistics
Role of sequencing depth	Obtain reads that overlap along the length of the transcript	Get enough counts of each transcript such that statistical inferences can be made
DSN	Useful for removing abundant transcripts so that more reads come from rarer transcripts	Not recommended since it can skew counts
Stranded library prep	Important for de Novo transcript assembly and identifying true anti-sense trancripts	Not generally required especially if there is a reference genome Actually important!
Long reads (>80 bp)	Important for de Novo transcript assembly and identifying transcriptional isoforms	Not generally required especially if there is a reference genome
Paired-end reads	Important for de Novo transcript assembly and identifying transcriptional isoforms	Not important Actually important!

From RNA-seqlopedia

3’ TAGSEQ- An Alternative to Whole RNA-Seq

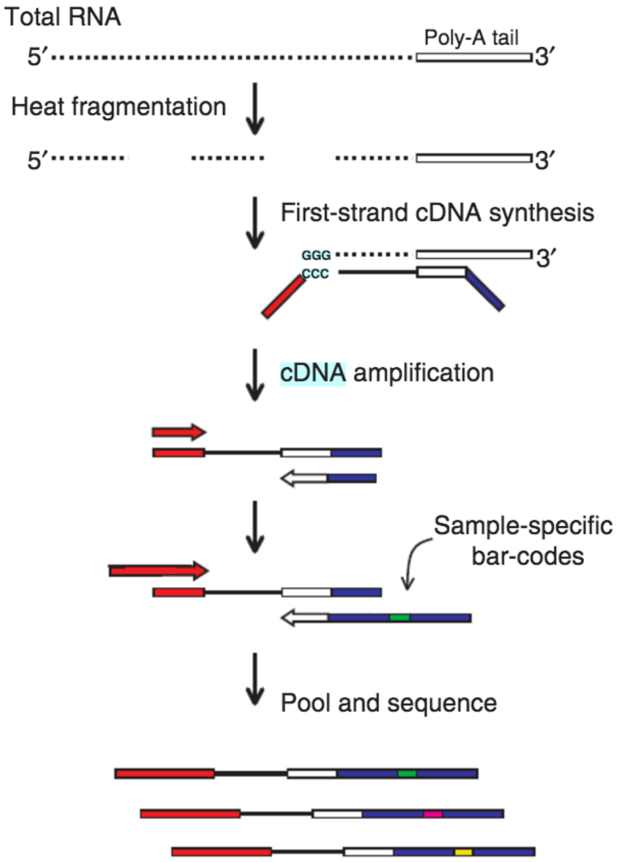
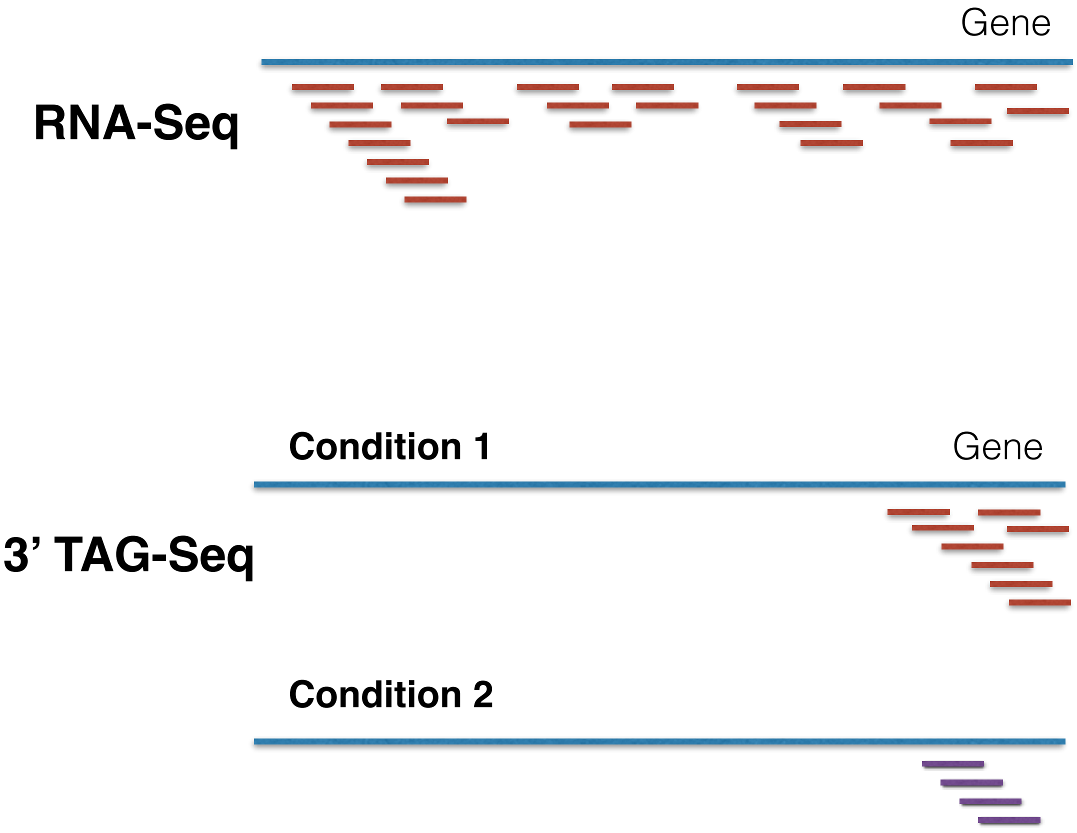


Fig. 1 Overview of the protocol used to prepare 3′ cDNA tag libraries from total RNA. RNA was fragmented at the beginning to eliminate biases resulting from differences in transcript lengths. First-strand cDNA was primed with a modified oligo-dT containing primer to target 3′ ends. Each sample was prepared with a sample-specific oligonucleotide barcode, then quantified and pooled prior to sequencing.

