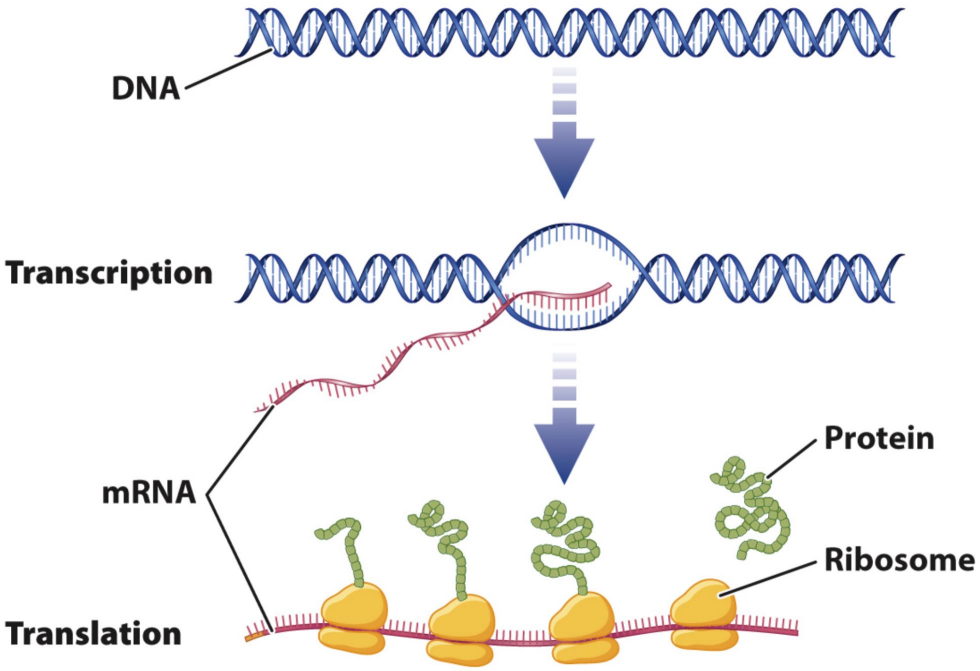
SevenBridges

# Intro to RNA-seq

# Beyond DNA

# Central dogma of molecular biology

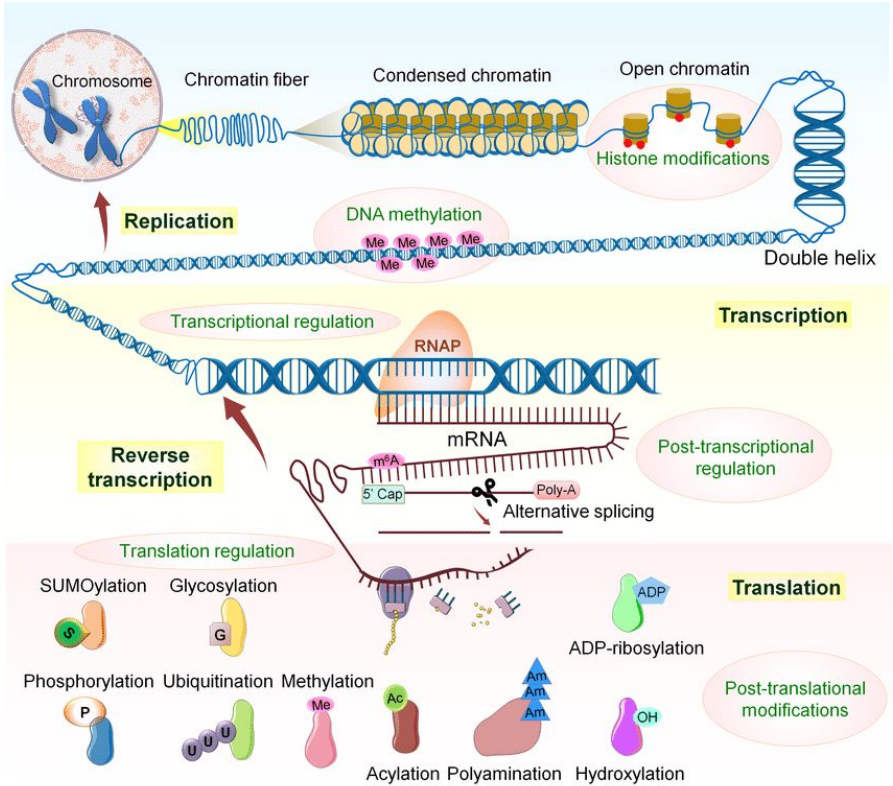



**Replication -** Before cell division DNA is replicated

**Transcription** - synthesis of an RNA molecule based on a segment of DNA

**Translation -** synthesis of a protein based on a sequence of an mRNA molecule

# Central dogma of molecular biology




**Replication -** Before cell division DNA is replicated

**Transcription** - synthesis of an RNA molecule based on a segment of DNA

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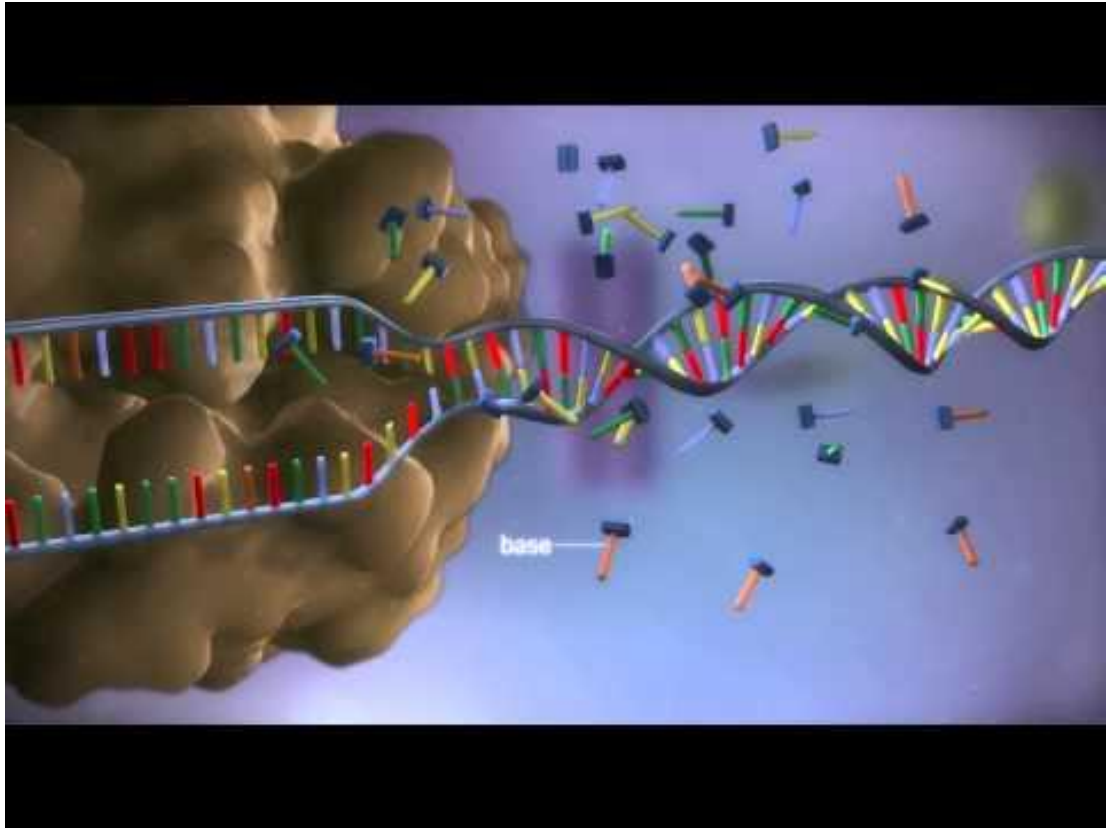
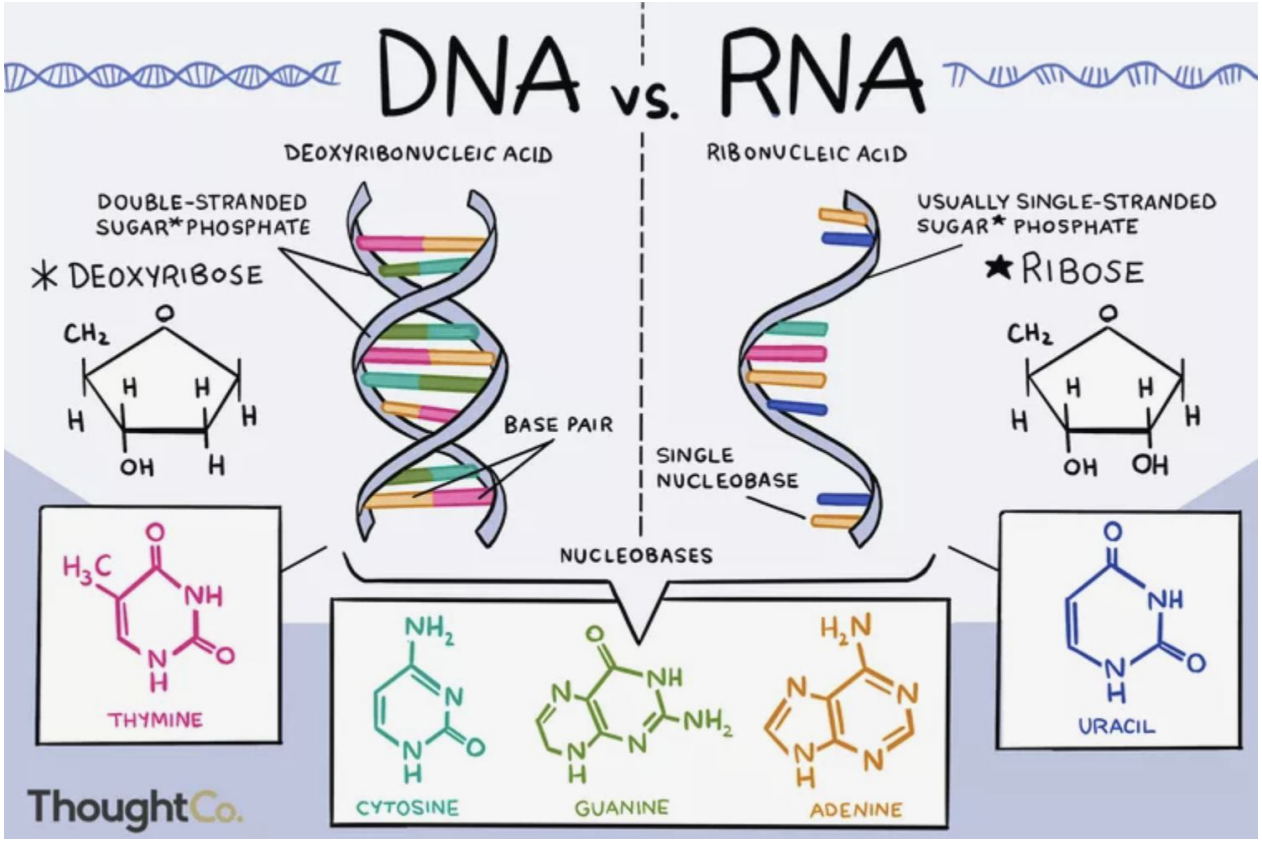
# Central dogma of molecular biology - Video



# Transcriptomics

Lots of RNAs, splicing, GTF, translation

# RNA vs DNA - difference?

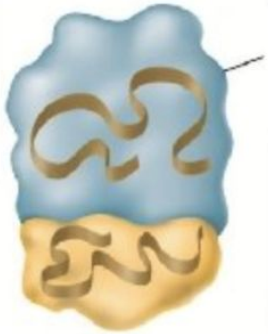



**DNA:**
- Deoxyribonucleic acid
- Double strand
