Intro to High-Throughput DNA Sequencing

Analysis of Next-Generation Sequencing Data

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Slides at https://bit.ly/2T3sjRg[1]

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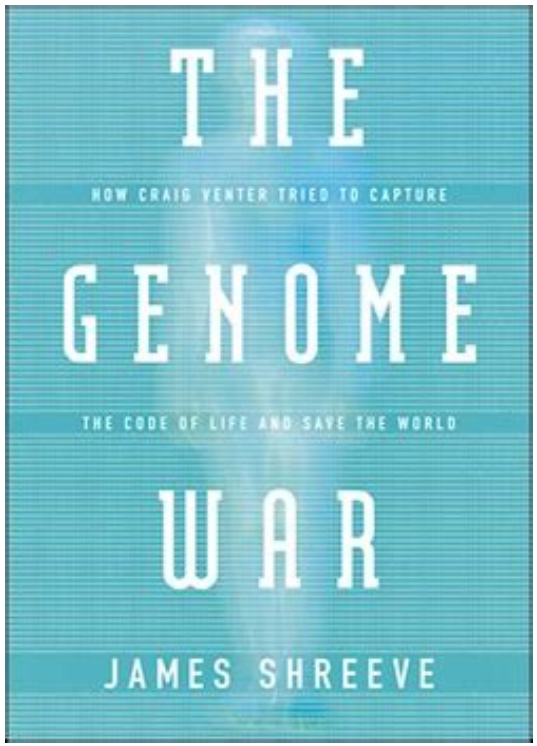
[1] https://physiology.med.cornell.edu/faculty/skrabanek/lab/angsd/schedule_2020/

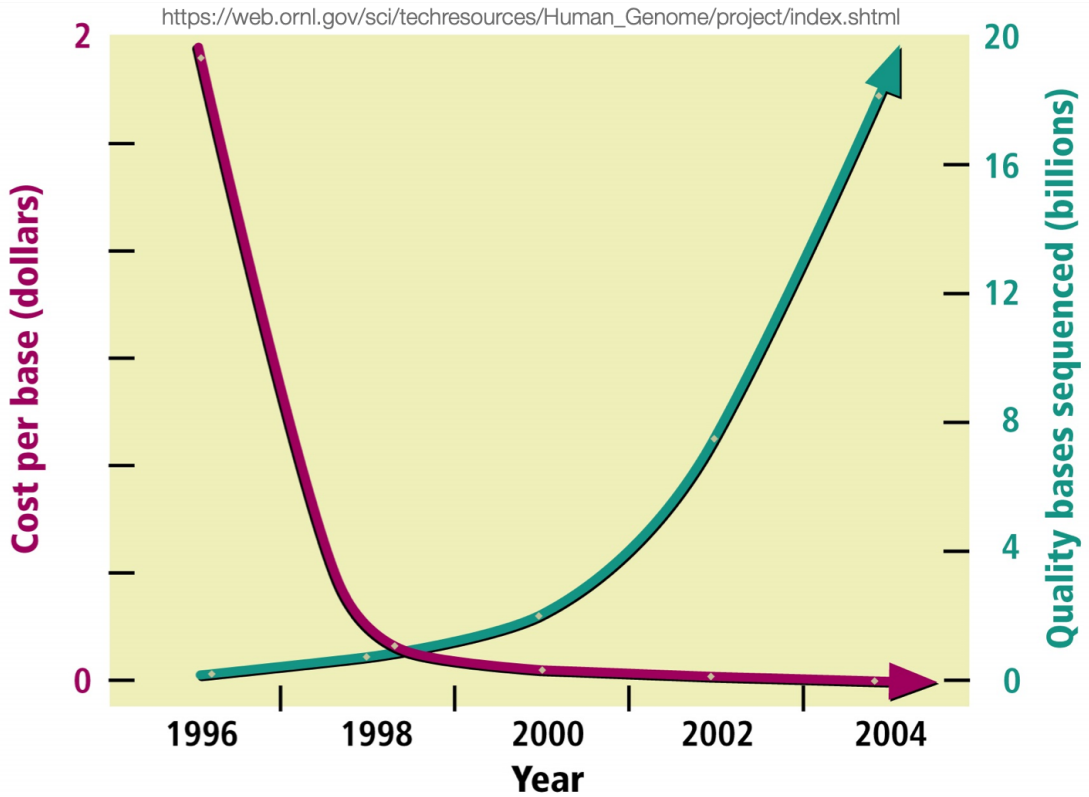
1. Why does sequencing data need bioinformatics?
2. What do we sequence?
3. How do we sequence?
4. Why do we sequence?
5. Experimental design
6. References

Why does sequencing data need bioinformatics?

Evolution of sequencing data

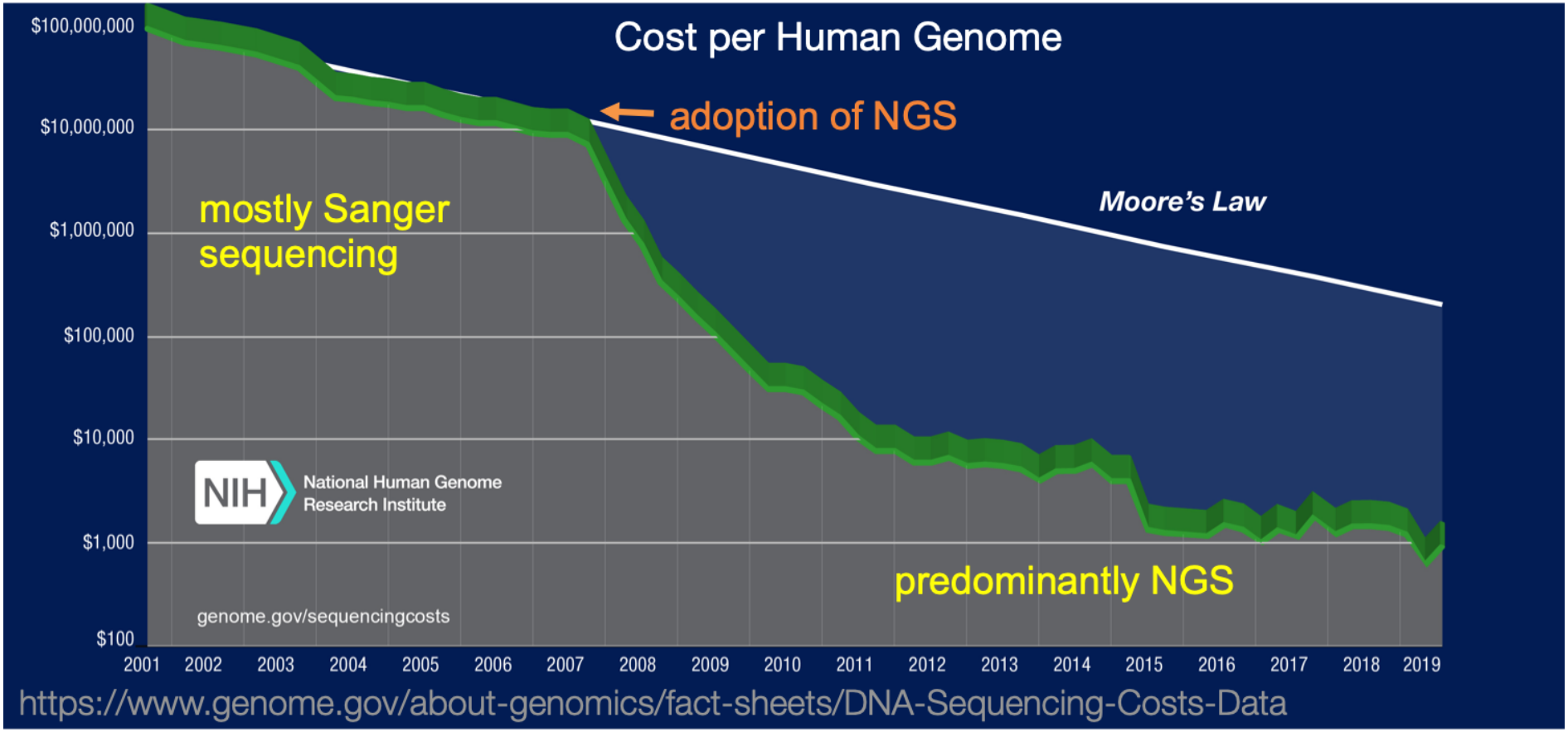
The Human Genome Project (1990-2003) ushered in the era of "next"-generation sequencing.





Evolution of sequencing data: NGS

NGS = "next generation sequencing"



ANGSD relies on bioinformatics

Next-generation sequencing	
High-throughput	millions of nucleotides can be sequenced at once
Relatively cheap*	experiments involving NGS have become abundant

relatively large data files are being generated on a regular basis

Bioinformatics

Processing

formatting, data wrangling

Alignment

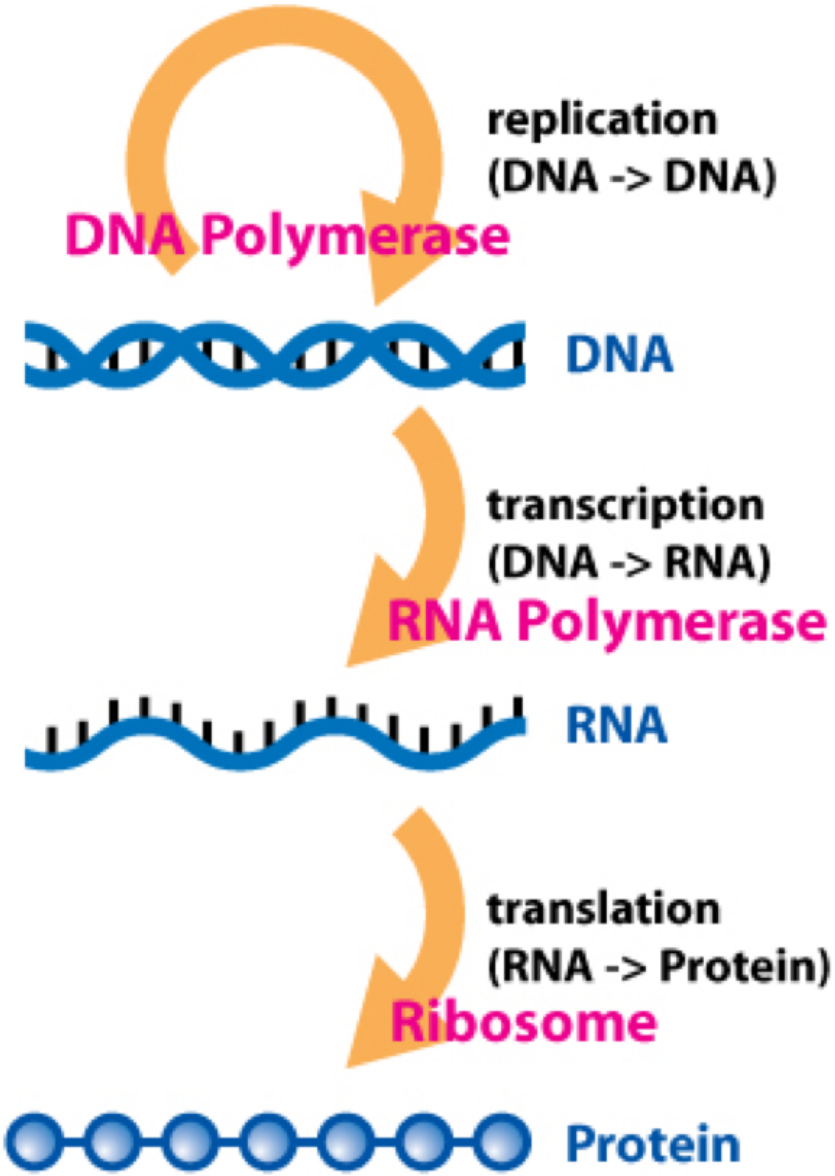
Statistical analyses

Interpretation

* The cost of analysis has remained high and is difficult to estimate!