Targeting the 3’ prime end of RNA

WHY TAGSEQ?

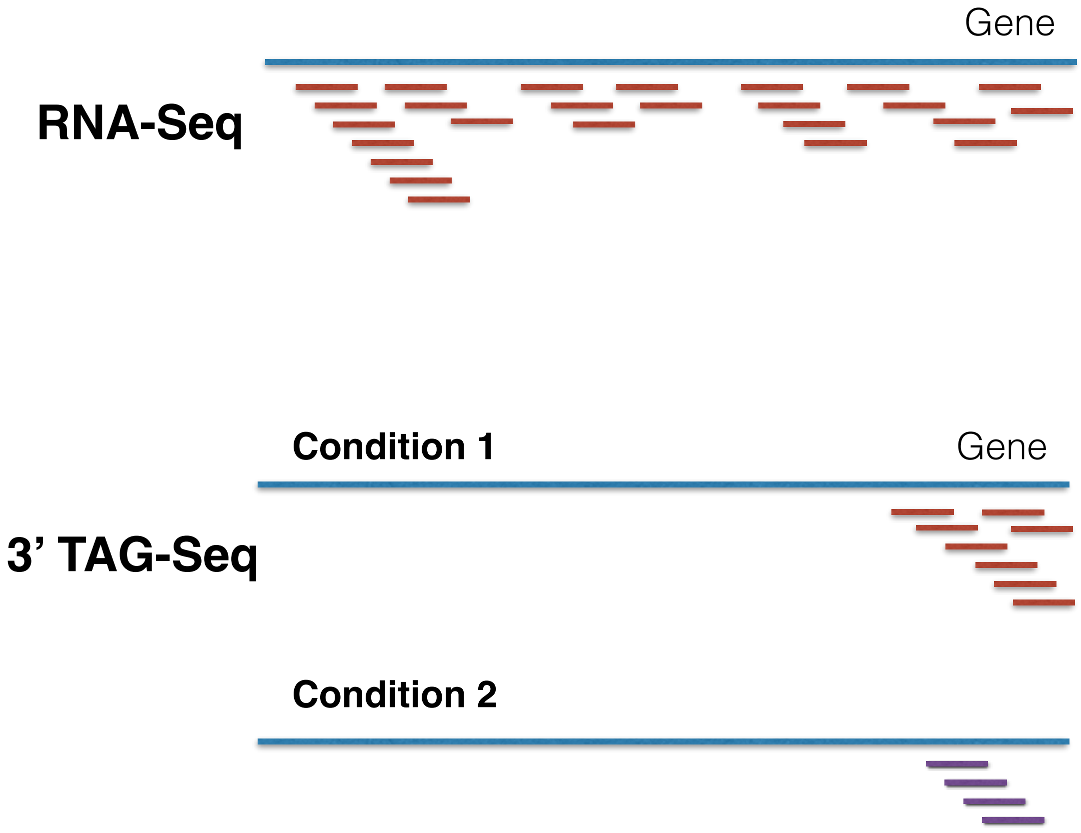
- Cheaper to sequence 3’ end instead of the entire RNA.
- Amount of input RNA required is less.
- You can still identify differential expression.



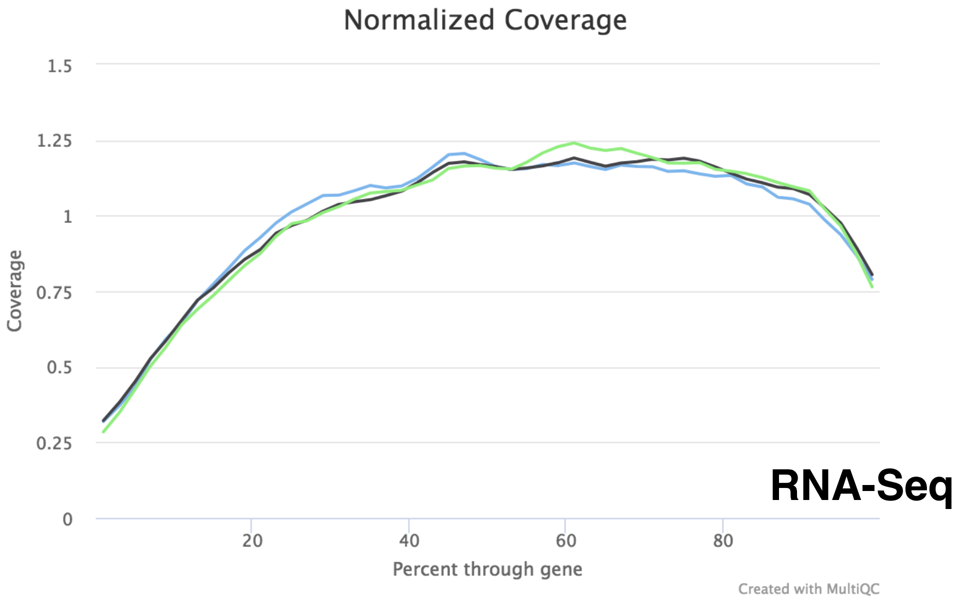
WHY NOT TAGSEQ?

