Basics of RNASeq

Outline

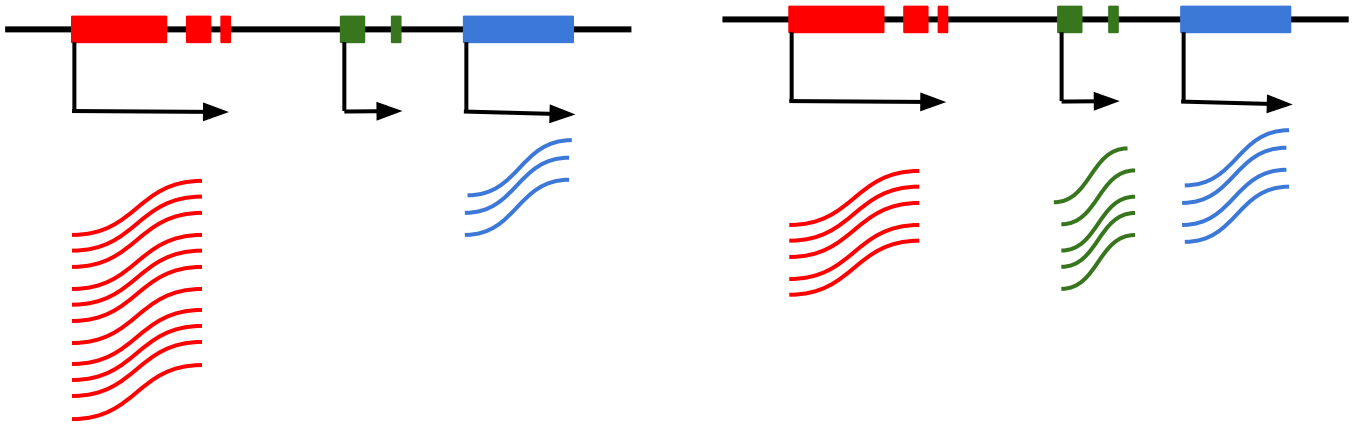
- The Big Picture
- Illumina platform
- Fasta format
- Fastq format
- RNASeq
- Data set for the class