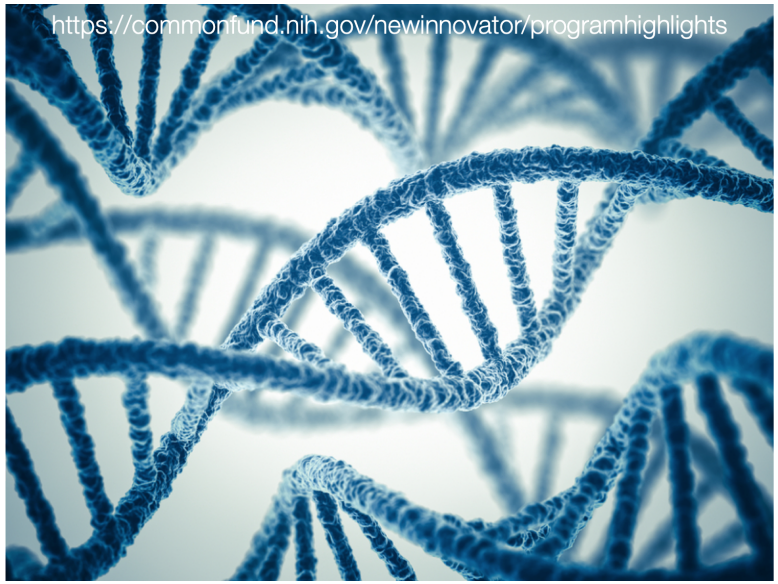
What do we sequence?

An essential macromolecule of life: DNA

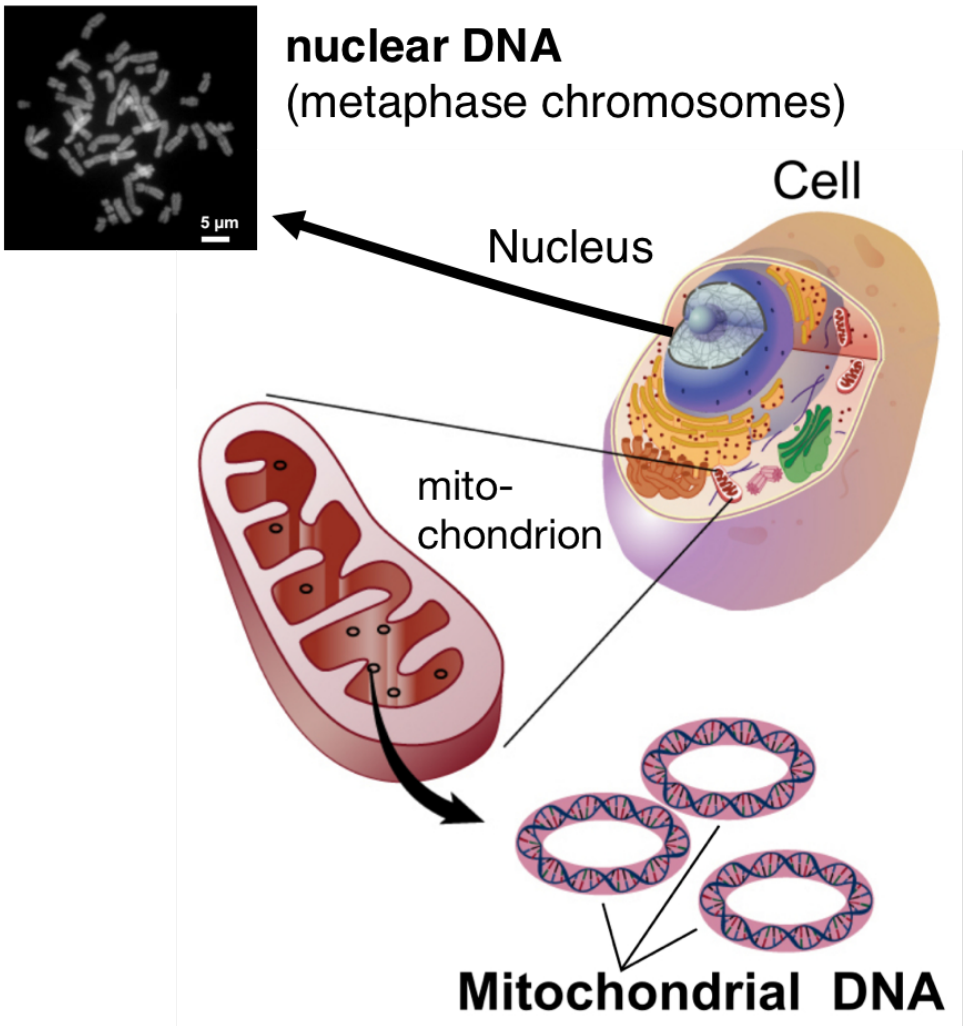


https://en.wikipedia.org/wiki/Central_dogma_of_molecular_biology

- the "hard drive" of all living organisms
- determines the traits of an organism
- contains the blueprint information for proteins

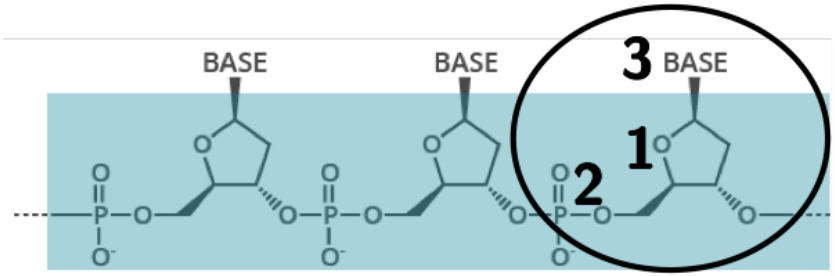


Two general types of eukaryotic DNA

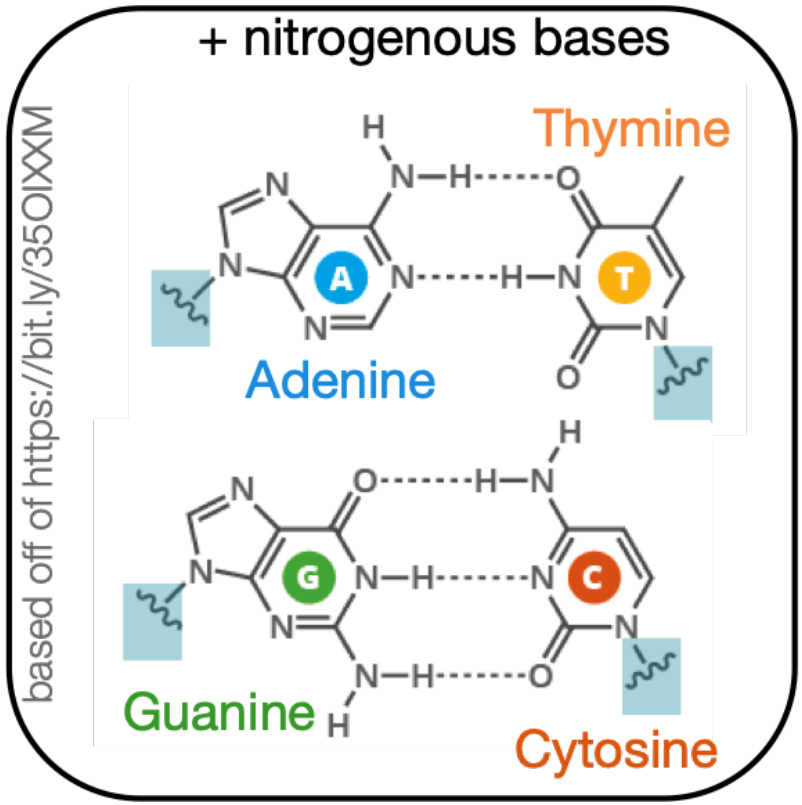


- GENOMIC (NUCLEAR) DNA
  - contained and replicated within the **nucleus**
  - "linear"
  - multiple chromosomes, which are inherited from both parents
- MITOCHONDRIAL DNA
  - contained and replicated within **mitochondria**
  - circular
  - represents 1 chromosome
  - inherited (only!) from the mother

DNA: Deoxyribonucleic acid



sugar-phosphate backbone



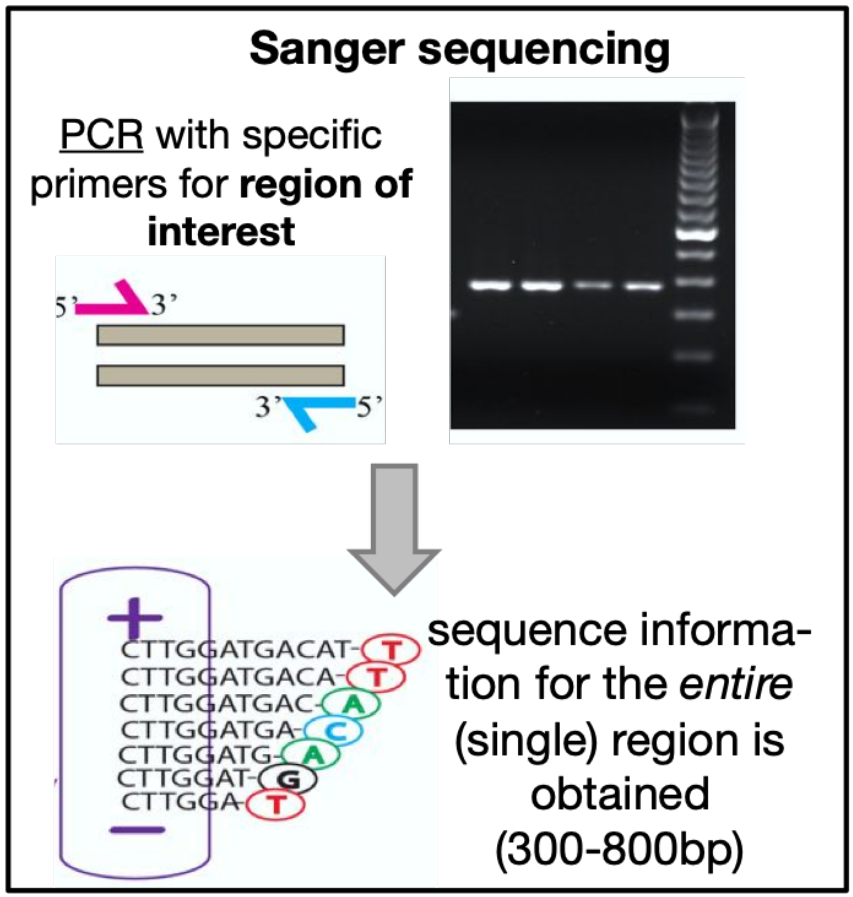
- each **nucleotide**:
  1. **sugar**: 2'-deoxyribose (5 carbon atoms = pentose)
  2. **phosphate**: 1-3 linked phosphate units attached to the 5'-carbon of the sugar
  3. **nitrogeneous base**: either a single-ring pyrimidine (cytosine, thymine) or a double-ring purine (adenine, guanine)