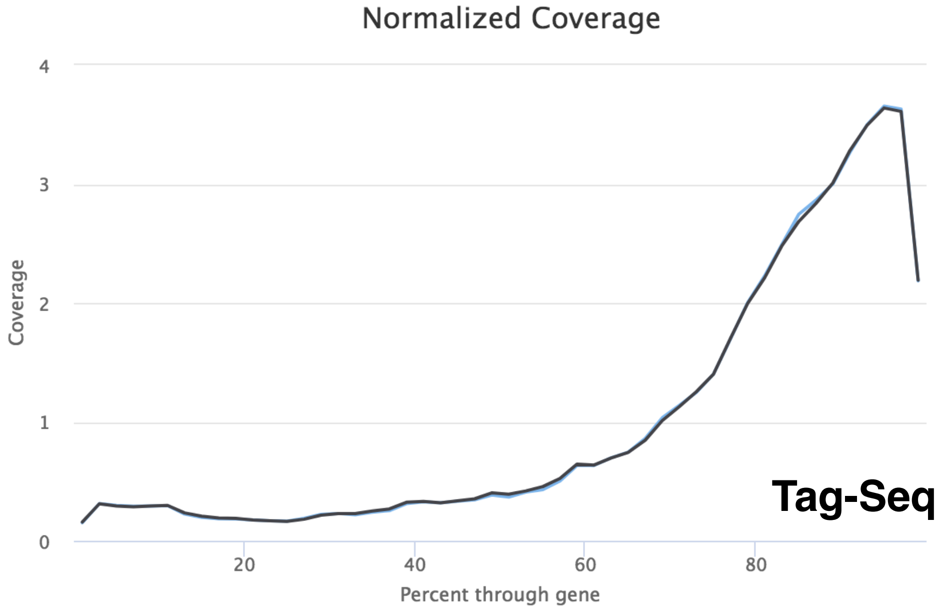
- If you want to look at differential splicing

- If you want to identify polymorphisms in gene sequences



Whole RNA-Seq vs TagSeq





Whole RNA-Seq vs TagSeq

TagSeq recovers known concentrations of mRNA (ERCC controls) with more accuracy than whole mRNASeq