The Big Picture

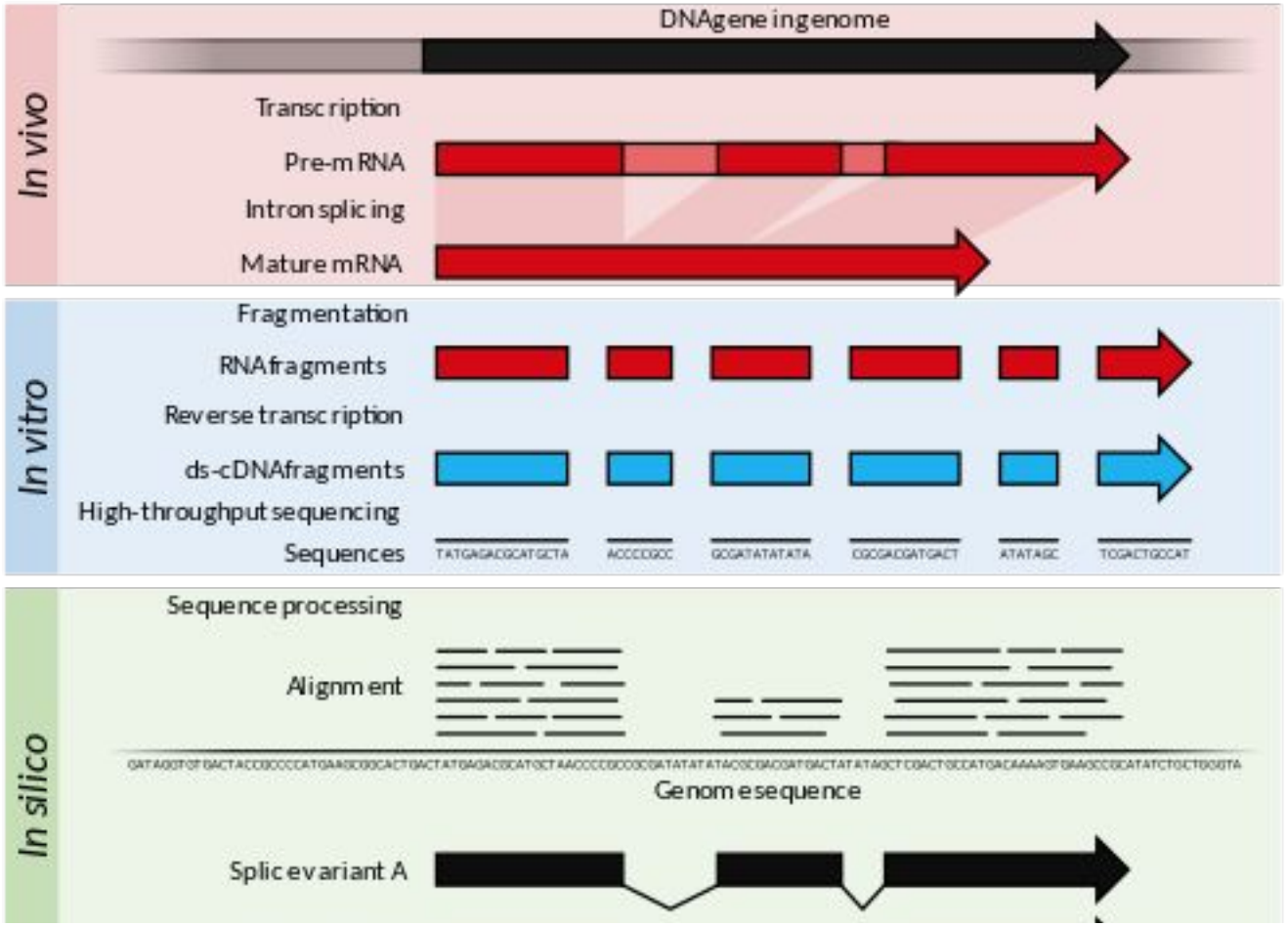
Genome

Genes

Transcripts



The Big Picture




illumina

Illumina Sequencing Technology

$3.3 billion in revenue in 2018

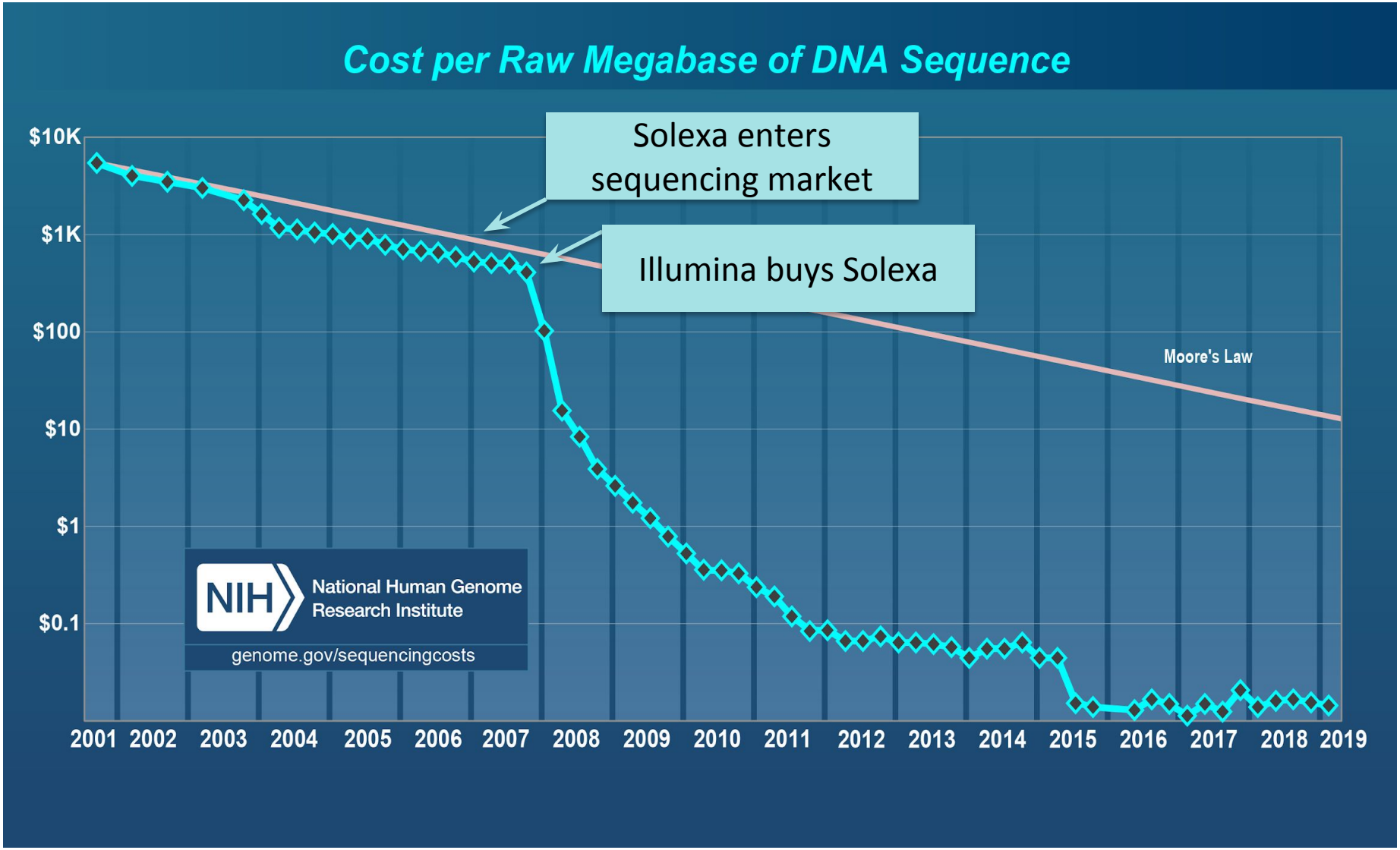
Market share (estimated):
90% in 2016
75% in 2018

Why are they so popular?

- Low price
- High throughput
- High base calling fidelity
- Paired end sequencing