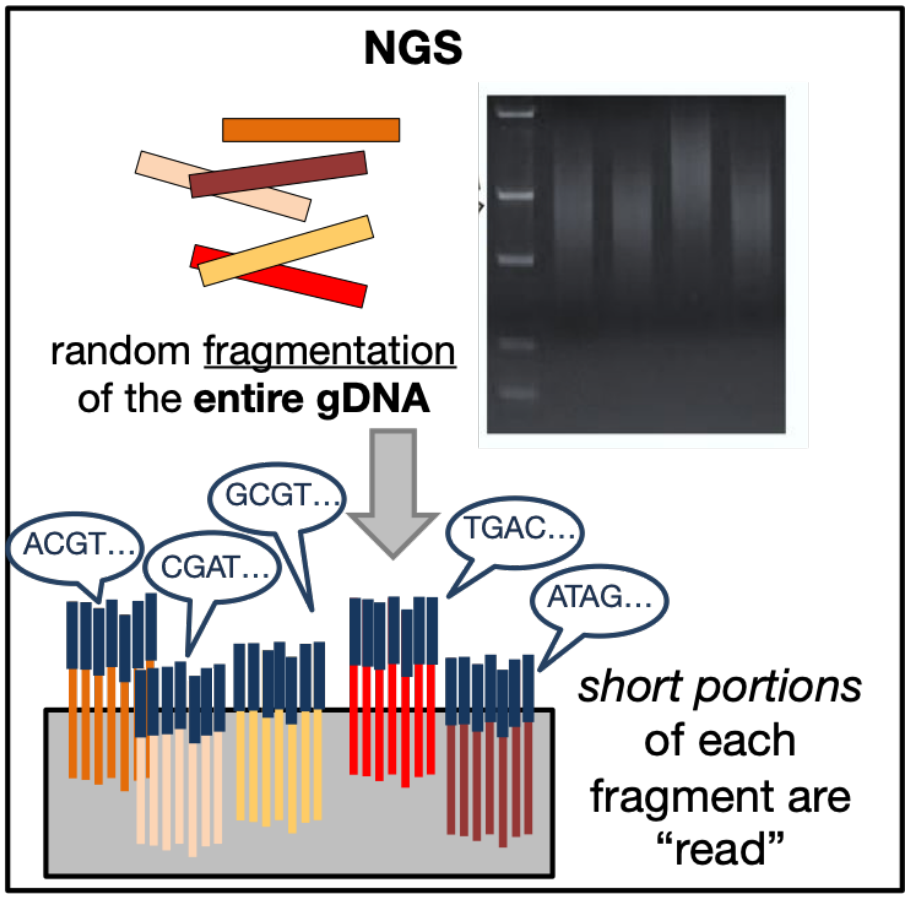
SEQUENCING = IDENTIFYING THE ORDER OF THE BASES

How do we sequence?

Next-generation sequencing

- refers to **highly parallelized sequencing** of millions of DNA fragments at the same time (in contrast to the traditional one-region-at-a-time approach)

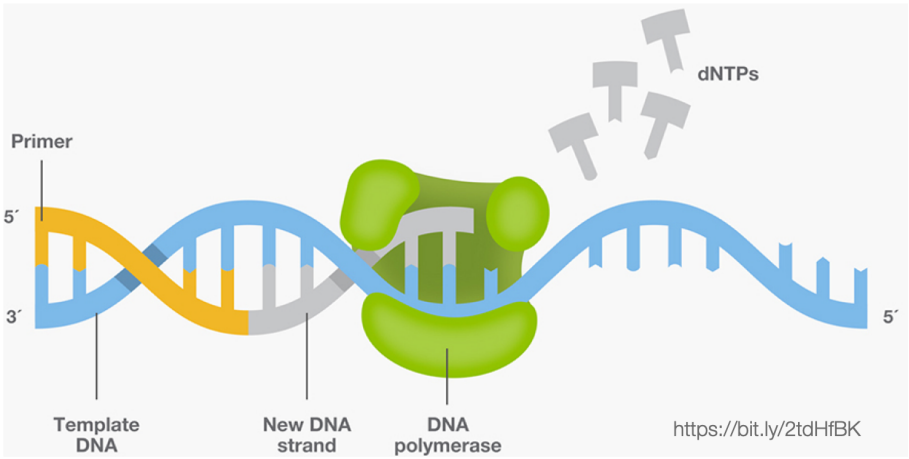


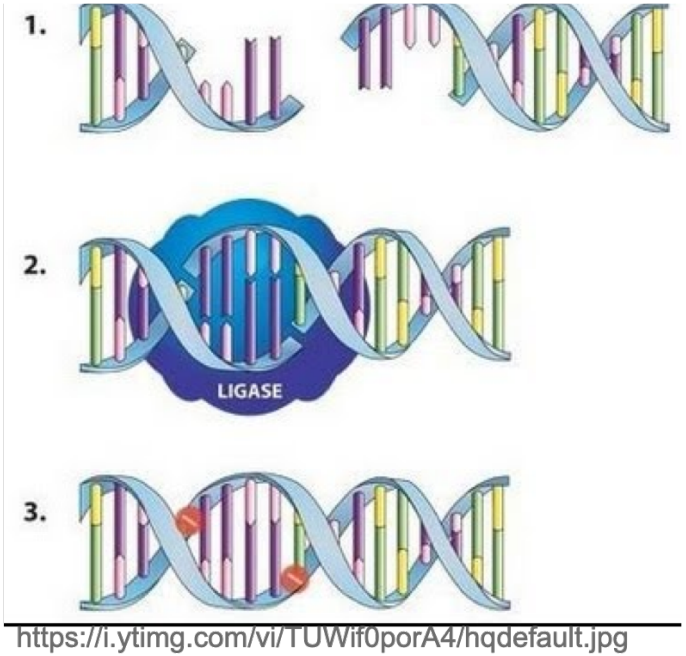


Decoding the DNA

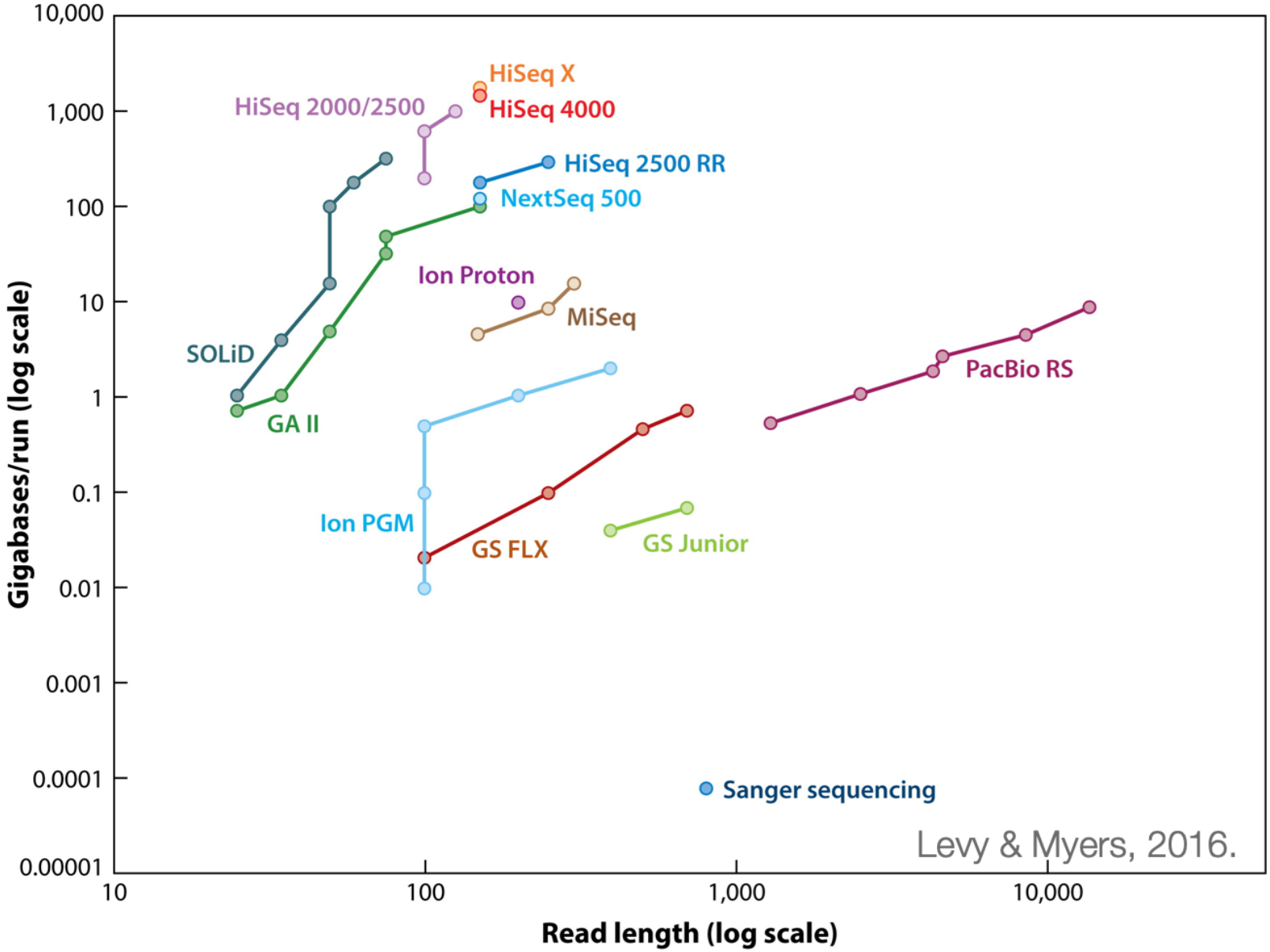
DNA sequencing mostly relies on **enzymes** that are DNA "readers":

- **DNA Polymerase**: synthesizes a new strand of DNA
  - **sequencing by synthesis** platforms: Solexa/Illumina, Ion Torrent
- **DNA Ligase**: joins the "sticky" ends of two strands of DNA together
  - **sequencing by ligation** NGS platforms: SOLiD, Complete Genomics





Next-generation sequencing platforms



Next-generation sequencing platforms

Unifying characteristics of the different NGS platforms:

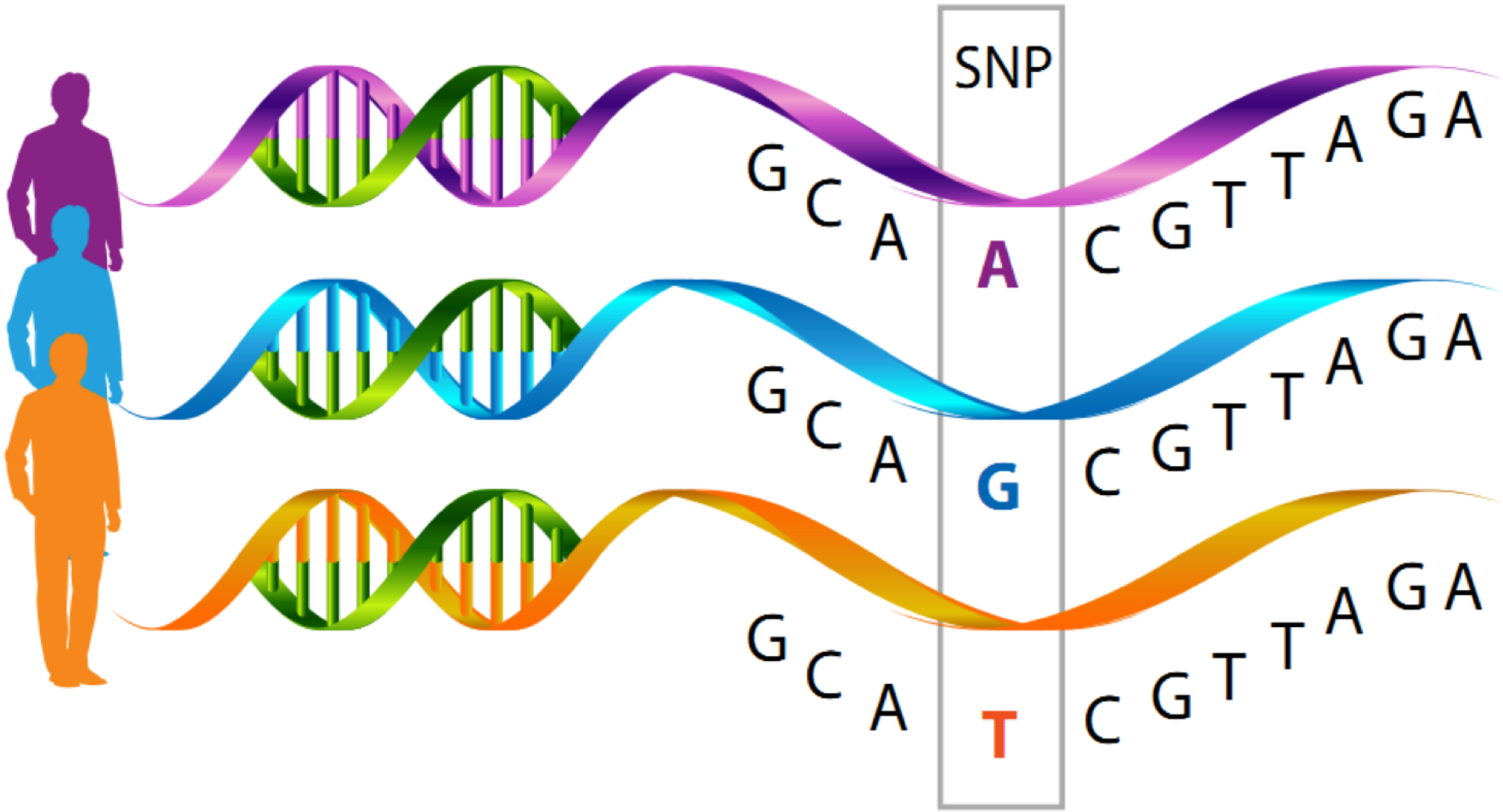
- **short fragments (250-1000 bp)** are assessed via **short reads (50-250 bp)**
- require **clonal amplification** of every single DNA fragment
- markedly **higher error rates** than Sanger sequencing of the 1980s-1990s (0.1–15%)

See Goodwin et al. [2016] for detailed descriptions of NGS platforms.

Why do we sequence?

Why do we sequence?

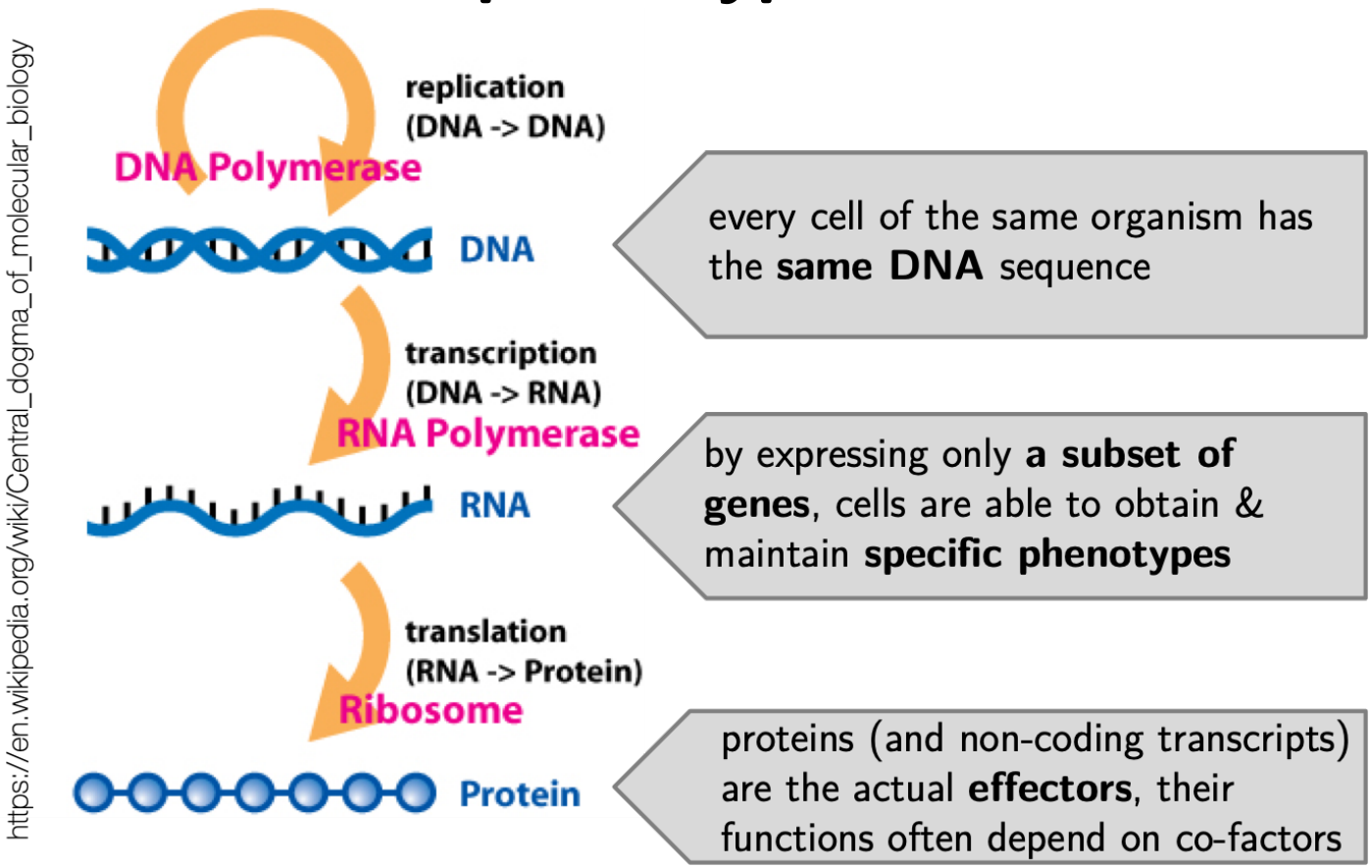
. . . to identify individuals

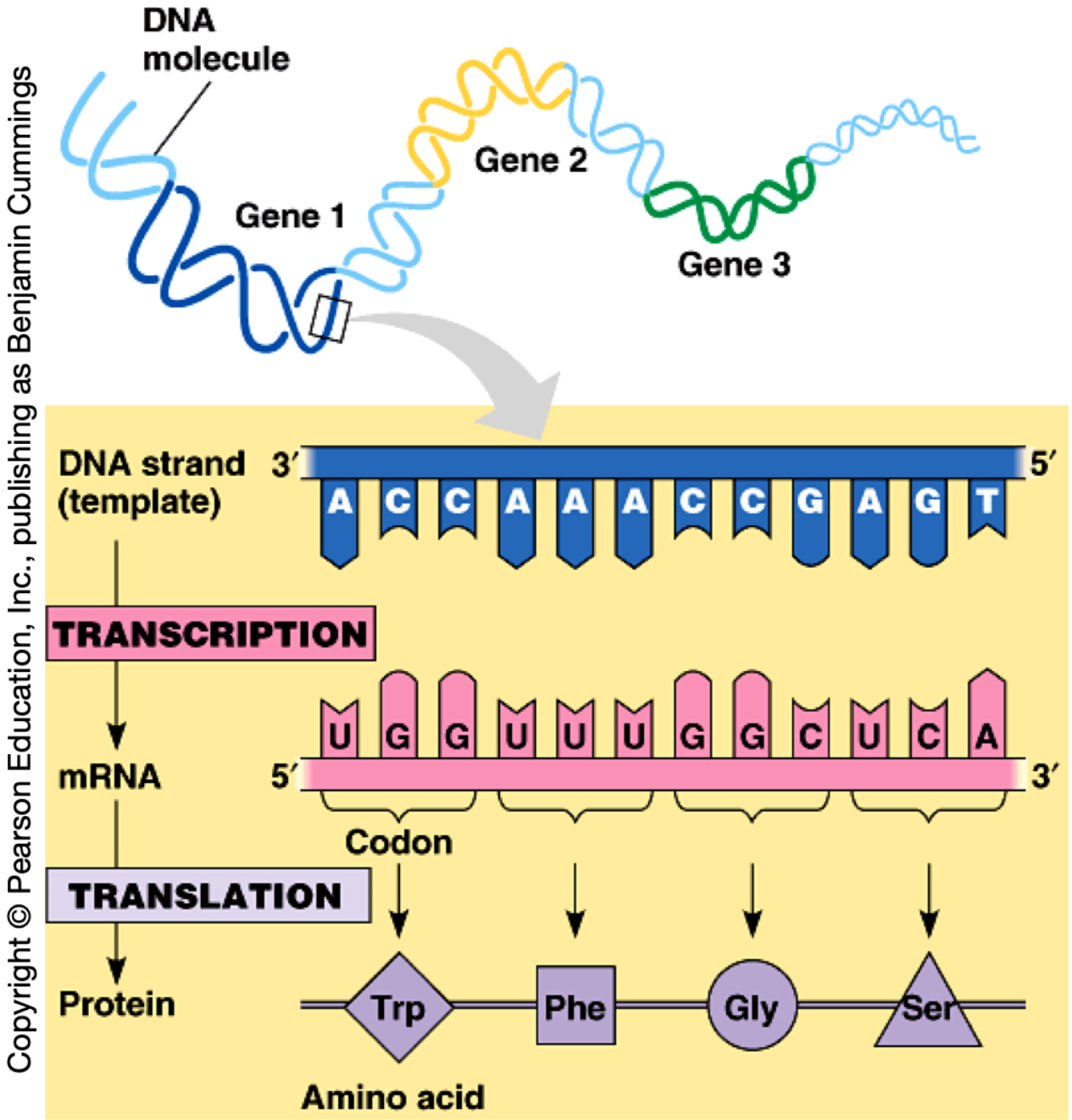


https://neuroendoimmune.wordpress.com/2014/03/27/dna-rna-snp-alphabet-soup-or-an-introduction-to-genetics/

Why do we sequence?