- T (thymine)

**RNA:**
- Ribonucleic acid
- Single strand
- U (uracil)

# Main types of RNA

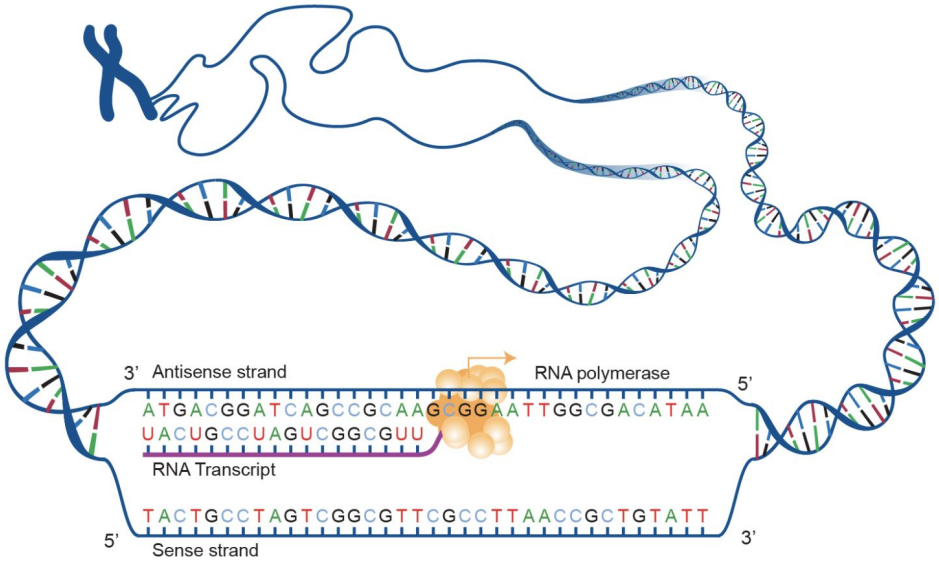
**Messenger RNA**
Carries instructions for polypeptide synthesis from nucleus to ribosomes in the cytoplasm.




**Ribosomal RNA**
Forms an important part of both subunits of the ribosome.


**Transfer RNA**
Carries amino acids to the ribosome and matches them to the coded mRNA message.

# Transcription




**Transcription** - **process of making an RNA copy of a gene sequence**. This copy, called a messenger RNA (mRNA) molecule, leaves the cell nucleus and enters the cytoplasm, where it directs the synthesis of the protein, which it encodes.

**Main transcription enzyme:** RNA polymerase

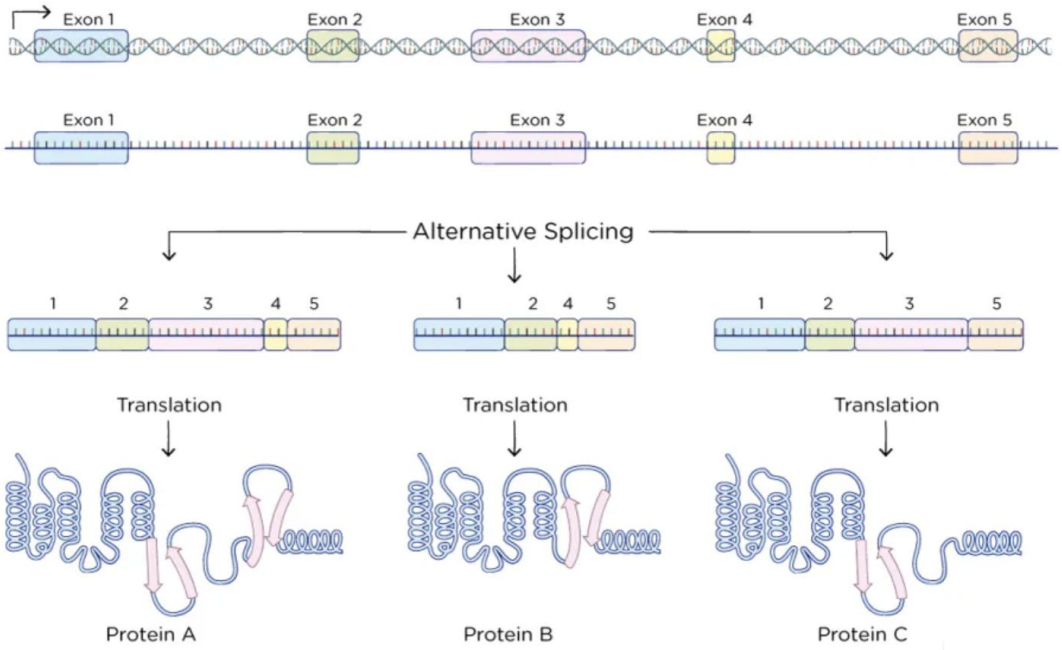
Transcription begins when RNA polymerase binds to a **promoter** sequence near the beginning of a gene (directly or through helper proteins).