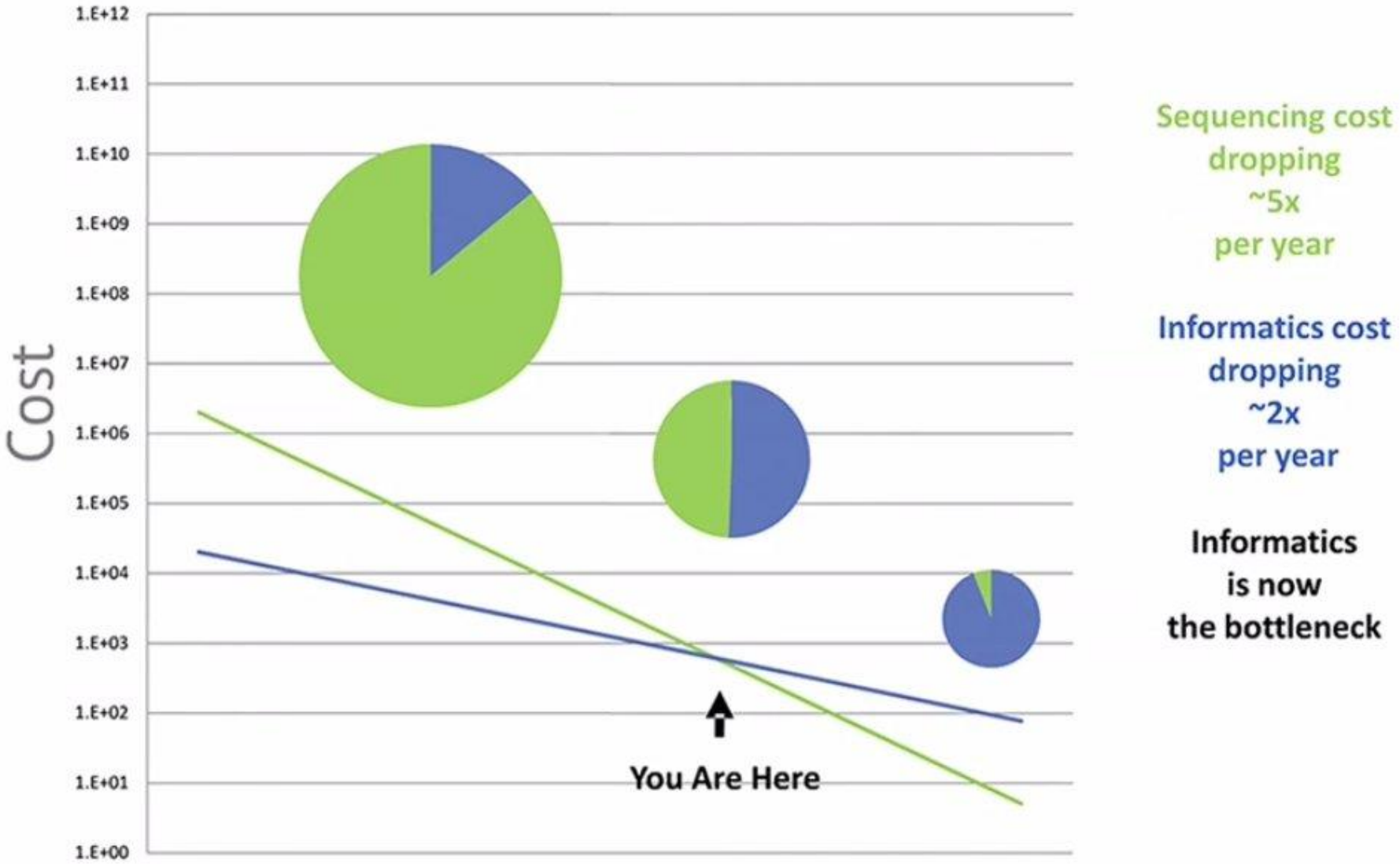
Announced acquisition of PacBio in late 2018 - may or may not go through.

More info: https://www.fool.com/investing/2019/07/22/what-happens-if-pacific-biosciences-isnt-acquired.aspx



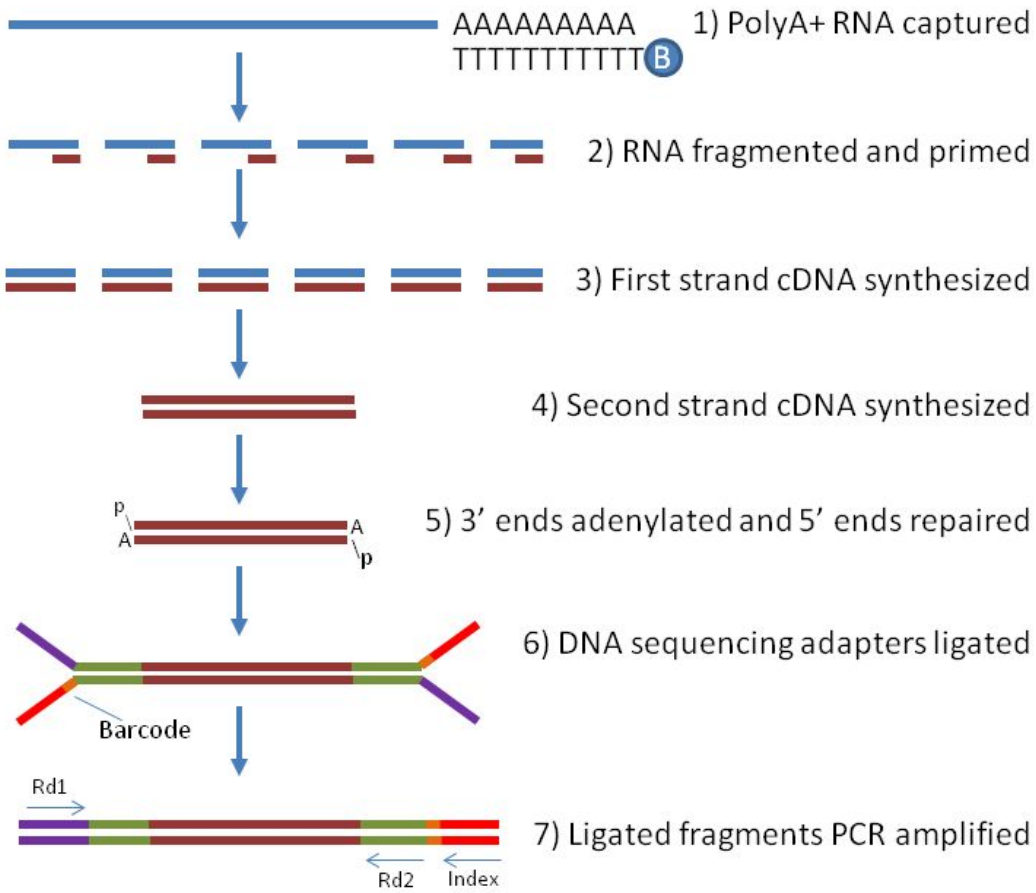
https://www.genome.gov/about-genomics/fact-sheets/DNA-Sequencing-Costs-Data

DNA Sequencing Economics



Raymond McCauley 2014 https://www.youtube.com/watch?v=j88APStUcp4&feature=youtu.be

How does it work?