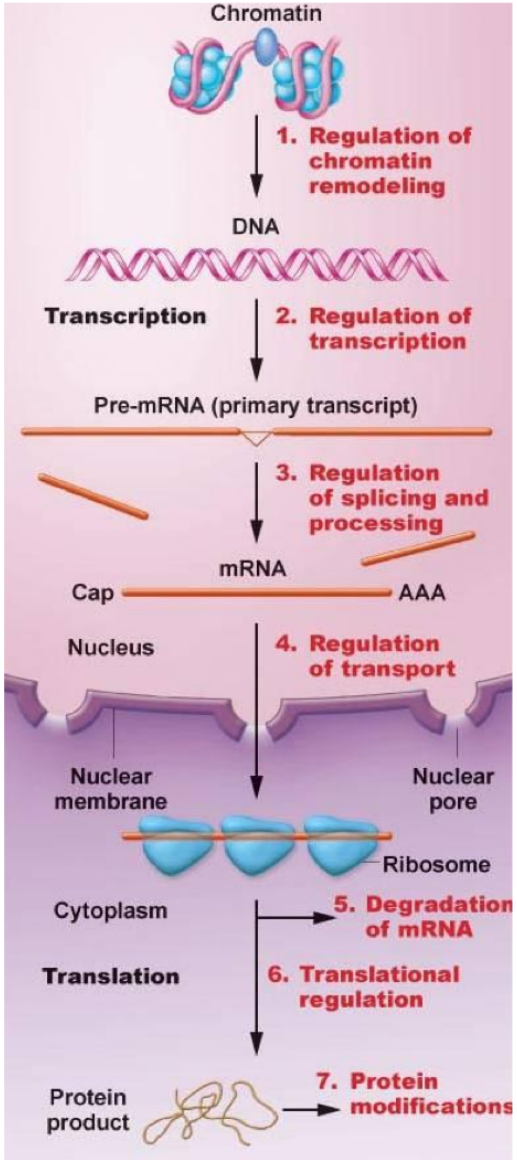
. . . to understand the molecular basis of different cellular **phenotypes**



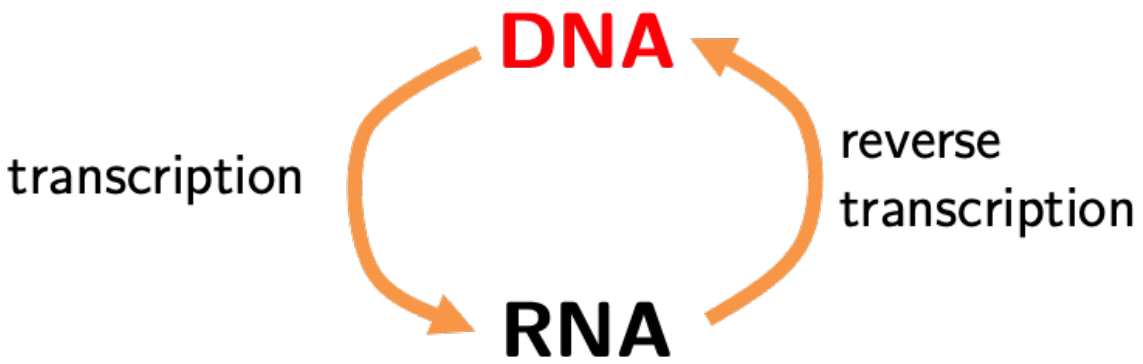


Central dogma: the genetic code serves as a manual for building different proteins.

Understanding the genetic code and its interpretation



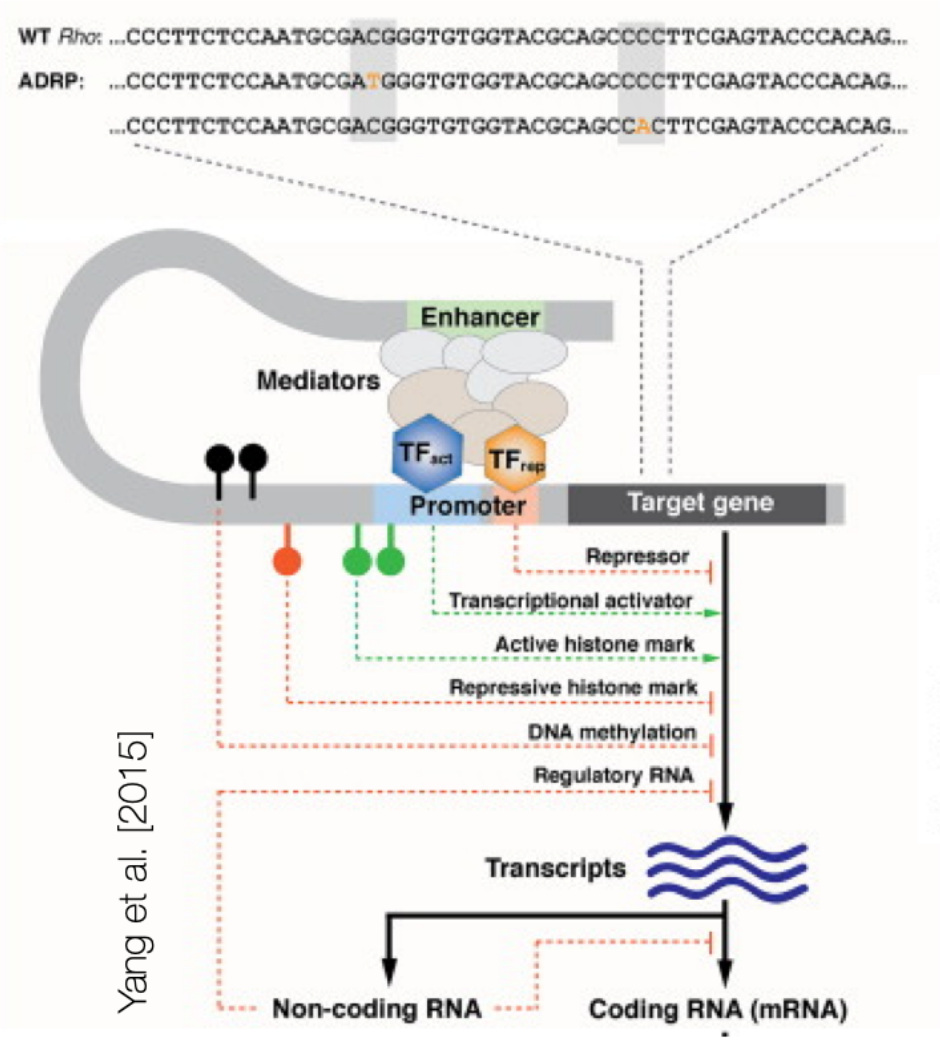
Both RNA and DNA molecules can be assessed through sequencing in a high-throughput manner.



Understanding the genetic code and its interpretation

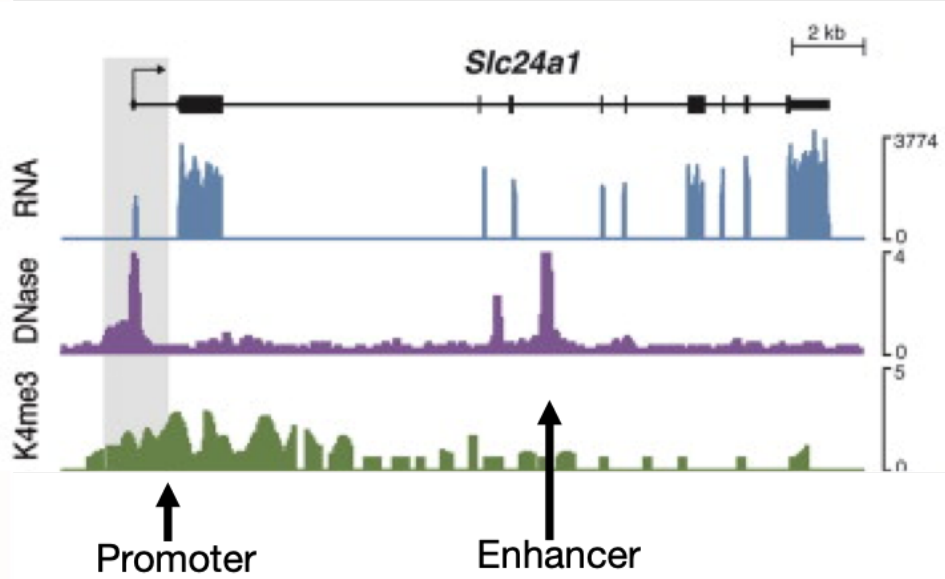
NGS can be used for (A) **qualitative** as well as (B) **quantitative** approaches.

(A) "Reading" the actual sequence

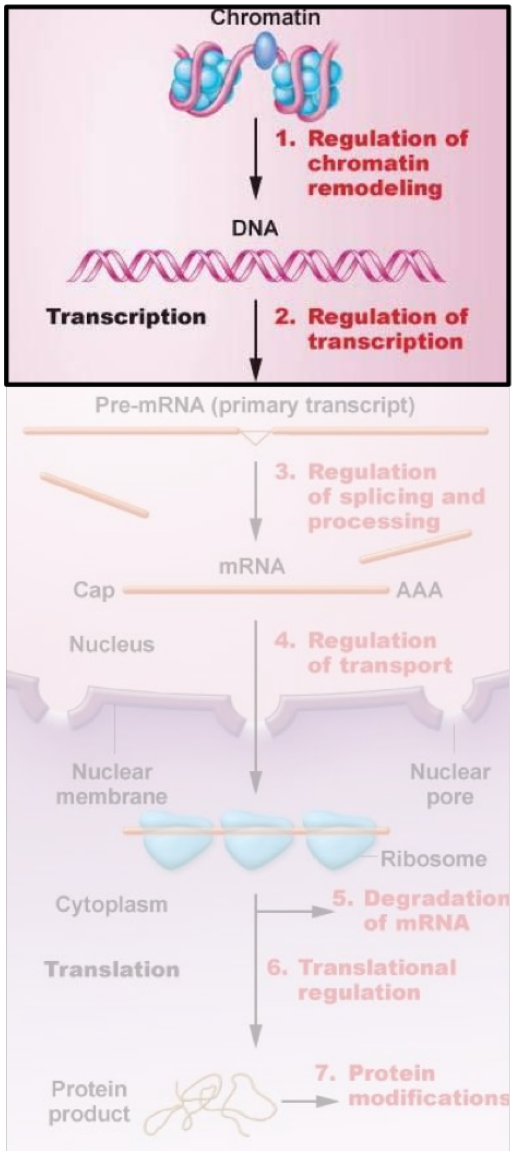


(B) Characterizing DNA regions with certain properties

that are first biochemically enriched in a sample of interest – their DNA sequence is only needed to identify the locus of origin, the information of interest is based on the abundance (= enrichment) of seq. reads