RNA polymerase uses one of the DNA strands (the **template strand**) as a template to make a new, complementary RNA molecule.

Transcription ends in a process called **termination**.

Termination depends on sequences in the RNA, which signal that the transcript is finished.

# Transcription




**GENE (DNA): consists of introns and exons**

**pre-mRNA: Initial transcription product**

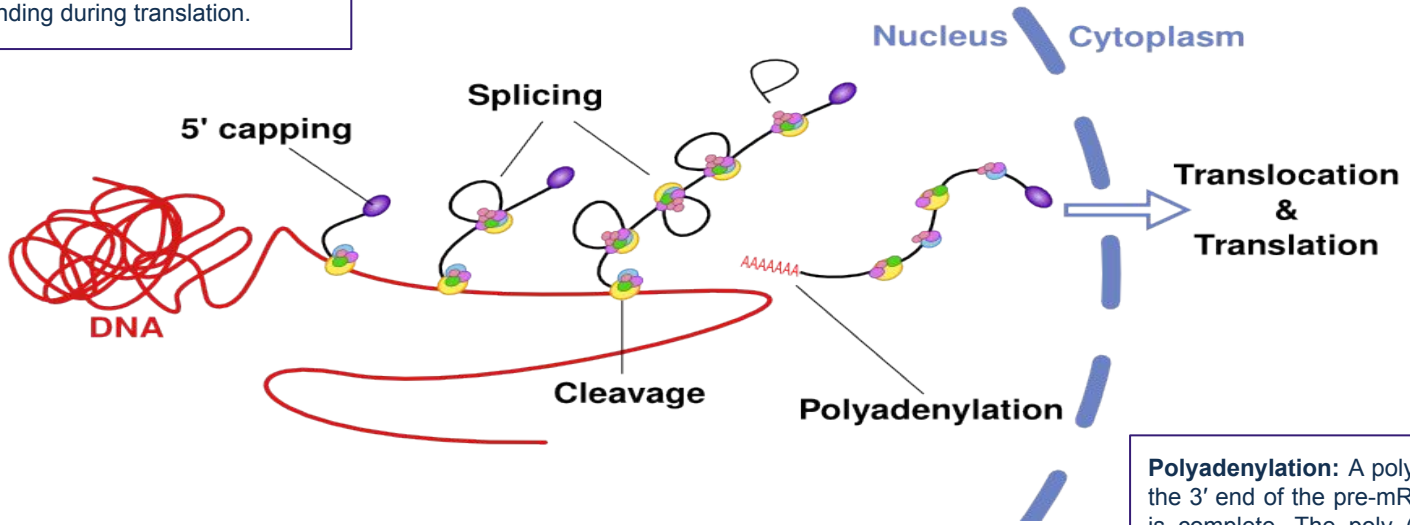
**After initial transcription maturation of RNA sequence is performed in a process of alternative splicing**

**Proteins: One gene (usually) code multiple proteins**

Alternative splicing is **the process of selecting different combinations of exons (splice sites) within a messenger RNA precursor (pre-mRNA) to produce variably spliced mRNAs**. These multiple mRNAs can encode proteins that vary in their sequence and activity, and yet arise from a single gene.

# Alternative splicing and maturation

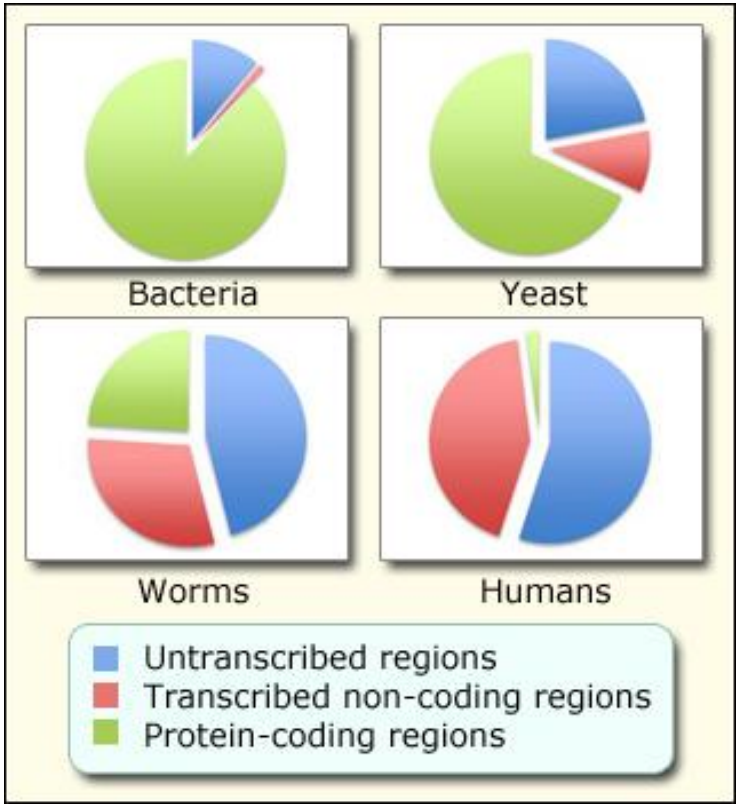
**5' capping:** A cap is added to the 5′ end of the pre-mRNA while elongation is still in progress. The 5′ cap protects the nascent mRNA from degradation and assists in ribosome binding during translation.




**Splicing:** Introns are removed from the pre-mRNA before the mRNA is exported to the cytoplasm.

**Polyadenylation:** A poly (A) tail is added to the 3′ end of the pre-mRNA once elongation is complete. The poly (A) tail protects the mRNA from degradation, aids in the export of the mature mRNA to the cytoplasm, and is involved in binding proteins involved in initiating translation.