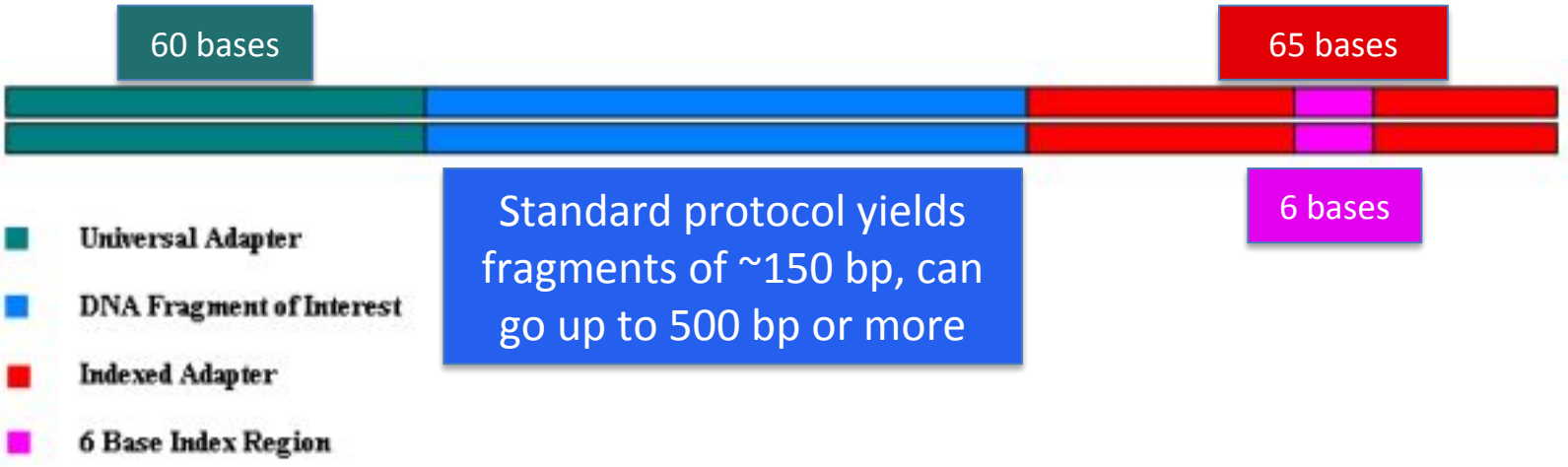


https://www.labome.com/method/RNA-seq.html

How does it work?

Library construction can vary by kit

TruSeq Example:



You will need the adapter sequences and a good understanding of adapter locations to later trim them out of your data

Illumina TruSeq DNA Adapters De-Mystified
https://www.med.unc.edu/pharm/calabreselab/files/tufts-sequencing-primer

Videos

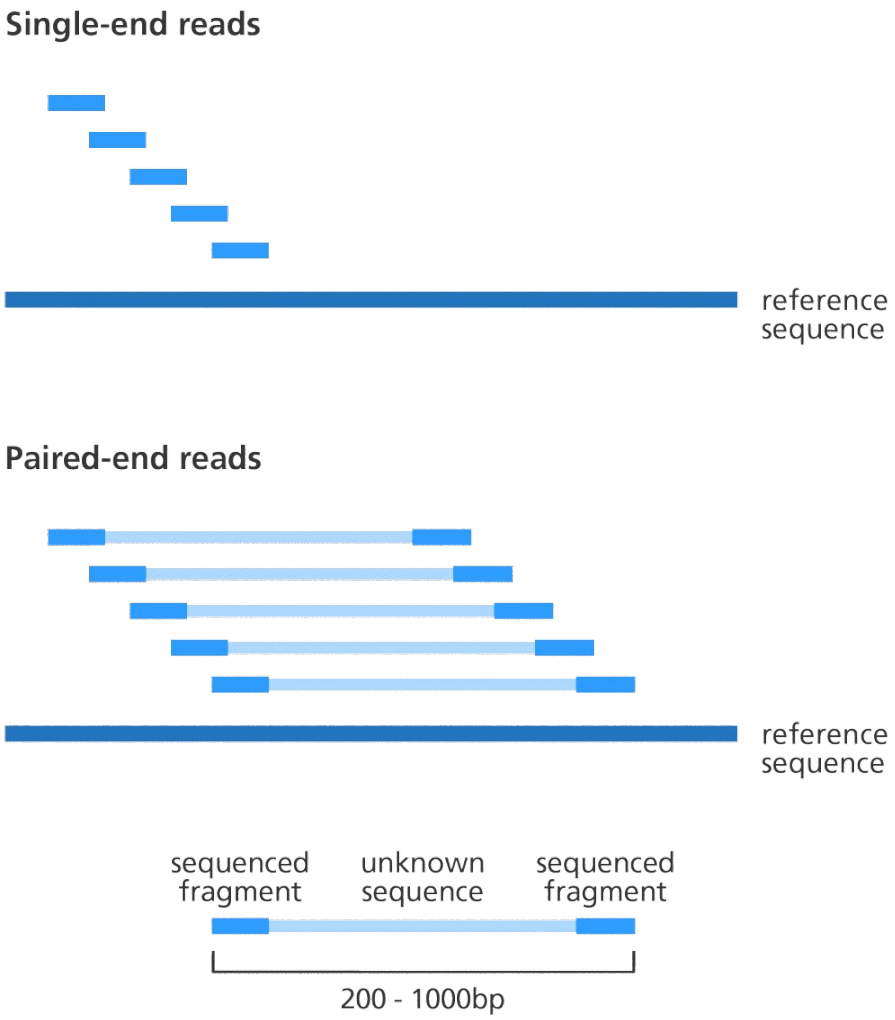
Illumina Sequencing by Synthesis by Illumina
https://www.youtube.com/watch?v=fCd6B5HRaZ8

Illumina Sequencing Technology by Illumina
https://www.youtube.com/watch?v=womKfikWlxM

Library Prep by ThermoFisher
https://www.youtube.com/watch?v=_yC0Bzw3WbQ

Also many platform specific videos

Paired End Sequencing



Devon Ryan https://www.biostars.org/p/267167/

Paired End Sequencing

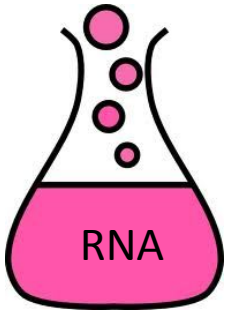
Why?