Understanding DNA: it's not just about the letters



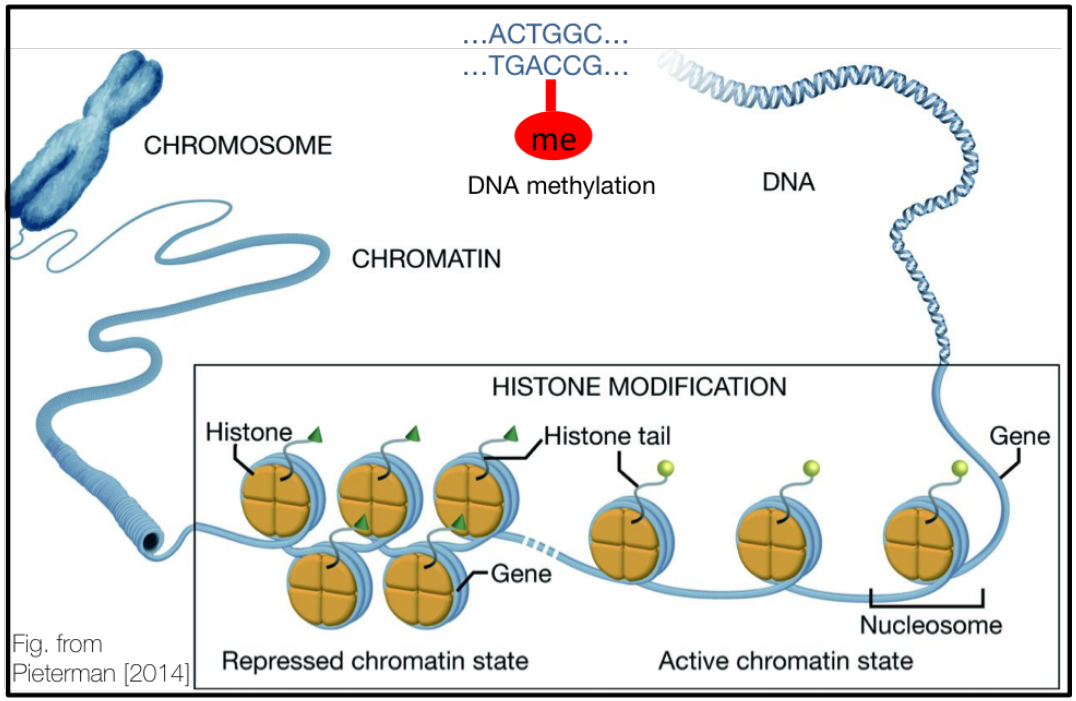
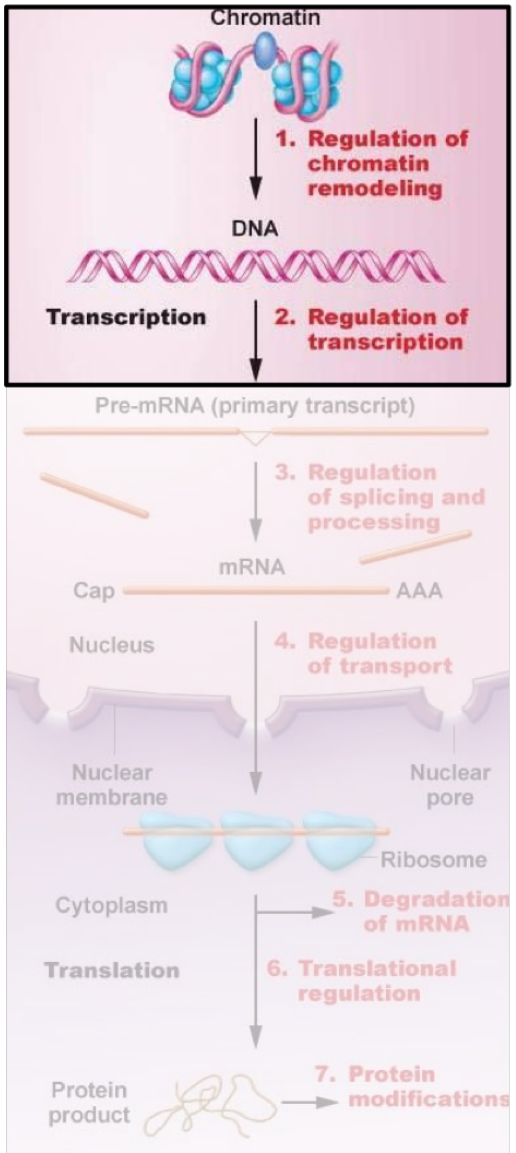
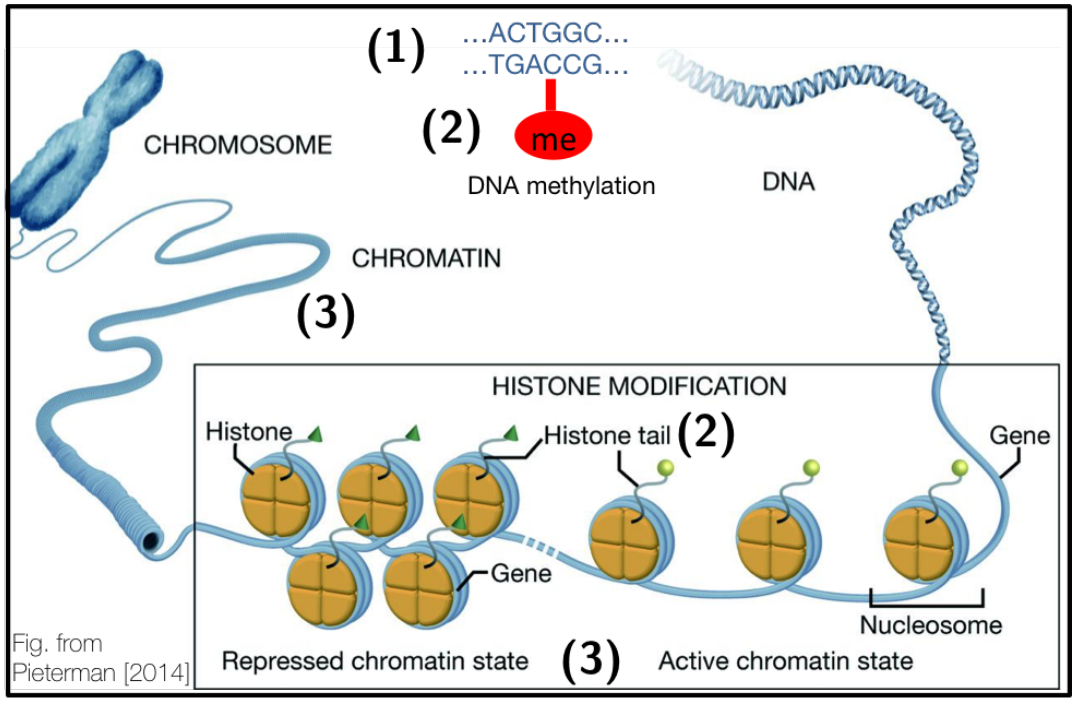


Fig. from Pieterman [2014]

Understanding DNA: it's not just about the letters



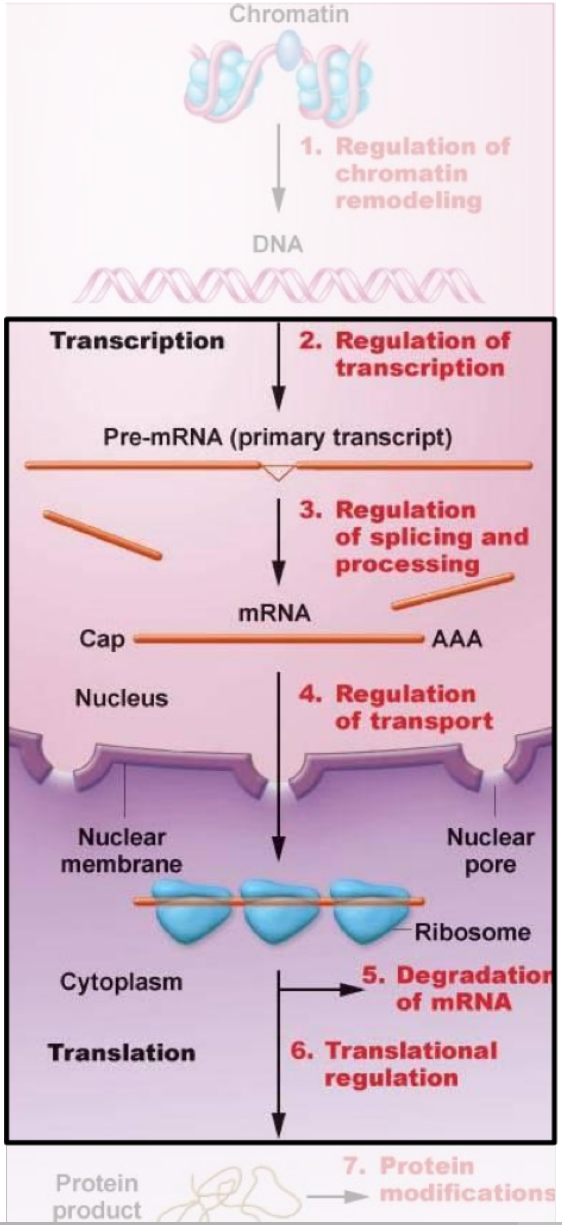


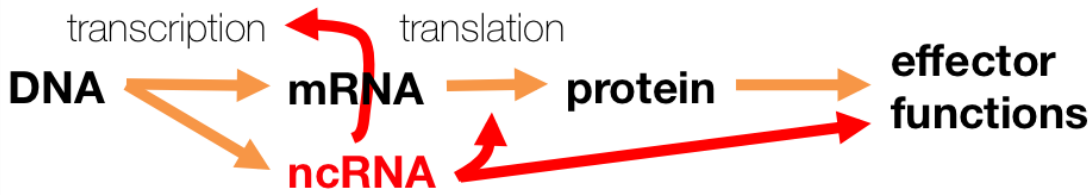
(1) DNA sequence: genome assembly, variant detection
- whole genome (WGS), whole exome (WES), amplicons

(2) Interrogating **epigenetic marks**
- histone marks, TF binding: ChIP-seq
- DNA methylation: bisulfite sequencing

(3) Understanding the **chromatin structure**
- active/repressed: ATAC-, DNase, MNase-seq, ...
- 3D interactions: Hi-C, ChIA-PET

Understanding **RNA**





(1) Gene expression: sequencing transcripts
- transcript identification & quantification (including non-coding transcripts): RNA-seq
- nascent transcripts: PRO-, GRO-seq

(2) Identifying **RNA-binding proteins**
- RIP-seq, CLIP-seq

(3) Determining **RNA structures**
- PARS, Structure-seq

(4) Assessing **RNA modifications**
- meRIP-seq, ICE (adenosine-inosine editing)