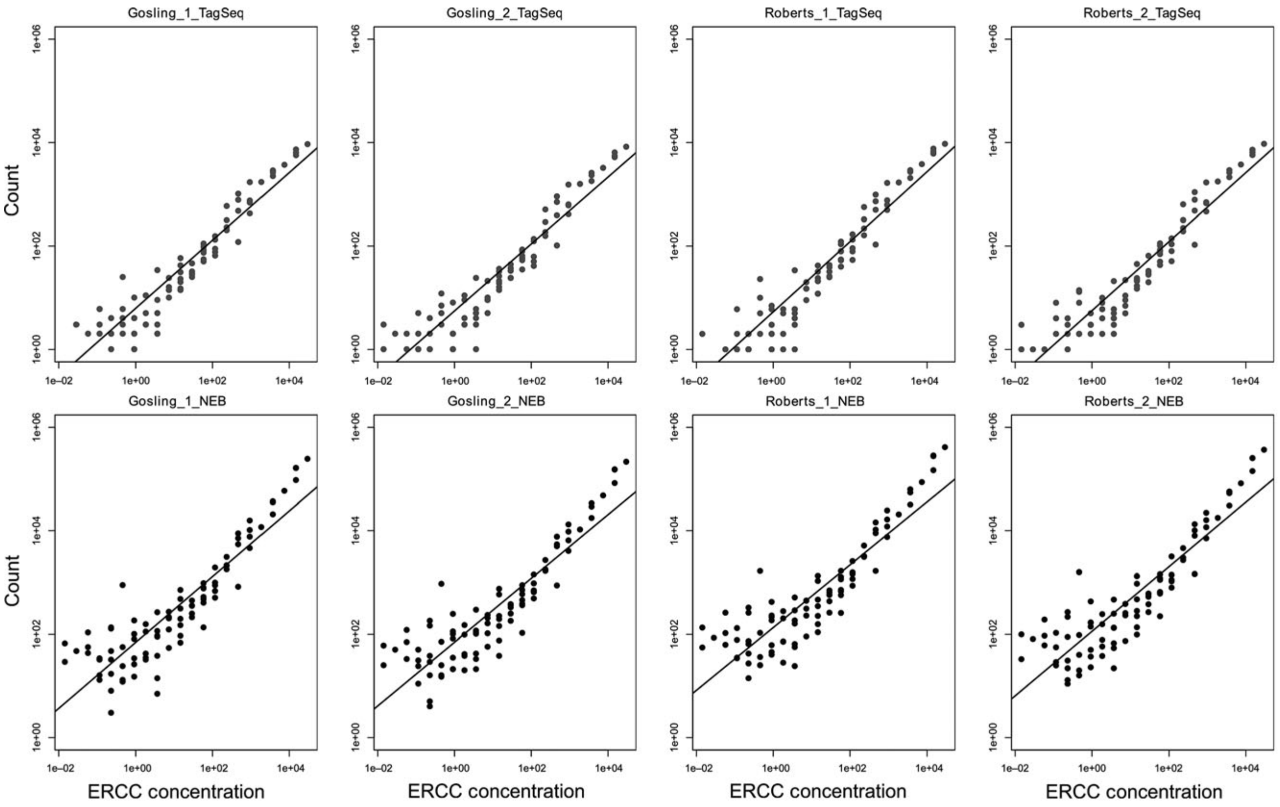


Fig. 1 Regression of observed vs. expected ERCC transcripts shows TagSeq has higher adjusted R^2 values for four different biological samples prepared with both methods (paired *t*-test, $t = 18.63$, d.f. = 3, $P < 0.001$).

Lohman et al, Molecular Ecology Resources, 2016

Comparing Stranded RNA-Seq Library Protocols

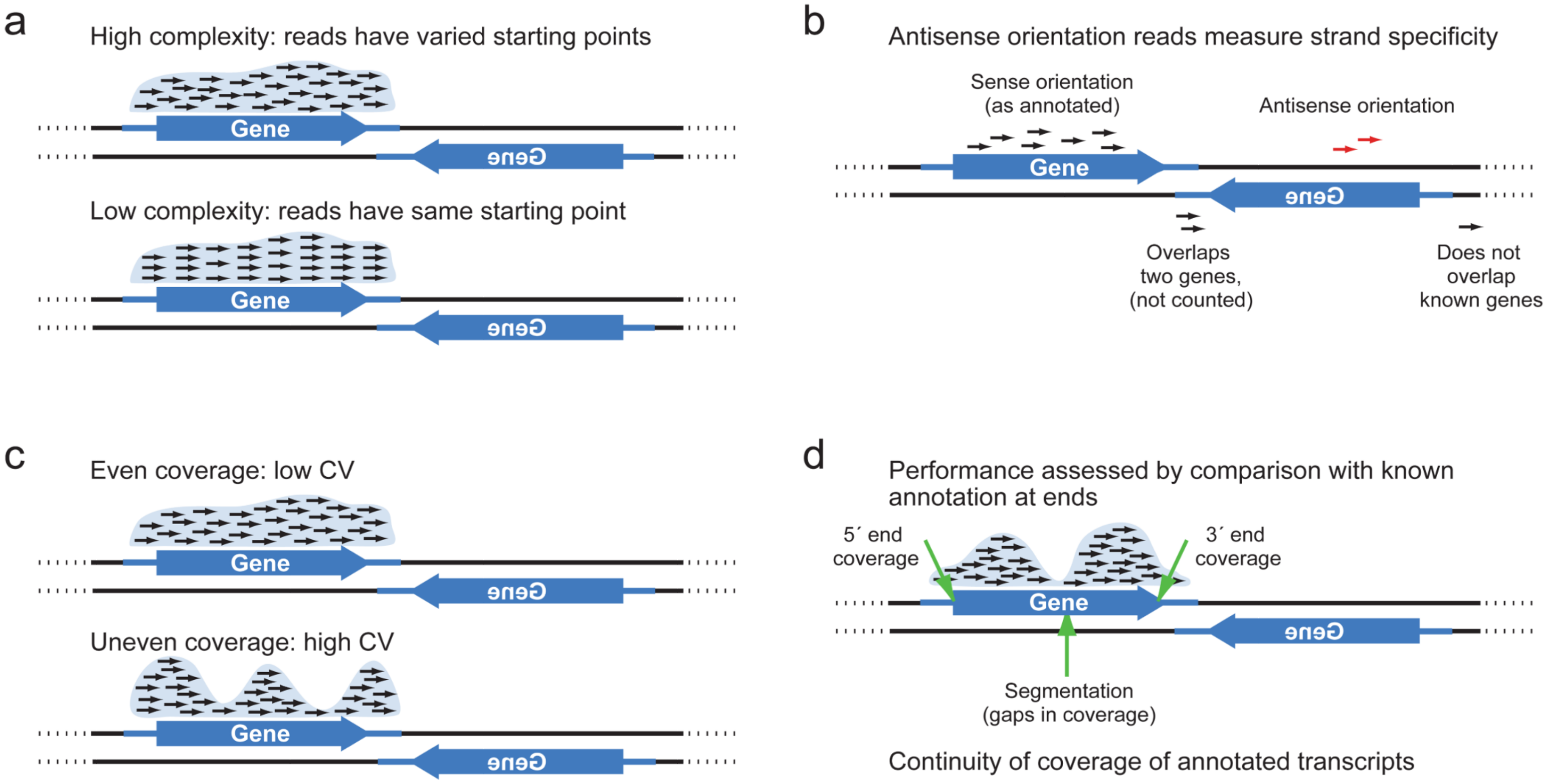
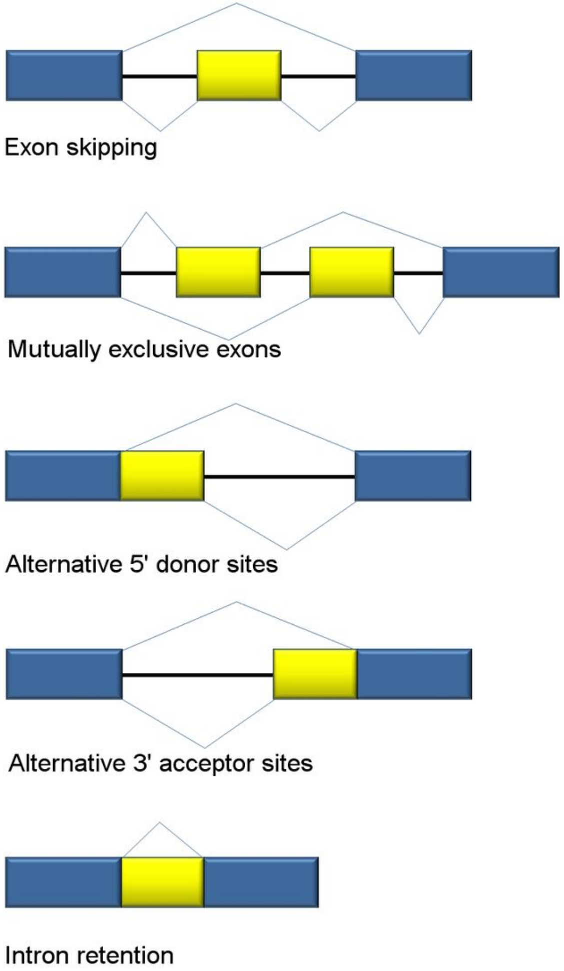