- Overcome lack of length.
- Map accurately to repetitive regions.
- Identify insertion/deletion mutations
- Better assembly.

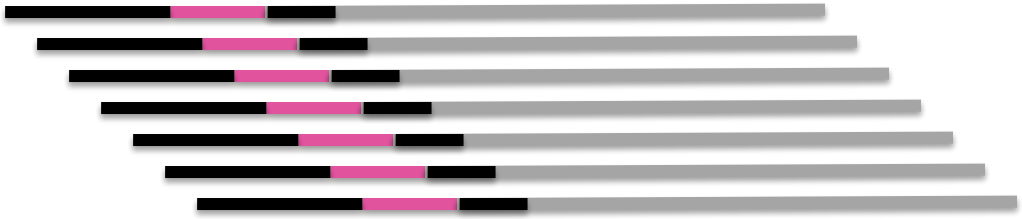
https://www.illumina.com/science/technology/next-generation-sequencing/plan-experiments/paired-end-vs-single-read.html

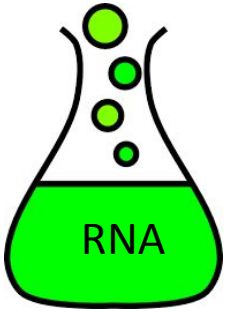
Multiplexing

Loading many samples into one lane.



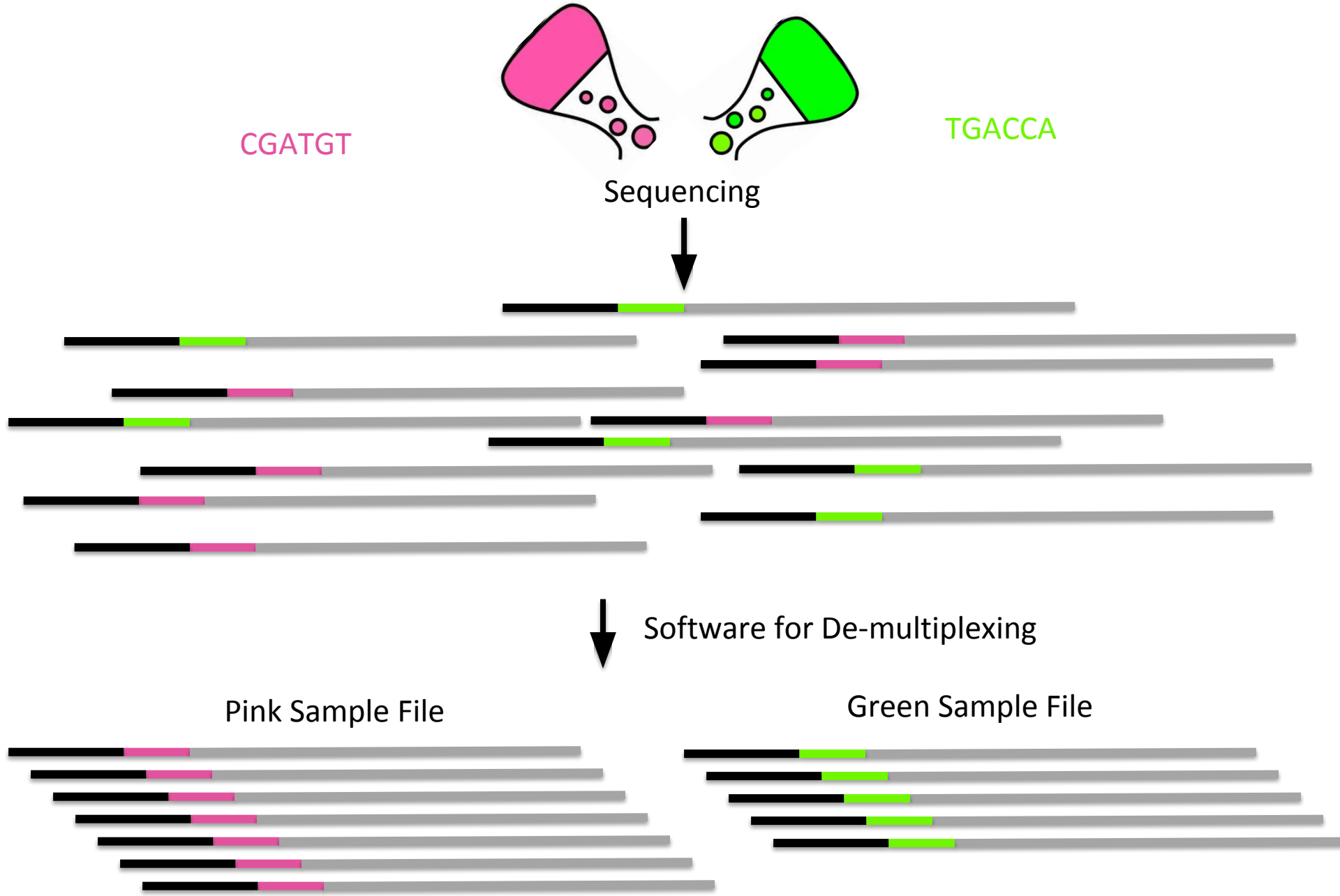
Pink Sample With CGATGT





Green Sample with TGACCA

http://www.illumina.com/technology/next-generation-sequencing/multiplexing-sequencing-assay.html



http://www.illumina.com/technology/next-generation-sequencing/multiplexing-sequencing-assay.html