(5) Understanding **translation**
- Ribo-seq

Applications of NGS: RNA-seq is the most common one

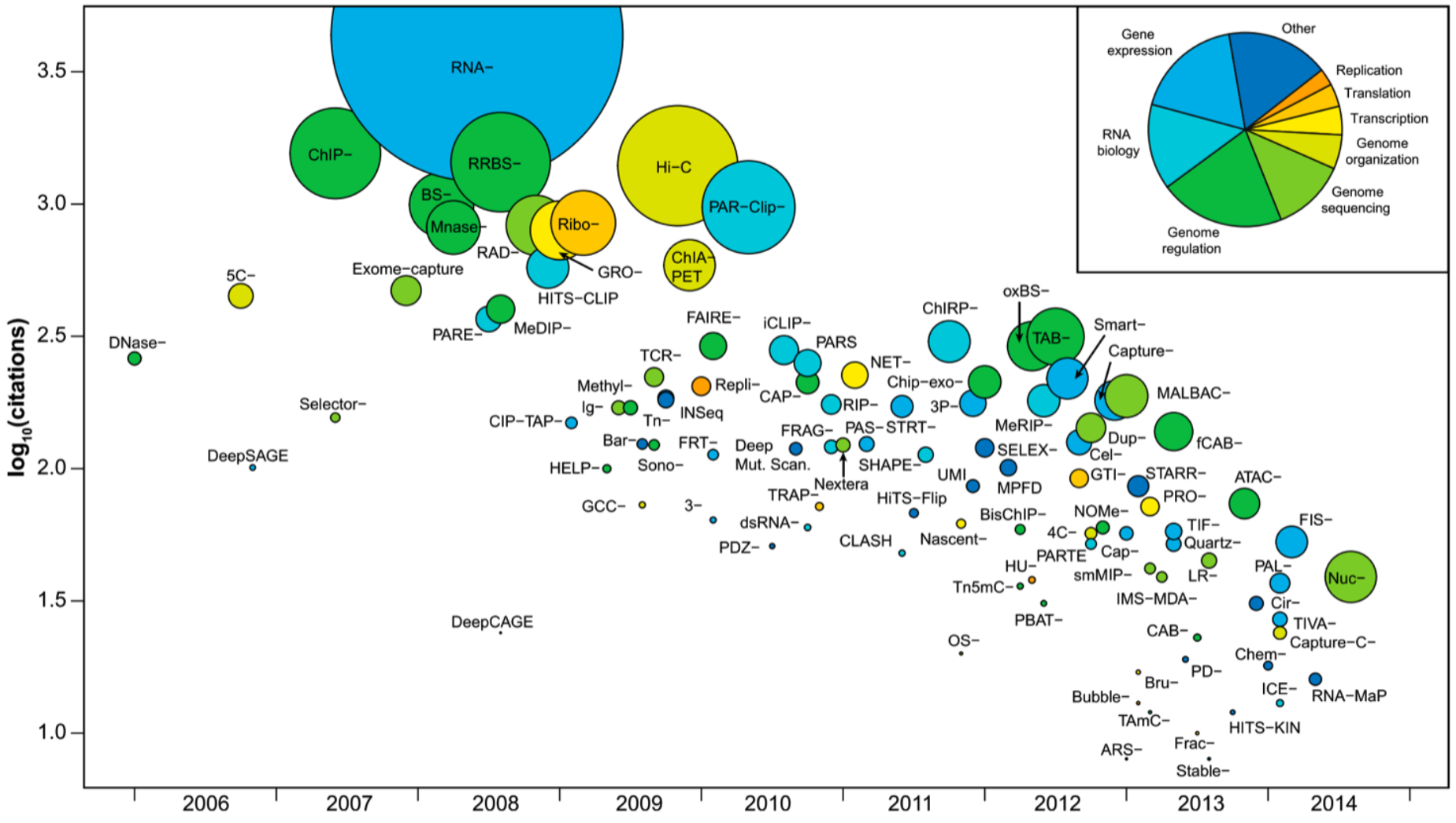


Fig. from Reuter, J. A., Spacek, D. V., & Snyder, M. P. (2015). Molecular Cell, 58(4), 586–597.

Main steps of typical NGS experiments

TEMPLATE PREP

Obtaining the molecules of interest:
DNA, RNA, nucleotide-protein complexes

⇓

Library preparation:
fragmentation and ligation of sequencing adapters

⇓

Amplification

SEQUENCING

Sequencing by **Synthesis**
vs.
Sequencing by **Ligation**

short reads vs. long reads

BIOINFORMATICS

Base calling

⇓

Alignment
Identifying loci of the sequenced fragments

⇓

Additional processing

⇓

Interpretation

Experimental design

Where to sequence at WCM?

Genomics and Epigenomics Sequencing Services

- highly experienced staff
- nevertheless: know the issues you need to discuss with them!

Jenny Xiang, M.D.
Director of Genomics Services
WCM CLC Genomics and Epigenomics Core Facility
(212) 746-4258
jzx2002@med.cornell.edu

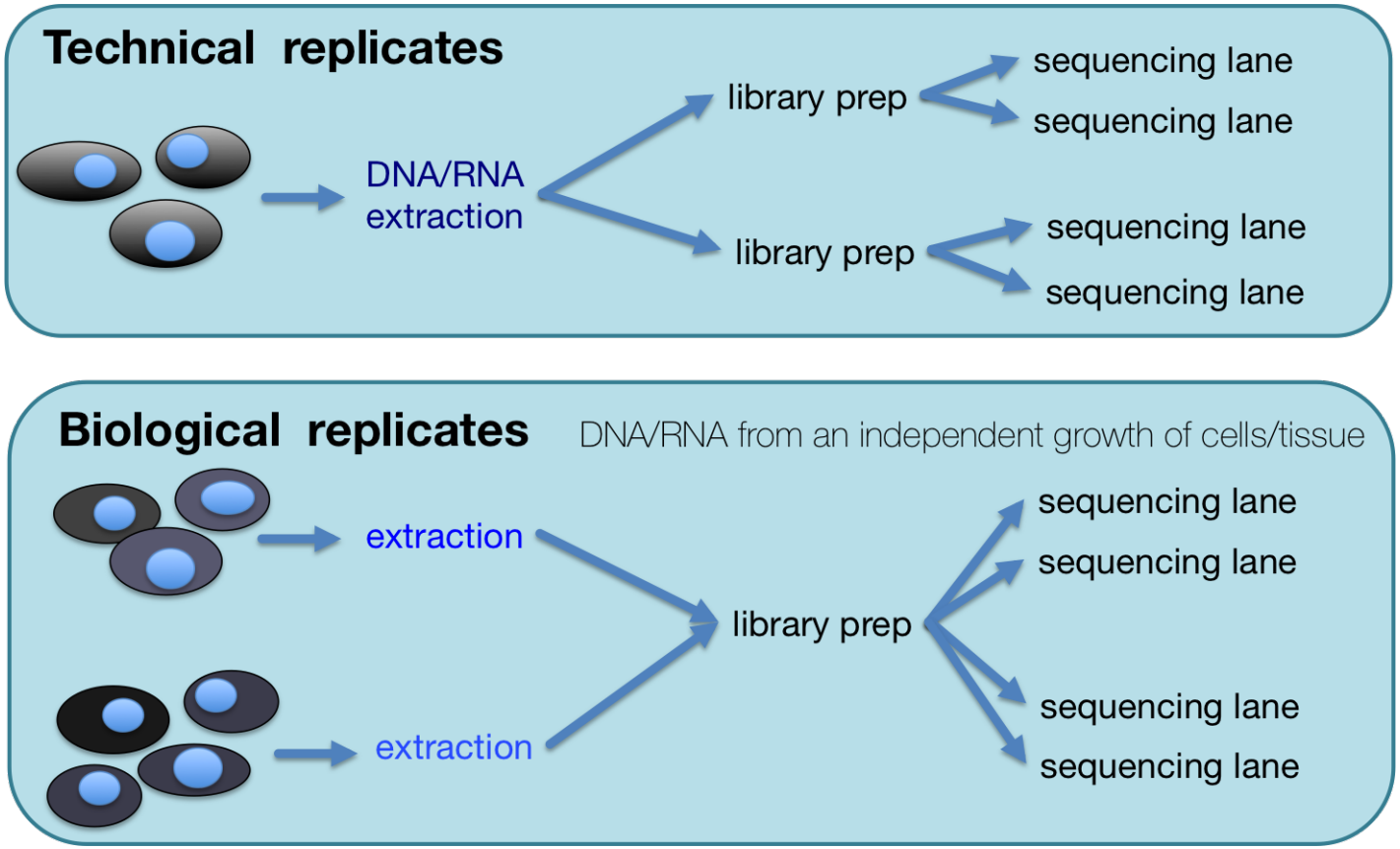
Alicia Alonso, Ph.D.
Director of Epigenomics Services
WCM CLC Genomics and Epigenomics Core Facility
(212) 746-3260
ala2035@med.cornell.edu

Experimental design considerations

- **How many replicates?**
- How to avoid batch effects?
- How many reads?

Why do we need replicates?

- replicates are needed to understand the **level of noise**

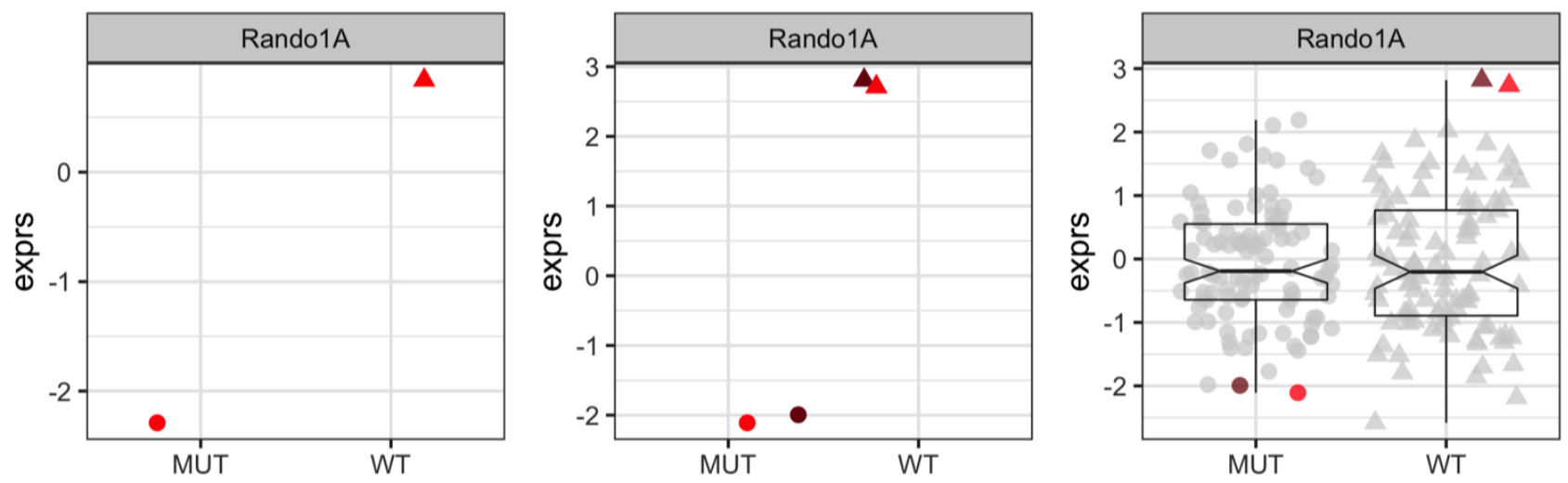


Cross-platform replicates sometimes may make sense, too.

Why do we need replicates?

"Samples are our windows to the population." [Krzywinski and Altman, 2013]

- definitely needed for quantitative assessments, e.g. RNA-seq for determining expression level differences [Schurch et al., 2016]




```
data.frame(exprs = rnorm(200), condition = c("WT","MUT"), gene_name = "Rando1A")
```

- qualitative approaches (e.g. variant calling) also benefit from technical replicates [Robasky et al., 2014, Derryberry et al., 2016]

Experimental design considerations

- How many replicates?
- **How to avoid batch effects?**
  - Understanding typical sources of noise and artifacts
- How many reads?