# Transcription - how much of DNA is transcribed?



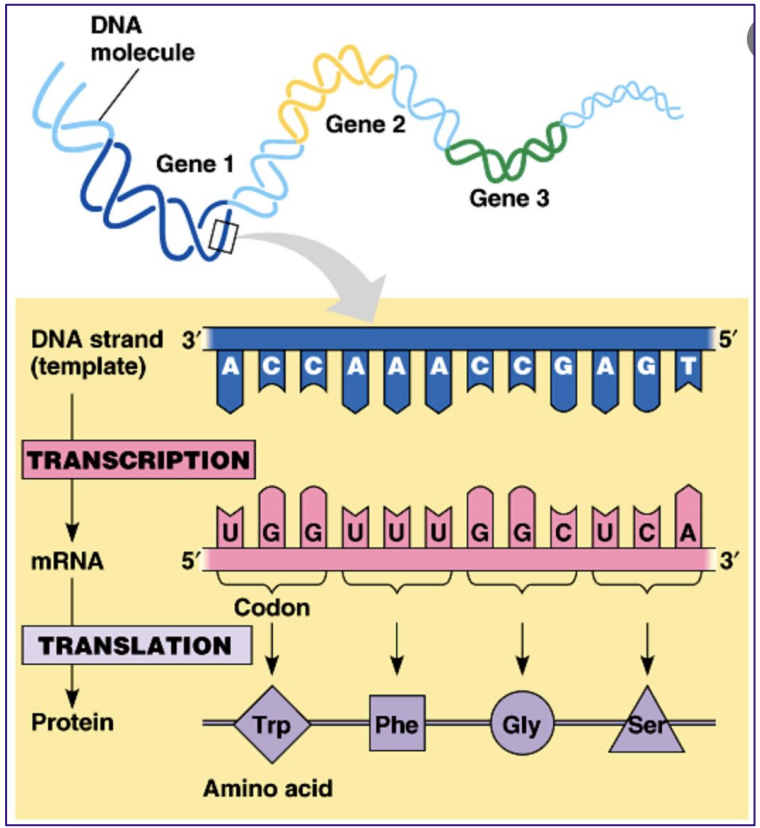

**Gene** - segment of DNA which is transcribed into RNA which then has a function in cell

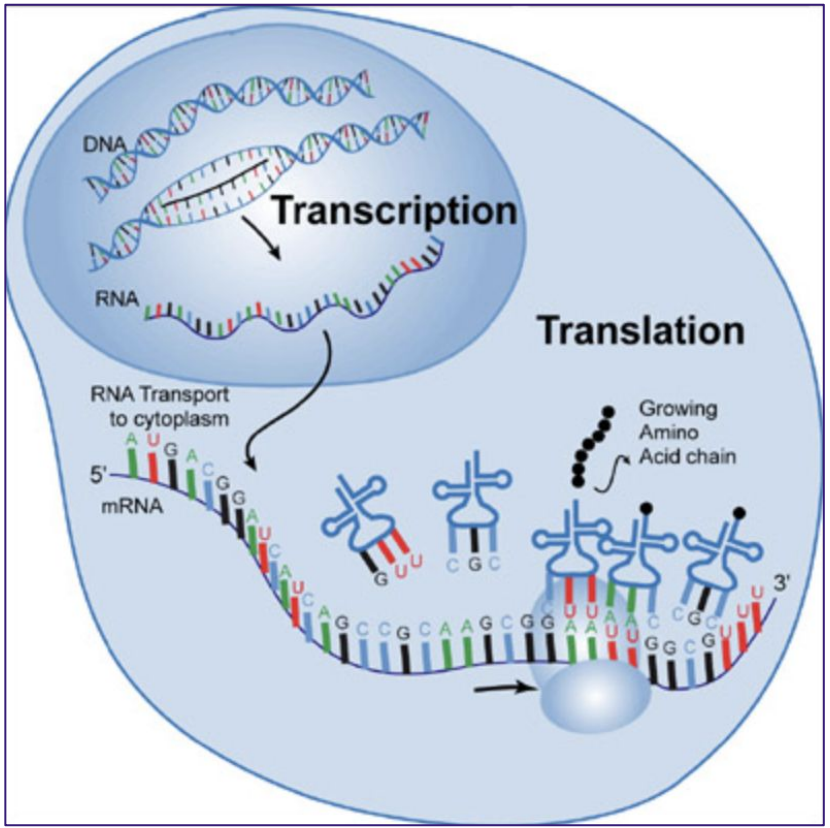
If RNA codes for protein that RNA is called **mRNA** and the region of genome from which it is transcribed is called **protein-coding gene** (green)

Genes which code for RNA with different functions other than protein coding - structural, regulatory, transport etc. - **non-coding genes** (red)

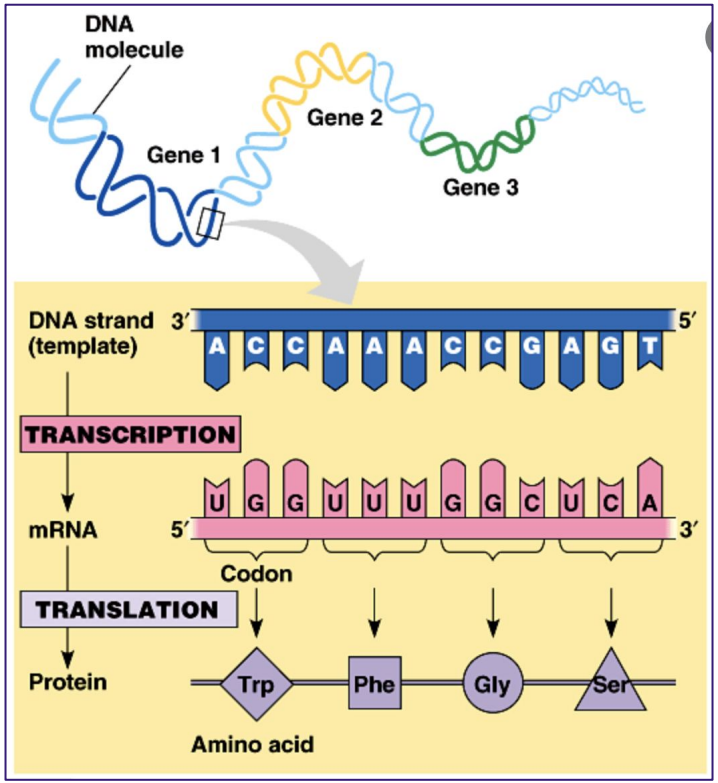
Some regions of DNA (most of it) are not transcribed at all (blue)

# mRNAs: translation to proteins



# mRNAs: translation to proteins




| First Letter | Second Letter: U | Second Letter: C | Second Letter: A | Second Letter: G | Third Letter |
|---|---|---|---|---|---|
| U | UUU, UUC: Phe; UUA, UUG: Leu | UCU, UCC, UCA, UCG: Ser | UAU, UAC: Tyr; UAA: Stop; UAG: Stop | UGU, UGC: Cys; UGA: Stop; UGG: Trp | U C A G |
| C | CUU, CUC, CUA, CUG: Leu | CCU, CCC, CCA, CCG: Pro | CAU, CAC: His; CAA, CAG: Gln | CGU, CGC, CGA, CGG: Arg | U C A G |
| A | AUU, AUC, AUA: Ile; AUG: Met (Start) | ACU, ACC, ACA, ACG: Thr | AAU, AAC: Asn; AAA, AAG: Lys | AGU, AGC: Ser; AGA, AGG: Arg | U C A G |
| G | GUU, GUC, GUA, GUG: Val | GCU, GCC, GCA, GCG: Ala | GAU, GAC: Asp; GAA, GAG: Glu | GGU, GGC, GGA, GGG: Gly | U C A G |

# RNA-seq

Library preparation

# RNA-seq library prep

**Step 1: Isolate the RNA from cells**

**Step 2: Break the RNA into small fragments**

**Step 3: Convert the RNA fragments into double stranded DNA**

We do this because RNA transcripts can be thousand of bases long, but the sequencing machine can only sequence short (200-300bp) fragments

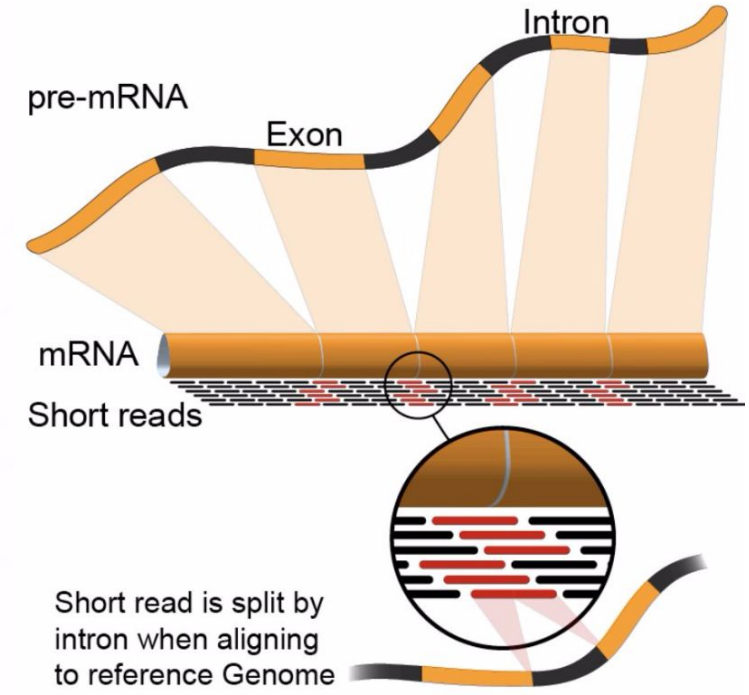
Double stranded DNA is more stable than RNA and can be easily amplified and modified. This leads us to the next step...