a
High complexity: reads have varied starting points
Gene
Gene
Low complexity: reads have same starting point
Gene
Gene
b
Antisense orientation reads measure strand specificity
Sense orientation (as annotated)
Antisense orientation
Gene
Gene
Overlaps two genes, (not counted)
Does not overlap known genes
c
Even coverage: low CV
Gene
Gene
Uneven coverage: high CV
Gene
Gene
d
Performance assessed by comparison with known annotation at ends
5´ end coverage
3´ end coverage
Gene
Gene
Segmentation (gaps in coverage)
Continuity of coverage of annotated transcripts

Figure 2. Key criteria for evaluation of strand-specific RNAseq libraries
Four categories of quality assessment. Double stranded genome (black parallel lines), with Gene ORF orientation (thick blue arrow) and UTRs (thin blue line), along with mapped reads (short black arrows – reads mapped to sense strand; red – reads mapped to antisense strand). (**a**) Complexity. (**b**) Strand Specificity. (**c**) Evenness of coverage. (**d**) Comparison to known transcript structure..

Comprehensive comparative analysis of strand-specific RNA sequencing methods, Levin et al, 2010

Why is RNA-Seq Difficult?

- Biases may mean what we are seeing is not reflective of true state of the transcriptome.
- Ugh, splicing!
- Gene level, exon level?
- Multimapping, partial mapping,, not mapping.
- Normalization issues
  - some datasets are larger than others, some genes are larger than others



From Wikipedia- alternative splicing

Illumina Fastq file

FASTQ Format

```
@HWI-EAS216_91209:1:2:454:192#0/1
GTTGATGAATTTCTCCAGCGCGAATTTGTGGCT
+HWI-EAS216_91209:1:2:454:192#0/1
B@BBBBBB@BBBBAAAA>@AABA?BBBAAB??>A?
```

Line 1: @read name

Line 2: called base sequence

Line 3: +read name (optional after +)

Line 4: base quality scores

Illumina Base Quality Scores

http://www.asciitable.com/

```
Quality character  !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHI
                   |         |         |         |         |
ASCII Value        33        43        53        63        73
Base Quality (Q)   0         10        20        30        40
```

Probability of Error = $10^{-Q/10}$

(This is a **Phred** score, also used for other types of qualities.)