Price and Throughput

NextSeq Series ✪

HiSeq 4000 System

HiSeq X Series‡

NovaSeq 6000 System

Instrument	NextSeq	HiSeq 2500	NovaSeq	NextSeq	NovaSeq		
Run	Mid-output	High-output	S Prime	High-output	S1	S2	S4
Read Length	2 x 150	2 x 125	2 x 150	2 x 150	2 x 150	2 x 150	2 x 150
Unit	Lane	Lane	Lane	Lane	Lane	Lane	Lane
# of reads	130 M	220 M	375 M	400 M	750 M	1,800 M	2,250 M
Output	39 Gb	55 Gb	112 Gb	120 Gb	225 Gb	540 Gb	675 Gb
Costs	$1,581	$3,044	$2,957	$5,629	$4,715	$10,034	$8,764
Costs/M reads	$12.16	$13.84	$7.89	$14.07	$6.29	$5.57	$3.90

University of Minnesota: http://genomics.umn.edu/nextgen-novaseq.php

UNIVERSITY OF MINNESOTA

Price and Throughput

Companies offer regular deals:

RNASeq library prep + sequencing of 20 million reads per library
$189

Long Read Technologies

PacBio IsoSeq and Nanopore

- full length transcripts
- no fragmentation, no amplification
- more expensive
- great if you don’t have a reference genome
- great for discovering and profiling alternative splicing variants

File Formats

Fasta Format

>gi|31563518|ref|NP_852610.1| microtubule-associated proteins 1A/1B light chain 3A isoform b [Homo sapiens]
MKMRFFSSPCGKAAVDPADRCKEVQQIRD
QHPSKIPVIIERYKGEKQLPVLDKTKFLVPDHV
NMSELVKIIRRRLQLNPTQAFFLLVNQHSMV
SVSTPIADIYEQEKDEDGFLYMVYASQETFGF
>FN640832
CCTGGTAGCTATGGCTTGCCTTTACTAAGA
CCCATCTCAAACAGGCTCAATTATTTTTGGT
TCCAAGGGCCTGAAACATTCTTAAAGAAGC
GAATAGAGAAACACAGGAGCACAGTTTTT
CGCACCAATATCCCTCCAACTTTCCCTTTCT
TCTCCAATGTTAATCCCAGCGTTGTTGCTGT
CCTTGACACCAAGTCTTTTGCACACCTC

A sequence must start with a header line

- Begins with a >
- First “word” is the sequence id
- Rest of line may contain more sequence descriptors

Fasta Format

>gi|31563518|ref|NP_852610.1| microtubule-associated proteins 1A/1B light chain 3A isoform b [Homo sapiens]
MKMRFFSSPCGKAAVDPADRCKEVQQIRD
QHPSKIPVIIERYKGEKQLPVLDKTKFLVPDHV
NMSELVKIIRRRLQLNPTQAFFLLVNQHSMV
SVSTPIADIYEQEKDEDGFLYMVYASQETFGF

>FN640832
CCTGGTAGCTATGGCTTGCCTTTACTAAGA
CCCATCTCAAACAGGCTCAATTATTTTTGGT
TCCAAGGGCCTGAAACATTCTTAAAGAAGC
GAATAGAGAAACACAGGAGCACAGTTTTT
CGCACCAATATCCCTCCAACTTTCCCTTTCT
TCTCCAATGTTAATCCCAGCGTTGTTGCTGT
CCTTGACACCAAGTCTTTTGCACACCTC

The header is followed by the sequence

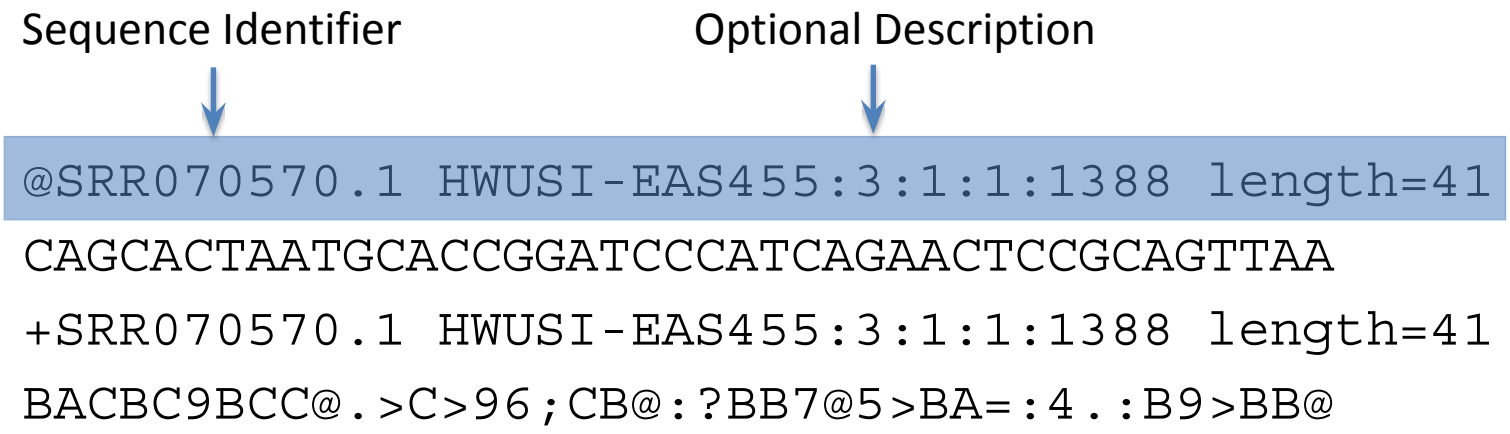
- May be amino acid or nucleotide
- May be a single line or multiple lines
- Should be consistent within a file