# RNA-seq

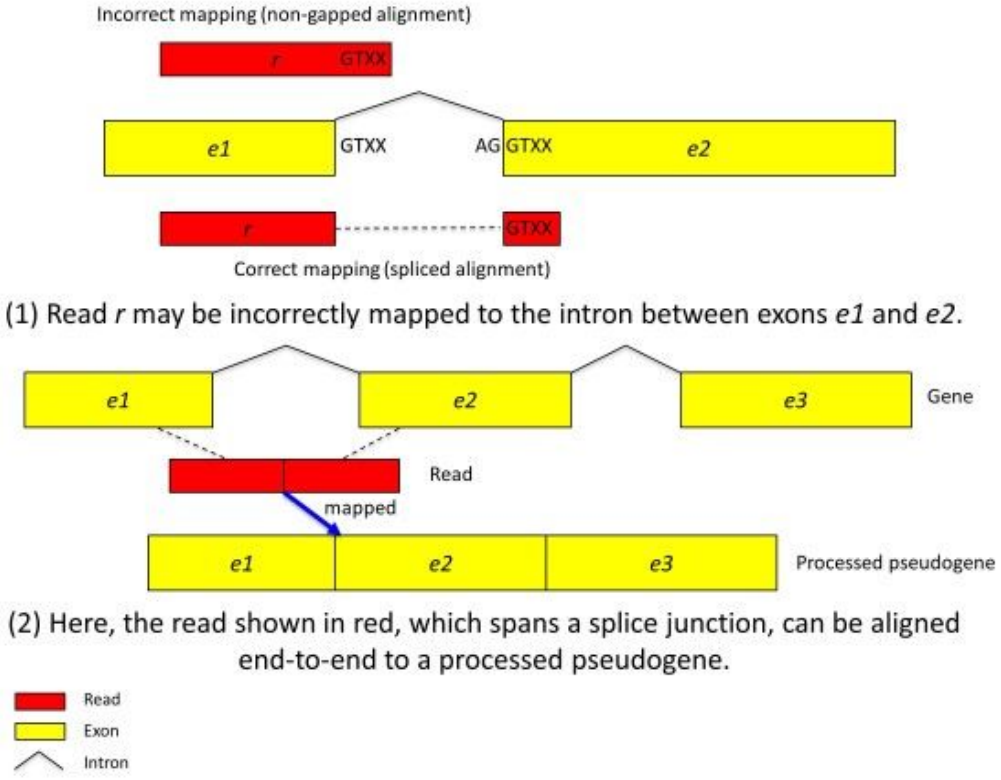
Splice-aware alignment

# Splice-aware alignment

- Average gene size ~ 10-15 kb
- Average length of mRNA ~ 2200b
- Average exon ~ 230b
- Average number of exons ~ 9.5
- For 100b reads ~ 35% of reads would span exons

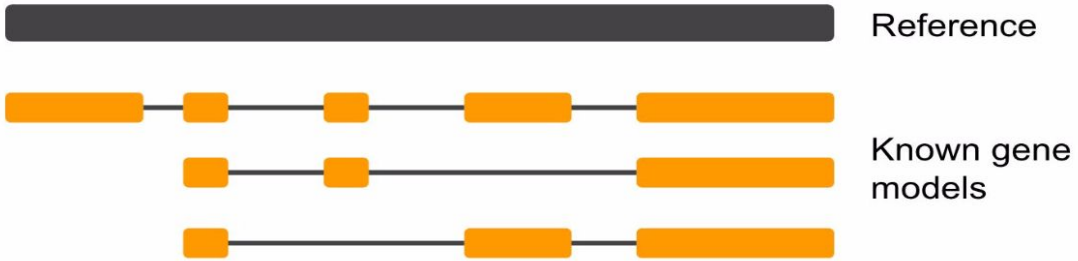


# Splice-aware alignment

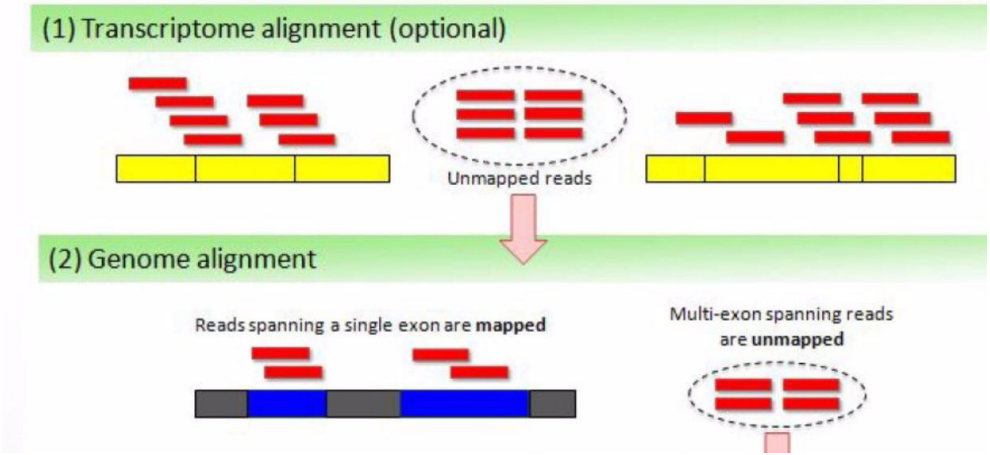


# GTF (gene transfer format)

| Col 1 | Col 2 | Col 3 | Col 4 | Col 5 | Col 6 | Col 7 | Col 8 | Col 9 |
|---|---|---|---|---|---|---|---|---|
| chr21 | HAVANA | transcript | 10862622 | 10863067 | . | + | . | gene_id "ENSG00000169.. |
| chr21 | HAVANA | exon | 10862622 | 10862667 | . | + | . | gene_id "ENSG00000169.. |
| chr21 | HAVANA | CDS | 10862622 | 10862667 | . | + | 0 | gene_id "ENSG00000169.. |
| chr21 | HAVANA | start_codon | 10862622 | 10862624 | . | + | 0 | gene_id "ENSG00000169.. |
| chr21 | HAVANA | exon | 10862751 | 10863067 | . | + | . | gene_id "ENSG00000169.. |
| chr21 | HAVANA | CDS | 10862751 | 10863064 | . | + | 2 | gene_id "ENSG00000169.. |
| chr21 | HAVANA | stop_codon | 10863065 | 10863067 | . | + | 0 | gene_id "ENSG00000169.. |
| chr21 | HAVANA | UTR | 10863065 | 10863067 | . | + | . | gene_id "ENSG00000169.. |