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%

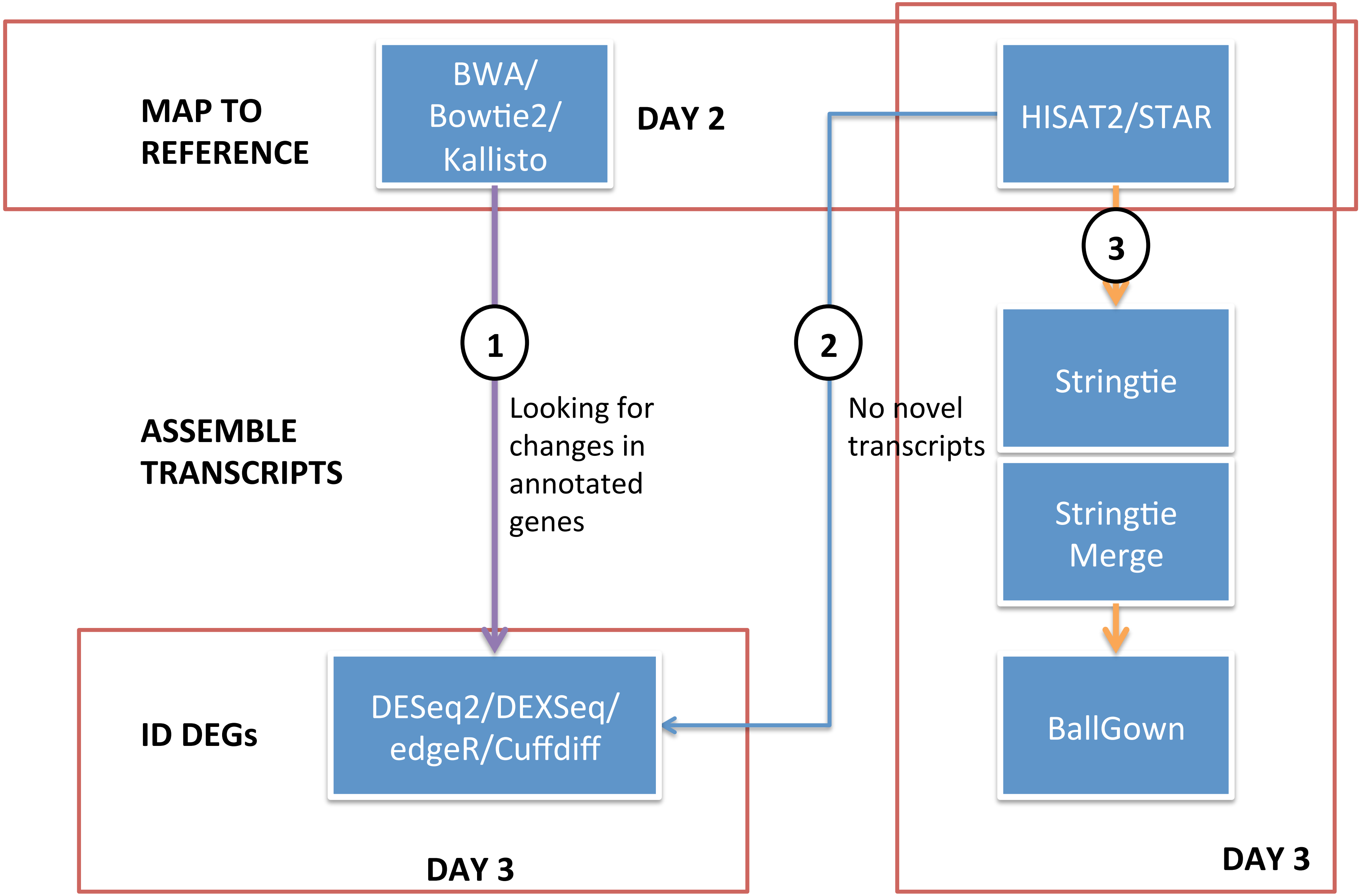
Quality scores are ASCII encoded in fastq files. Different platforms/older sequencing data can have different encoding! Illumina HiSeq 2500 produces Sanger encoded data.

Phred +33 =ASCII

How do we analyze RNA-Seq data?

- **STEP 1**: EVALUATE AND MANIPULATE RAW DATA
- **STEP 2**: MAP TO REFERENCE, ASSESS RESULTS
- **STEP 3**: ASSEMBLE TRANSCRIPTS
- **STEP 4**: QUANTIFY TRANSCRIPTS
- **STEP 5**: TEST FOR DIFFERENTIAL EXPRESSION
- **STEP 6**: VISUALIZE AND PERFORM OTHER DOWNSTREAM ANALYSIS