No empty line between sequence entries

Fastq Format

```
@SRR070570.1 HWUSI-EAS455:3:1:1:1388 length=41
CAGCACTAATGCACCGGATCCCATCAGAACTCCGCAGTTAA
+SRR070570.1 HWUSI-EAS455:3:1:1:1388 length=41
BACBC9BCC@.>C>96;CB@:?BB7@5>BA=:4.:B9>BB@
@SRR070570.2 HWUSI-EAS455:3:1:1:1785 length=41
CCAGAACACAAAGCTCATGACACGTTCACCTCCTGGAAGTT
+SRR070570.2 HWUSI-EAS455:3:1:1:1785 length=41
>AB@ACBB<BCA:>B;AA;@<B=;-=;<?@?<?=1-?B<8A
@SRR070570.3 HWUSI-EAS455:3:1:1:1679 length=41
ATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAAT
+SRR070570.3 HWUSI-EAS455:3:1:1:1679 length=41
BA=:==4?:8>A:8:>6:4:;2<07,<:@582+22'-';@>
```

Fastq Format



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

Fastq Format

The Sequence

```
@SRR070570.1 HWUSI-EAS455:3:1:1:1388 length=41
CAGCACTAATGCACCGGATCCCATCAGAACTCCGCAGTTAA
+SRR070570.1 HWUSI-EAS455:3:1:1:1388 length=41
BACBC9BCC@.>C>96;CB@:?BB7@5>BA=:4.:B9>BB@
```

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

Fastq Format

Totally useless line that begins with a + but does not need anything else; id and description are sometimes repeated.

```
@SRR070570.1 HWUSI-EAS455:3:1:1:1388 length=41
CAGCACTAATGCACCGGATCCCATCAGAACTCCGCAGTTAA
+SRR070570.1 HWUSI-EAS455:3:1:1:1388 length=41
BACBC9BCC@.>C>96;CB@:?BB7@5>BA=:4.:B9>BB@
```

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

Fastq Format

Quality values for each base.

```
@SRR070570.1 HWUSI-EAS455:3:1:1:1388 length=41
CAGCACTAATGCACCGGATCCCATCAGAACTCCGCAGTTAA
+SRR070570.1 HWUSI-EAS455:3:1:1:1388 length=41
BACBC9BCC@.>C>96;CB@:?BB7@5>BA=:4.:B9>BB@
```

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

FASTQ Quality Scores

Scores are encoded as a single character. From lowest score to highest score:

```
!"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHI
0…                                    …41
```

Can calculate the likelihood of a base being wrong with a logarithmic formula.

An I is 99.99% likely be correct.

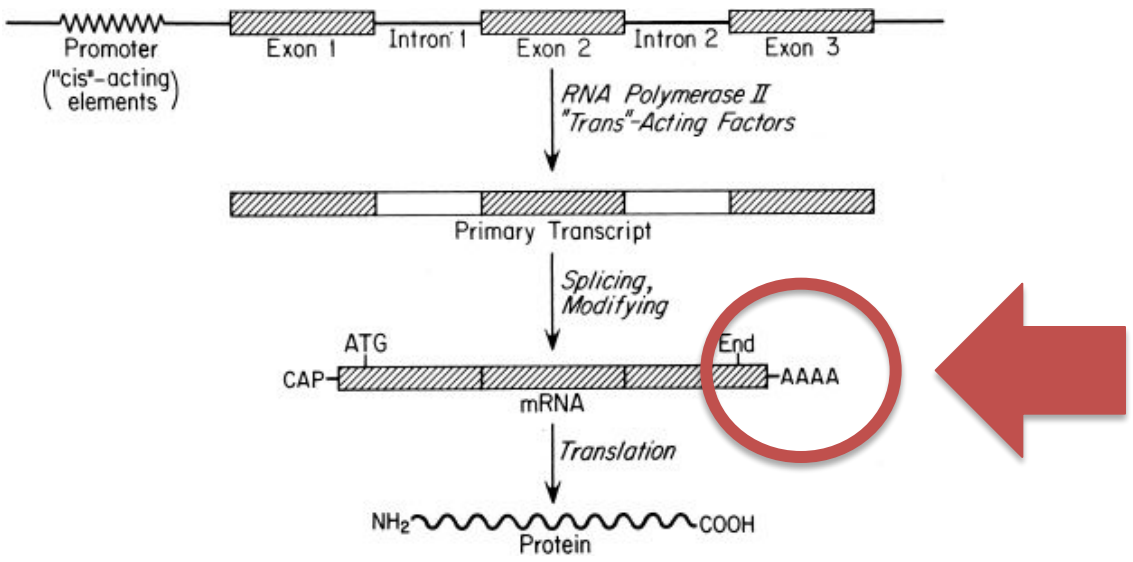
A * is only 90% likely to be correct.

https://en.wikipedia.org/wiki/Phred_quality_score
Ewing et al, 1998

RNA Sequencing

Targeting mRNA for sequencing

- To target mRNA
  - Poly-A enrichment - purify the poly-A containing mRNA molecules using poly-T oligo attached magnetic beads
  - Only works for eukaryotes