# Splice-aware alignment



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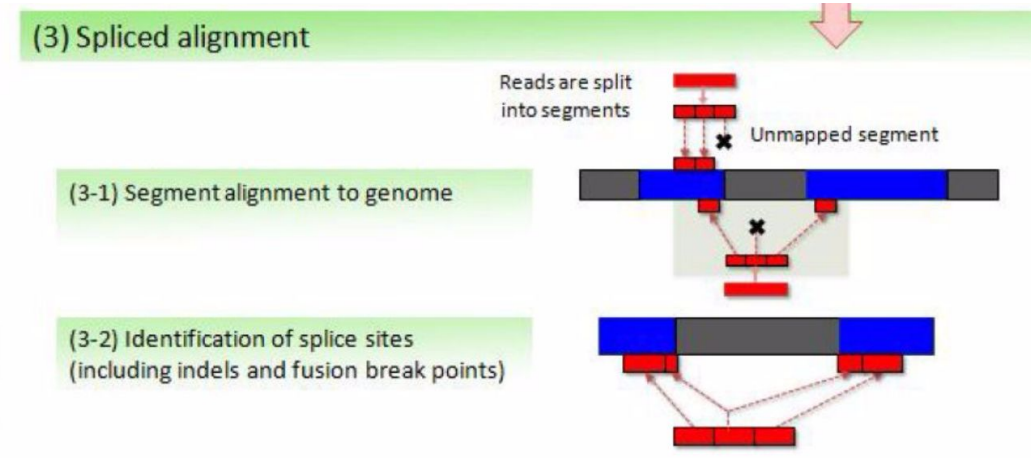


# Why do RNA-Seq?

# RNA-seq analysis

- RARELY: (splice-aware) alignment -> variant calling
- EVEN MORE RARELY: transcriptome assembly

# RNA-seq analysis

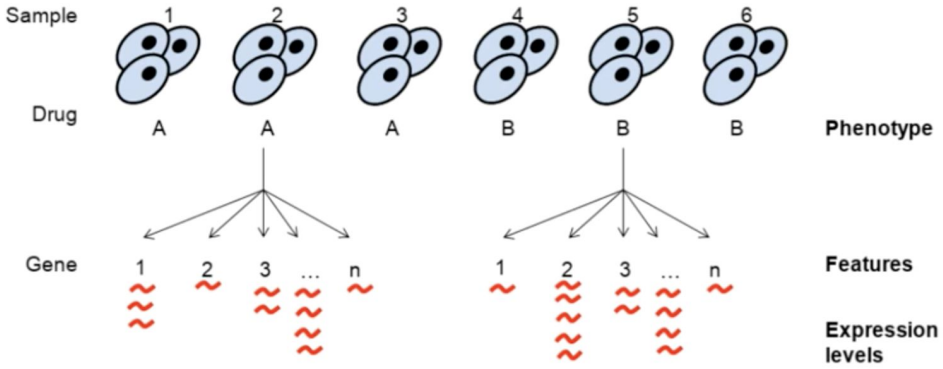
- OFTEN: **relative abundance (quantification)** of RNAs and testing for **differential expression**

**New term:**

- When gene products are created (through transcription and translation) we say that gene is **expressed**

# Why we analyze RNA

- All cells in the body have the same DNA
- However, set of RNA molecules between different cell types significantly differ



# Motivation for RNA quantification

- We (usually) want to check if there is **change in transcription (expression)** between conditions (healthy/sick, treated/untreated, different tissues, etc..)

# Transcriptomics

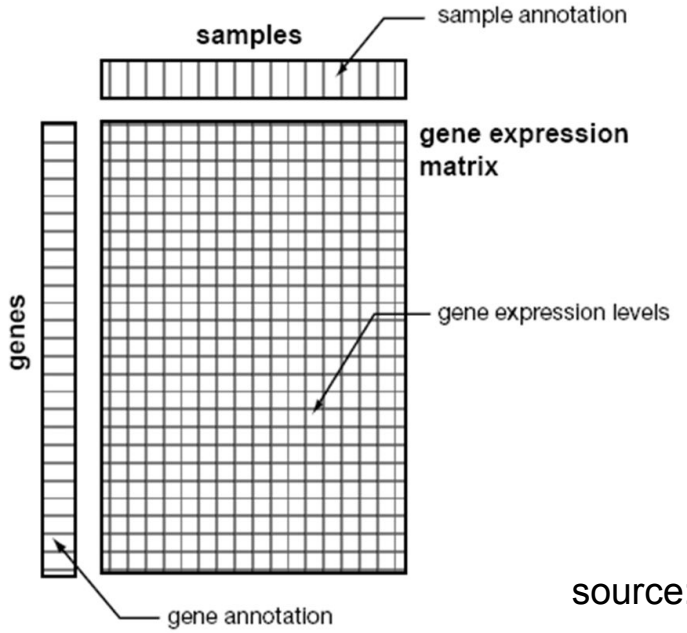
## Quantification

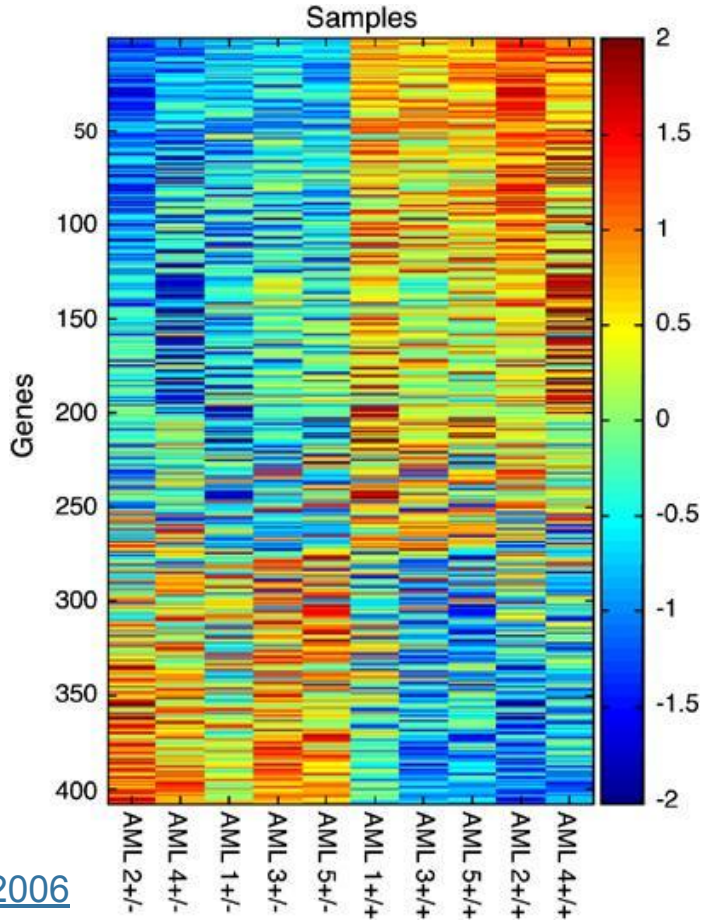
# We will talk about:

- RNA quantification
- Differential expression

# RNA quantification result

- Expression profiles




source: [Nature Leukemia 2006]

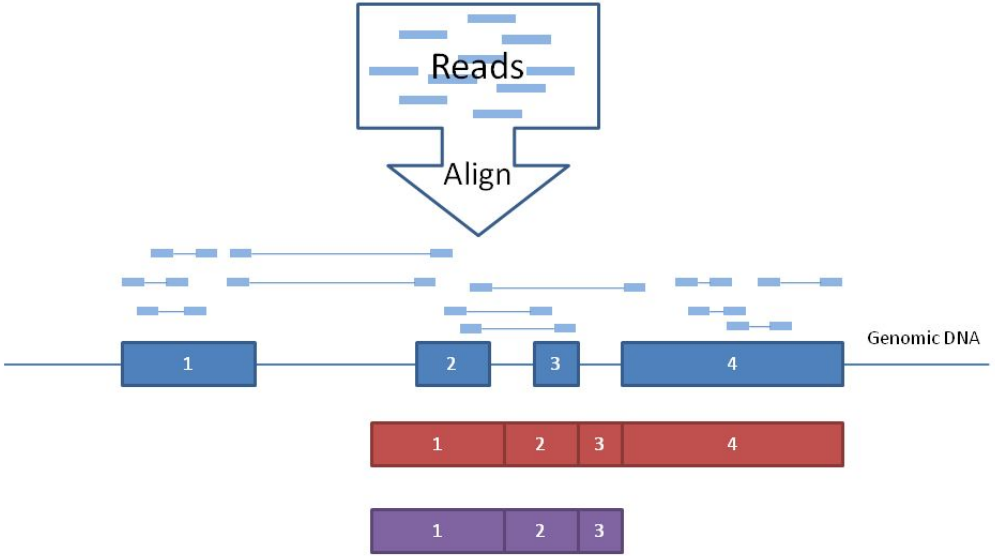
# Quantification - problems

- Quantification = Counting reads?
- We can be interested in gene expression quantification, but also in transcript quantification

# (1) RNA-seq: abundance estimation

*Problem statement:*

How to resolve alignment ambiguity?