The Big Picture



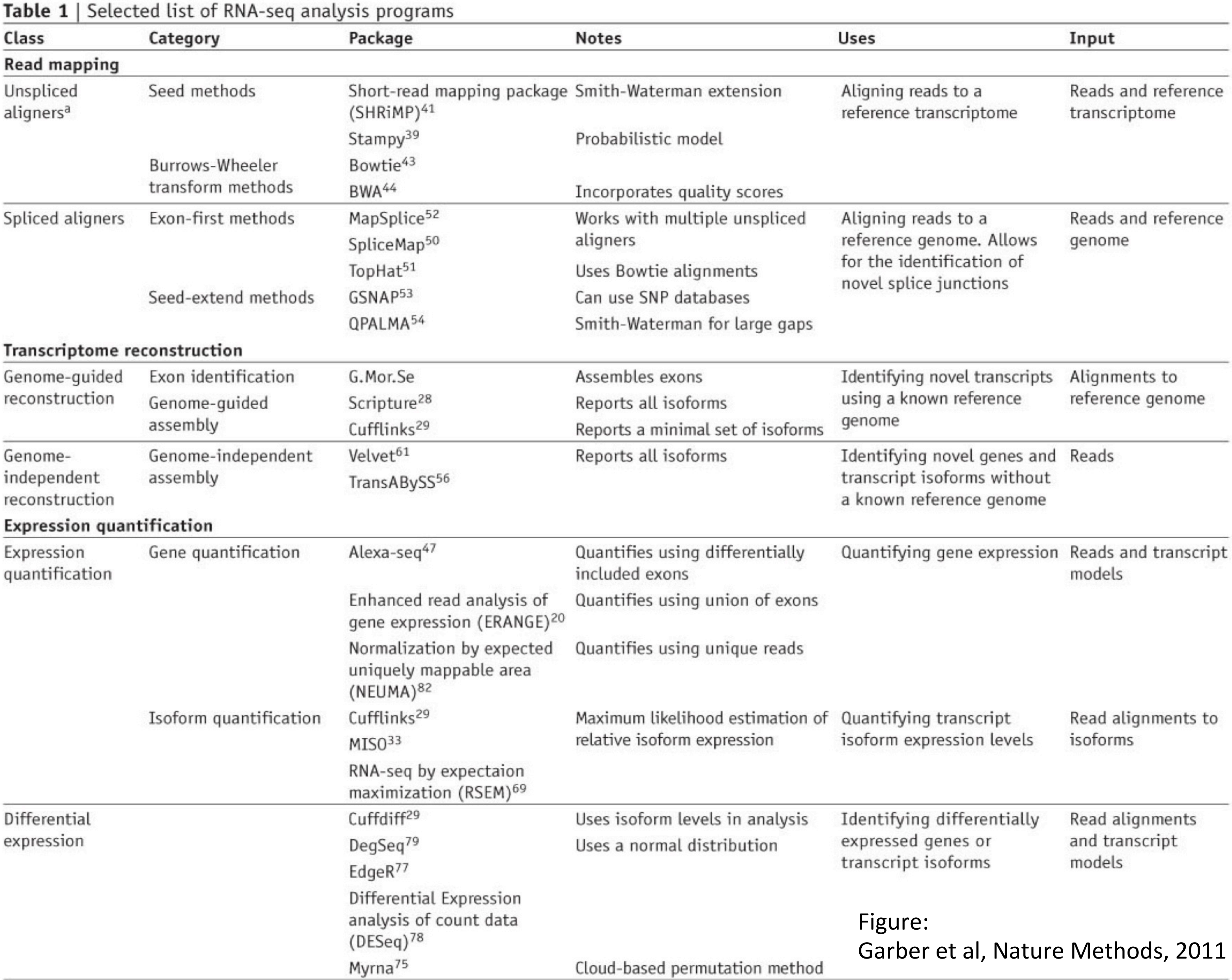
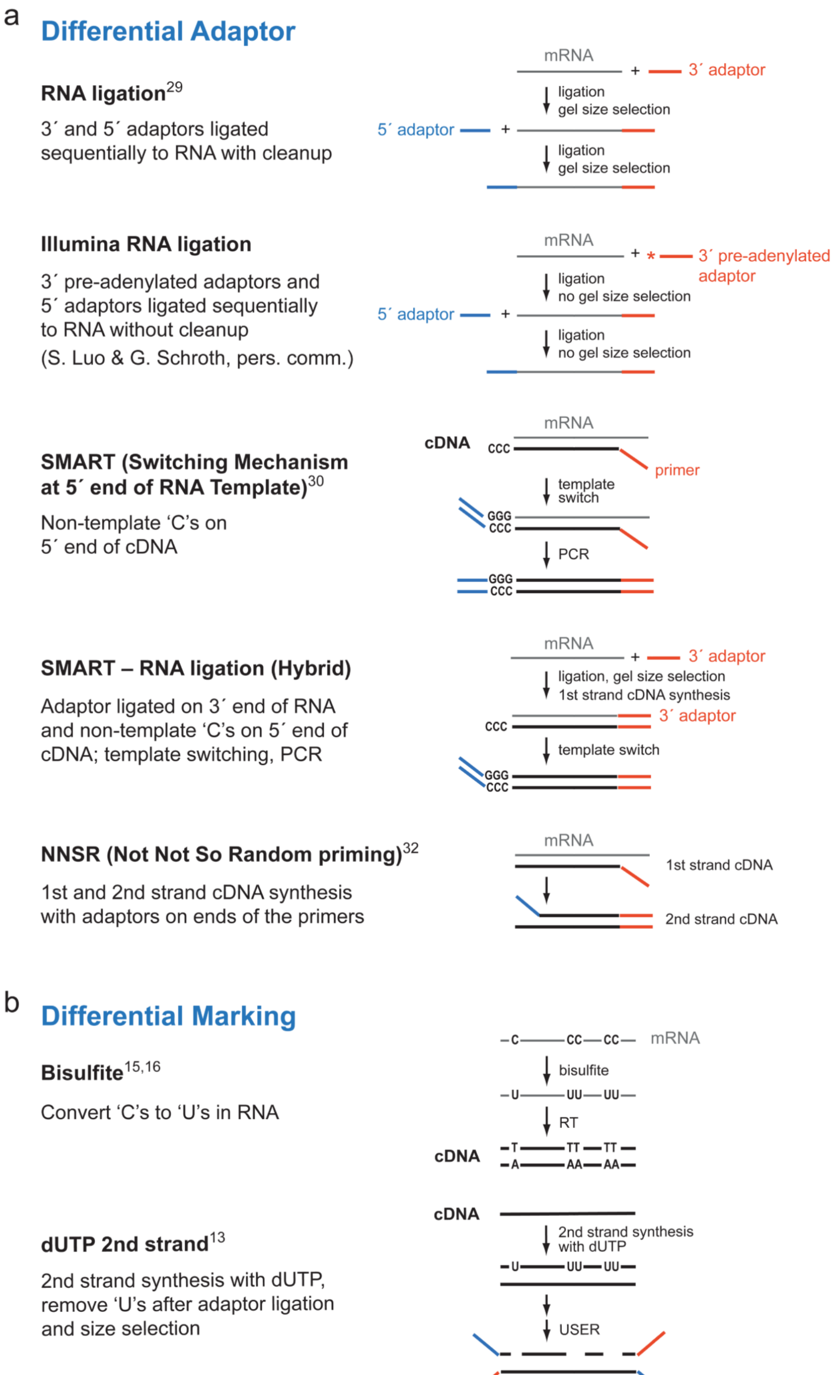