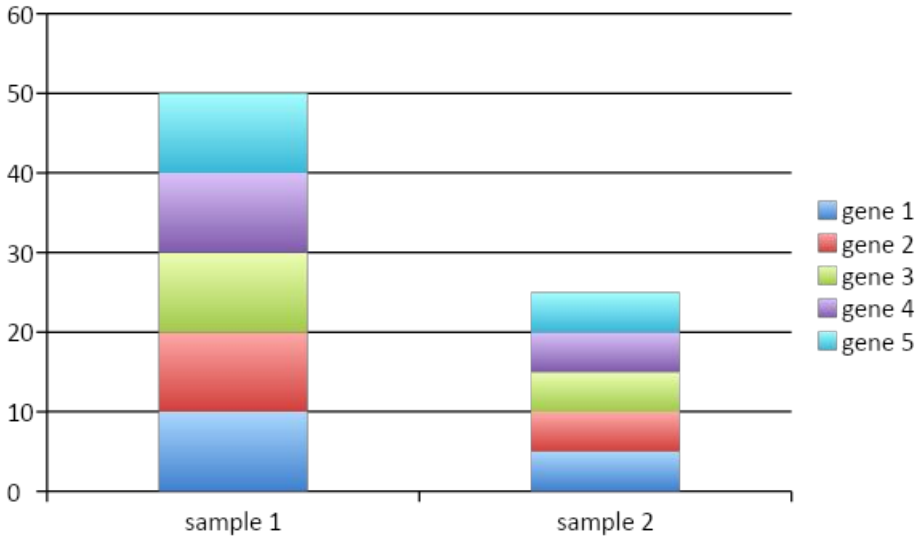
Experimental Goals for mRNA Seq

- Catalog of genes
- Gene expression levels
- Differential gene expression levels
- All of the above for alleles and splice variants
- Annotating the genes in a reference genome
- Variant (Genetic marker) discovery
- Post-transcriptional modifications, RNA-editing

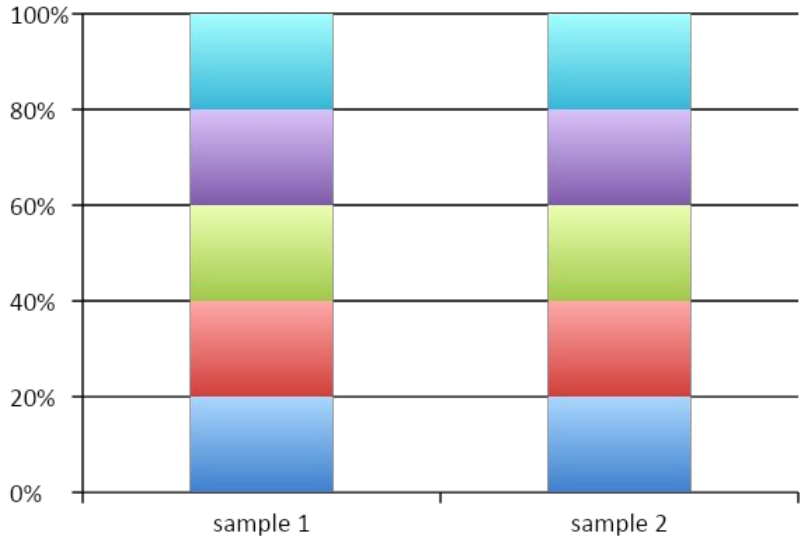
Limitations

RNASeq gives you relative abundance only

Absolute Quantities



Relative Quantities



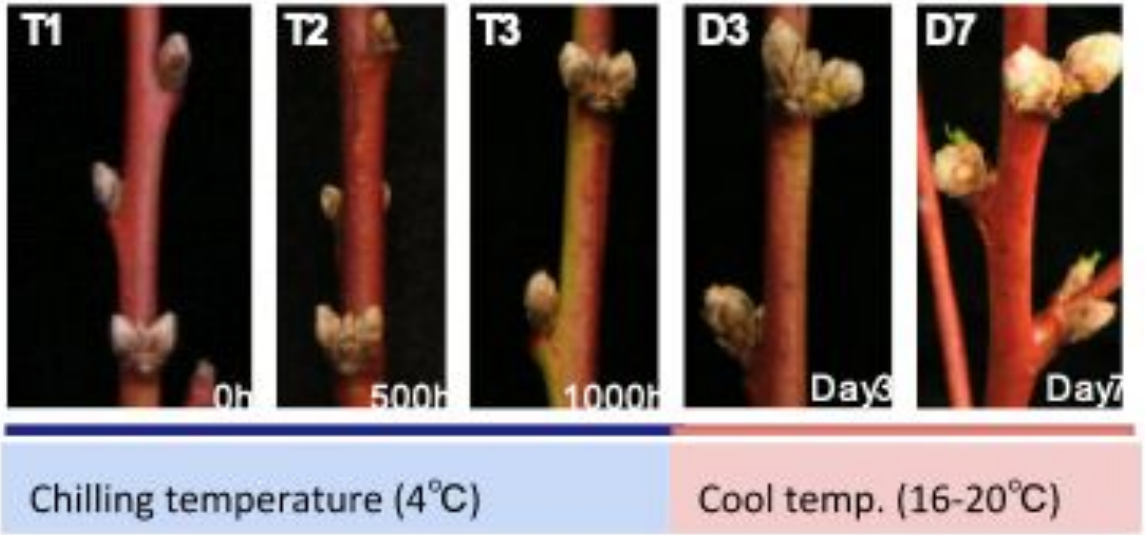
Limitations

- Reverse transcription, PCR and fragmentation steps can introduce biases
  - depletion of reads at both 5’ and 3’ ends
    - Difficult to identify the true start and end of novel transcripts
    - May underestimate expression level of short genes
  - GC bias, length bias
- PCR-free preps are available

Hansen KD, Brenner SE, Dudoit S. Biases in Illumina transcriptome sequencing caused by random hexamer priming. Nucleic Acids Res. 2010 Jul;38(12):e131.
Roberts A, Trapnell C, Donaghey J, Rinn JL, Pachter L. Improving RNA-Seq expression estimates by correcting for fragment bias. Genome Biol.2011;12(3):R22.

Data

- USDA grant “Abiotic Stress Response And Adaptive Phenology In Fruit Trees”
- Dormancy in apricots (*Prunus armeniaca*) and peaches (*Prunus persica*)
- Late blooming (high chill) variety – adapted to northern climates
- Early blooming (low chill) variety – adapted to southern climates
-



Questions before we begin?