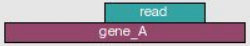
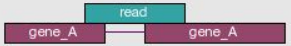
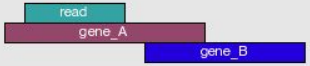
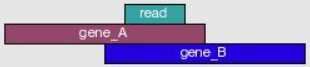

Source: http://dx.doi.org/10.13070/mm.en.3.203

# (1) RNA-seq: abundance estimation

Raw counting

vs.

**probabilistic** estimation

| | union | intersection_strict | intersection_nonempty |
|---|---|---|---|
|   | gene_A | gene_A | gene_A |
|   | gene_A | no_feature | gene_A |
|   | gene_A | no_feature | gene_A |
|   | gene_A | gene_A | gene_A |
|   | gene_A | gene_A | gene_A |
|   | ambiguous | gene_A | gene_A |
|   | ambiguous | ambiguous | ambiguous |

HTSeq counting model

# (2) RNA-seq: abundance estimation

- For transcript quantification we usually use different probabilistic methods

- E.g. Expectation Maximization algorithm (EML or EM), Maximum Likelihood estimation

# (2) RNA-seq: abundance estimation

## Maximum likelihood example

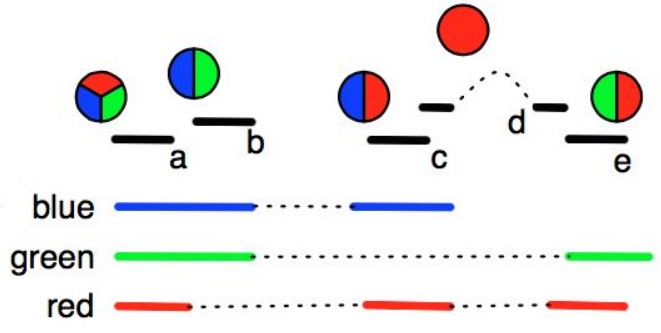
$i = 5$ single-end, equal-length reads (a,b,c,d,e)
$k = 3$ transcripts (blue, green, red)
$\rho = (\rho_{blue}, \rho_{green}, \rho_{red})$ relative abundances of transcripts
$\sum_k \rho_k = 1$, multinomial distribution

$P_i = \sum_k y_{i,k} \cdot \rho_k$, probability of detecting $i$-th read

where $y_{i,k} = 1$ if $i$-th read aligns to $k$-th transcript, otherwise 0

$$L(\rho) = \prod_i \sum_k y_{i,k} \cdot \rho_k$$

Analytical solution $\rho = (0.18, 0.18, 0.64)$




Adapted from: Lior Pachter 2011, arxiv: 1104.3889v2

# (2) RNA-seq: abundance estimation

## EM example

$(\rho_{blue}, \rho_{green}, \rho_{red}) = (\frac{1}{3}, \frac{1}{3}, \frac{1}{3})$, uniform prior

**E1 step:** Proportional assignment

$p_a = (1/3, 1/3, 1/3),\ p_b = (1/2, 1/2, 0),$

$p_c = (1/2, 0, 1/2),\ p_d = (0, 0, 1),\ p_e = (0, 1/2, 1/2)$

**M1 step:** recalculate abundances

$\rho_{blue} = (1/3 + 1/2 + 1/2 + 0 + 0)/5 = 0.27$

**E2 step:** prior $= (0.27, 0.27, 0.46)$

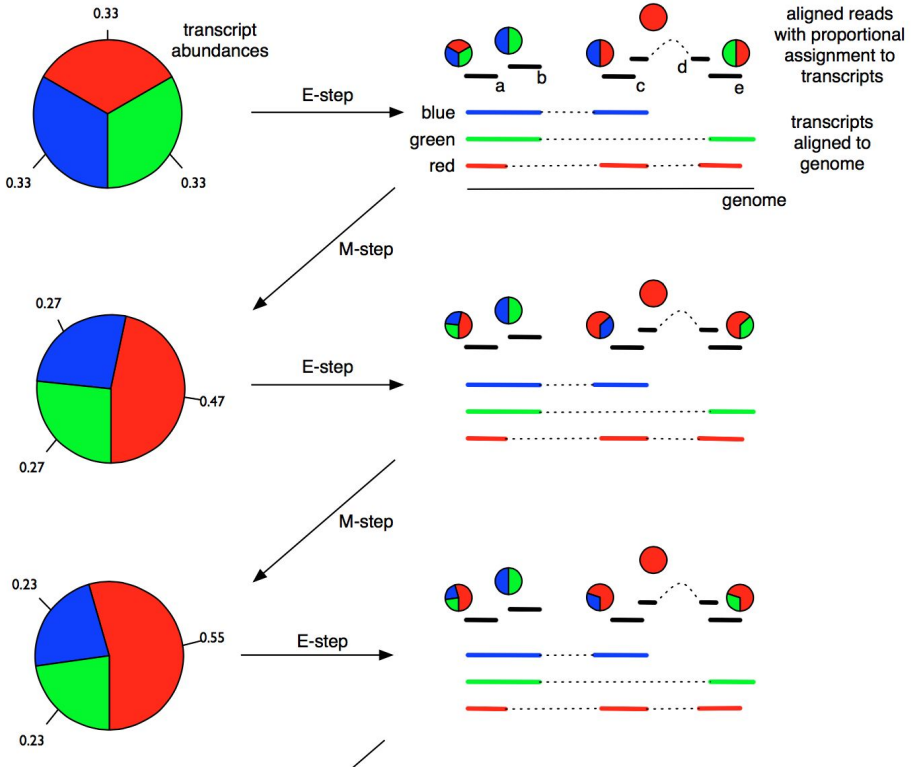
$p_a = (0.27, 0.27, 0.46),\ p_b = (1/2, 1/2, 0),$

$p_c = (\frac{0.27}{0.46 + 0.27}, 0, \frac{0.46}{0.46 + 0.27}),\ p_d = (0, 0, 1), ...$

**M2 step:**

$\rho_{blue} = (0.27 + 1/2 + 0.37 + 0 + 0)/5 = 0.23$

Iterative convergance $\rho_{blue} = 0.33, 0.27, 0.23, ..., 0.18$



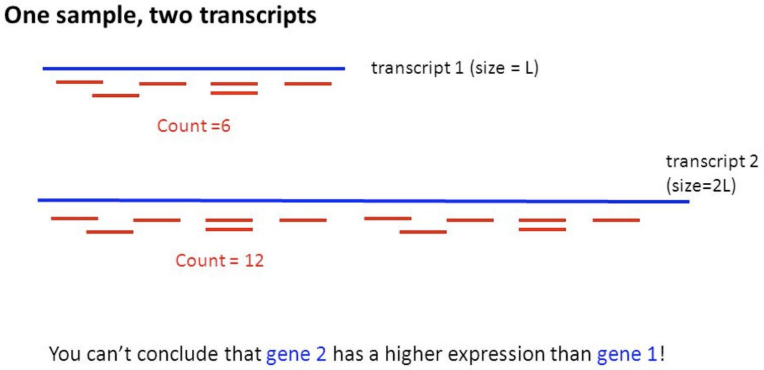
# RNA-seq: data normalization

*Problem statement:*

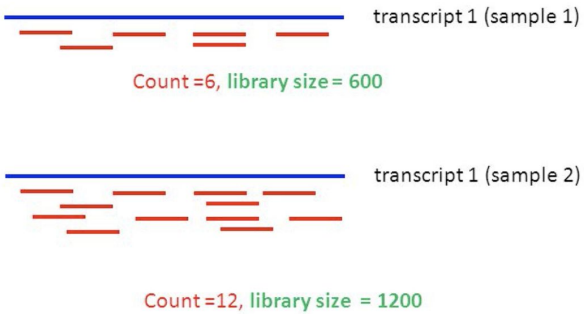
Can we compare expression of genes (within and between samples)

if we observe reads from sampled transcripts?