Table 1 | Selected list of RNA-seq analysis programs

Class	Category	Package	Notes	Uses	Input
Read mapping					
Unspliced aligners[a]	Seed methods	Short-read mapping package (SHRiMP)[41]	Smith-Waterman extension	Aligning reads to a reference transcriptome	Reads and reference transcriptome
		Stampy[39]	Probabilistic model		
	Burrows-Wheeler transform methods	Bowtie[43]			
		BWA[44]	Incorporates quality scores		
Spliced aligners	Exon-first methods	MapSplice[52]	Works with multiple unspliced aligners	Aligning reads to a reference genome. Allows for the identification of novel splice junctions	Reads and reference genome
		SpliceMap[50]			
		TopHat[51]	Uses Bowtie alignments		
	Seed-extend methods	GSNAP[53]	Can use SNP databases		
		QPALMA[54]	Smith-Waterman for large gaps		
Transcriptome reconstruction					
Genome-guided reconstruction	Exon identification	G.Mor.Se	Assembles exons	Identifying novel transcripts using a known reference genome	Alignments to reference genome
	Genome-guided assembly	Scripture[28]	Reports all isoforms		
		Cufflinks[29]	Reports a minimal set of isoforms		
Genome-independent reconstruction	Genome-independent assembly	Velvet[61]	Reports all isoforms	Identifying novel genes and transcript isoforms without a known reference genome	Reads
		TransABySS[56]			
Expression quantification					
Expression quantification	Gene quantification	Alexa-seq[47]	Quantifies using differentially included exons	Quantifying gene expression	Reads and transcript models
		Enhanced read analysis of gene expression (ERANGE)[20]	Quantifies using union of exons		
		Normalization by expected uniquely mappable area (NEUMA)[82]	Quantifies using unique reads		
	Isoform quantification	Cufflinks[29]	Maximum likelihood estimation of relative isoform expression	Quantifying transcript isoform expression levels	Read alignments to isoforms
		MISO[33]			
		RNA-seq by expectaion maximization (RSEM)[69]			
Differential expression		Cuffdiff[29]	Uses isoform levels in analysis	Identifying differentially expressed genes or transcript isoforms	Read alignments and transcript models
		DegSeq[79]	Uses a normal distribution		
		EdgeR[77]			
		Differential Expression analysis of count data (DESeq)[78]			
		Myrna[75]	Cloud-based permutation method		

Figure:
Garber et al, Nature Methods, 2011

Appendix



a **Differential Adaptor**

RNA ligation[29]

3´ and 5´ adaptors ligated sequentially to RNA with cleanup

Illumina RNA ligation

3´ pre-adenylated adaptors and 5´ adaptors ligated sequentially to RNA without cleanup (S. Luo & G. Schroth, pers. comm.)

SMART (Switching Mechanism at 5´ end of RNA Template)[30]

Non-template 'C's on 5´ end of cDNA

SMART – RNA ligation (Hybrid)

Adaptor ligated on 3´ end of RNA and non-template 'C's on 5´ end of cDNA; template switching, PCR

NNSR (Not Not So Random priming)[32]

1st and 2nd strand cDNA synthesis with adaptors on ends of the primers

b **Differential Marking**

Bisulfite[15,16]

Convert 'C's to 'U's in RNA

dUTP 2nd strand[13]

2nd strand synthesis with dUTP, remove 'U's after adaptor ligation and size selection

Levin et al.
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Figure 1. Methods for strand-specific RNA-Seq

Salient details for seven protocols for strand-specific RNA-Seq, differential adaptor methods (a) and differential marking methods (b). mRNA is shown in grey, and cDNA in black. For differential adaptor methods, 5' adaptors are shown in blue, and 3' adaptors in red.

Third generation sequencing

- Next, next generation sequencing?
- Single molecule sequencing- takes care of all above mentioned issues.
- Much longer reads (1-100kb)
- Many issues- high error rate and expensive
- Two categories:
    - Sequencing by synthesis (pacbio)
        - WATCH DNA as it is sequenced in realtime
        - ZMW technology lets smallest amount of light to be detected.
    - Direct sequencing
        - Oxford nanopore
        - Hydrogen ion changes ph in well. Change in ph indicates base has been incorporated.

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