# RNA-seq: data normalization




You can't conclude that gene 2 has a higher expression than gene 1!



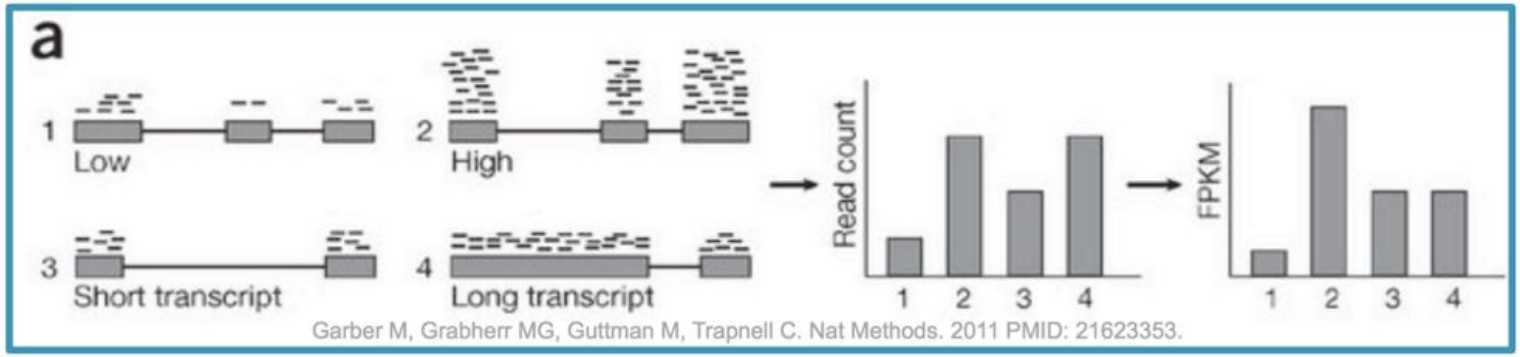

You can't conclude that gene 1 has a higher expression in sample 2 compared to sample 1!

- We need to account for gene length and library size

# RNA-seq: data normalization

Let $X_i$ be number of reads aligned to $i$th transcript

$$\sum_i X_i \neq \text{expression of a gene}$$




Garber M, Grabherr MG, Guttman M, Trapnell C. Nat Methods. 2011 PMID: 21623353.

# (2) RNA-seq: data normalization

Relative units (adjust for transcript length and sequencing depth):

- Transcripts per million (TPM)
- Fragments per kilobase of exon per million reads (FPKM)

$$FPKM_i = \frac{X_i}{\frac{N}{10^6} \cdot \widetilde{l_i}}$$

$$TPM_i = \frac{\frac{X_i}{\widetilde{l_i}} \cdot 10^6}{\sum_i \frac{X_i}{\widetilde{l_i}}}$$

$X_i$ - number of reads aligned to transcript ‘i’

$N$ - total number of reads

$l_i$ - read length

$\widetilde{l_i} = l_i/10^3$ - read length in kilobases

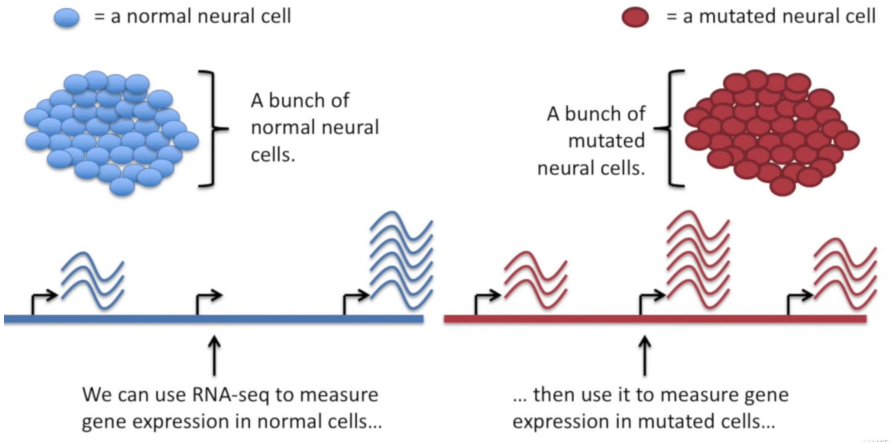
# Transcriptomics

Differential expression

# Differential expression:

*Problem statement:*

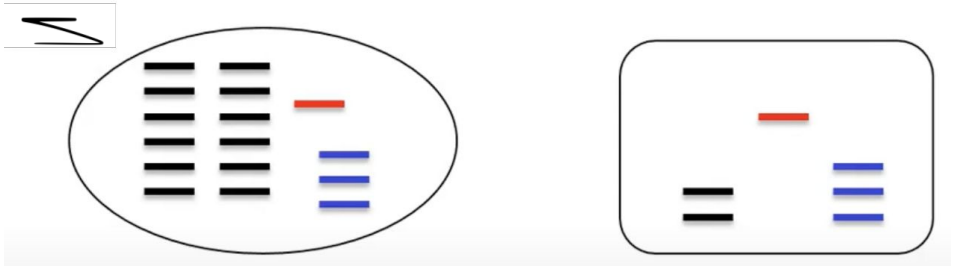
From thousands of genes, how do we know which ones are really differentially expressed and not observed changed by coincidence?

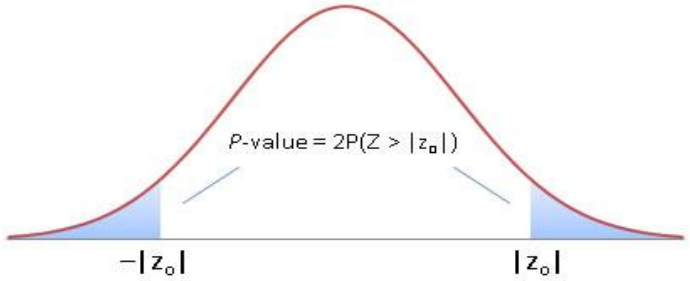


# (3) RNA-seq: multiple testing

## Measure of statistical significance

- **Null hypothesis**: there is no significant difference between specified populations, any observed difference being due to sampling or experimental error.
- The **p-value** is defined as the probability of obtaining a result equal to or "more extreme" than what was actually observed, when the null hypothesis is true.
- The **alternative hypothesis** is considered true if the statistic observed would be an unlikely realization of the null hypothesis according to the p-value.





# (3) RNA-seq: multiple testing

- In genomic studies you don't usually fit just one regression model or calculate just one p-value.  You calculate many p-values.
- *human_hg19_genes_2015.gtf* has about 26,000 genes and 54,000 transcripts.
- Suppose 1200 out of 20,000 genes are found significant at 0.05 level.
    - No correction: you should expect 0.05 * 20,000 = 1000 false positives
    - Solution: Multiple testing correction

# (3) RNA-seq: multiple testing

Multiple testing correction procedures:

- Bonferroni correction
  - p_value * total_number_of_tests_performed

For more info see also:

- BH (Benjamini-Hochberg) procedure
- BY (Benjamini–Yekutieli) procedure