General problems for NGS

Problems = sources of technical noise

Sample preparation

- DNA/RNA extraction with varying degrees of degradation
- contaminations
- mislabelling, mishandling

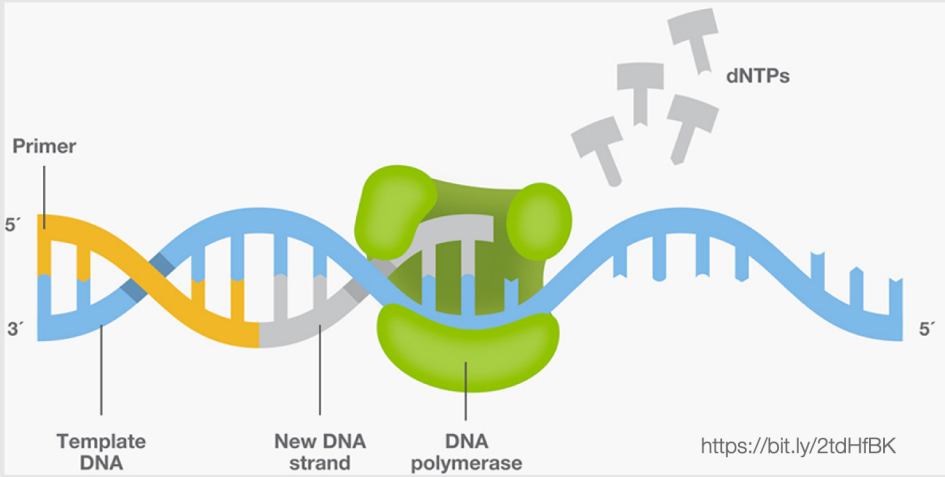
Biases of Illumina-based DNA sequencing

Somewhat **sequencing-machine**-specific problems

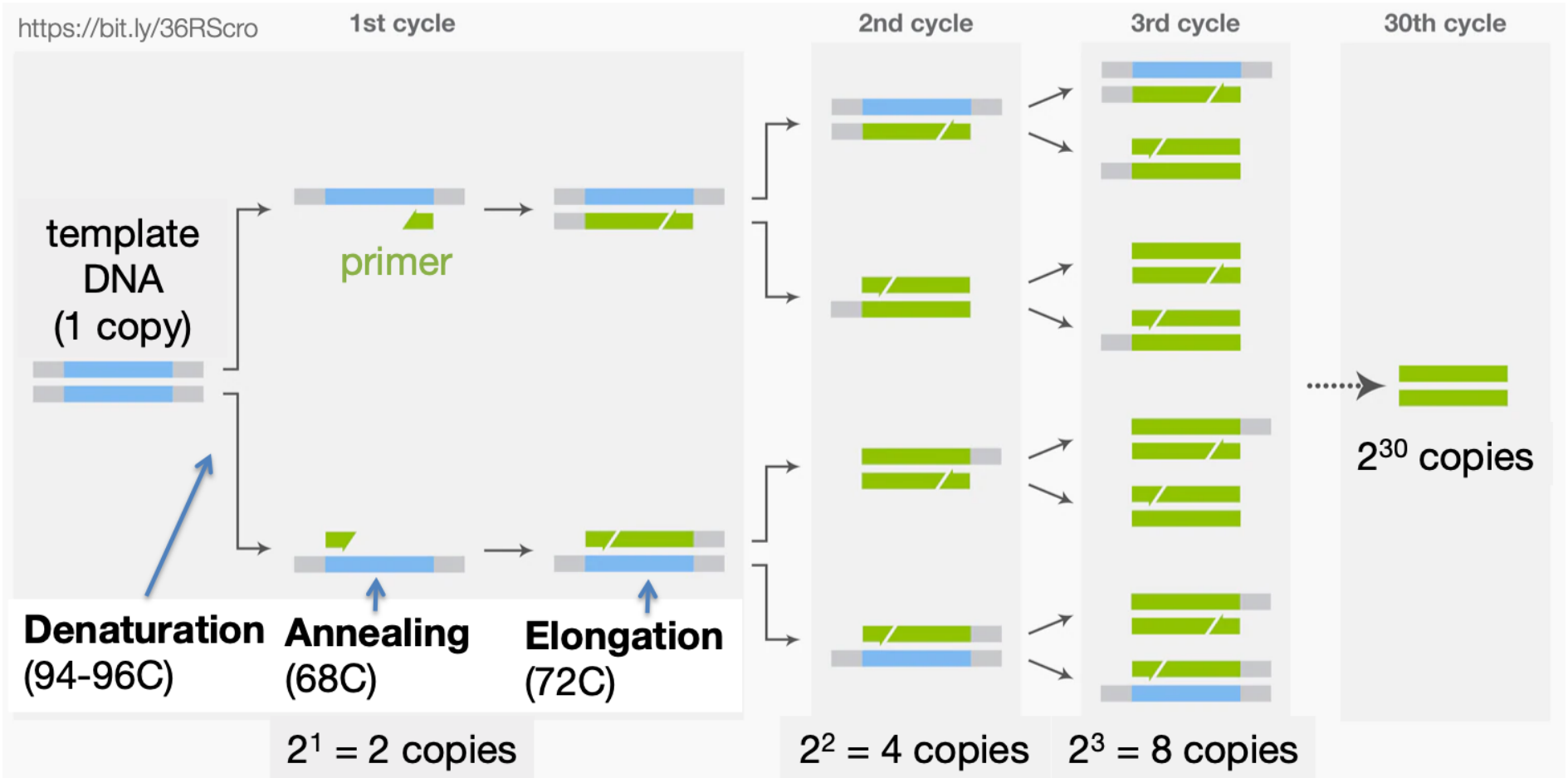
- sequencing errors
- miscalled bases

Sample-specific problems: **PCR artifacts**

- duplicated fragments (low library complexity)
- GC bias: fragments with moderate GC content are preferably amplified
- length bias: fragments between 250-700bp are strongly favored



The most important biochemical assay for NGS: PCR



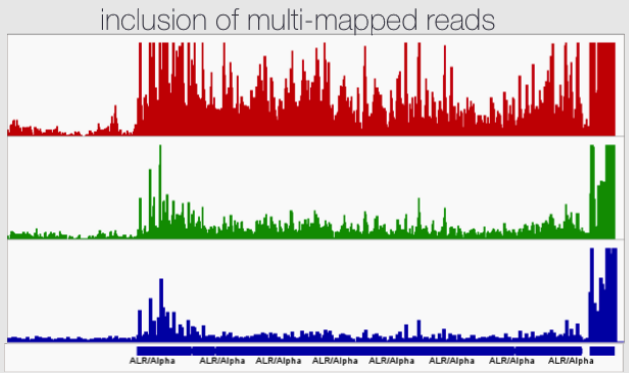
For NGS applications, template DNA fragments vary in size and GC content!
Exponential nature of the amplification process $\Rightarrow$ small differences in the starting population can lead to strongly skewed final populations.

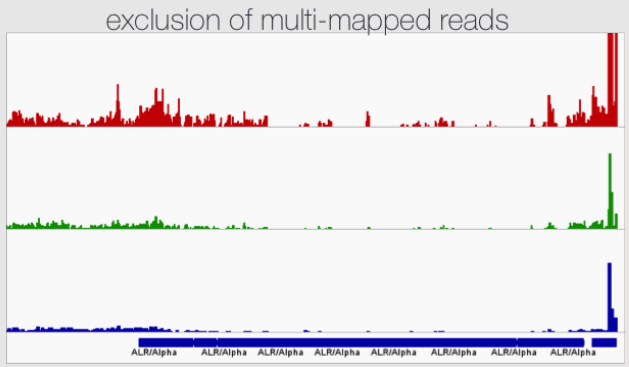
Always keep the number of PCR cycles to an absolute minimum!

Biases of Illumina-based DNA sequencing

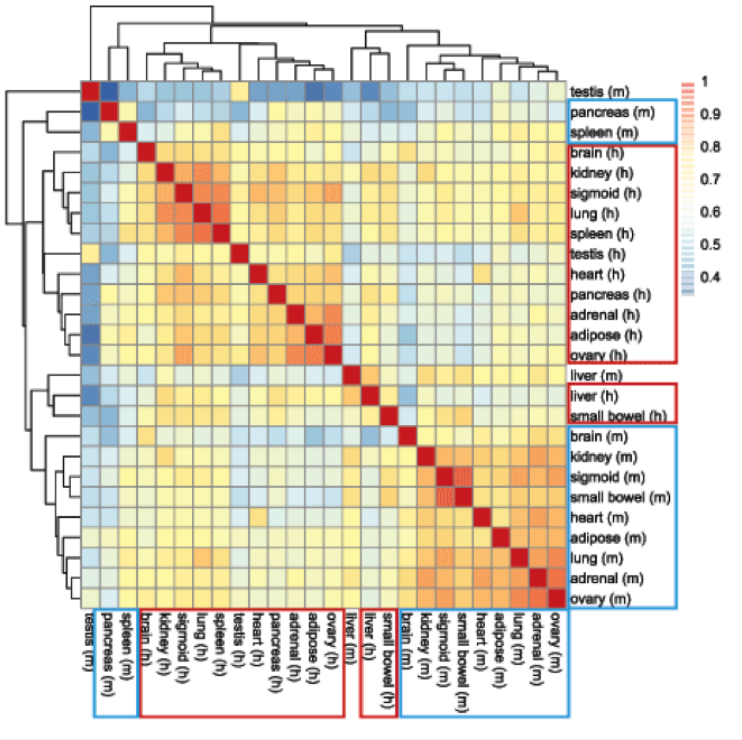
Bioinformatics problems

- **DNA**: long, repetitive elements are difficult to align to with short reads ("mappability" issue)
  - abundance of (structural) variants may complicate alignments
- **RNA**: great dynamic range (lowly expressed to extremely abundant)
  - saturation point is hardly reached: number of distinct transcripts depends on the overall make-up of the library
  - strongly affected by contaminations (DNA, rRNA, ...)
- inappropriate **data processing**, e.g. wrong parameter choices





Case study: ENCODE's comparison of mouse and human tissues



"Overall, our results indicate that there is ***considerable RNA expression diversity between humans and mice****, well beyond what was described previously, likely reflecting the fundamental physiological differences between these two organisms. "*

Lin, Lin, and Snyder (2014). PNAS 111:48

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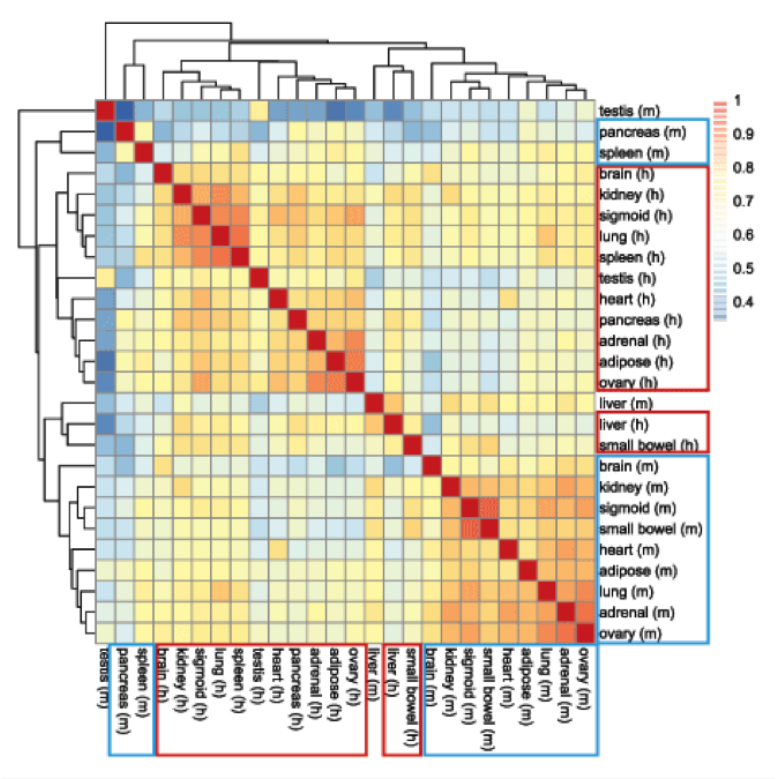

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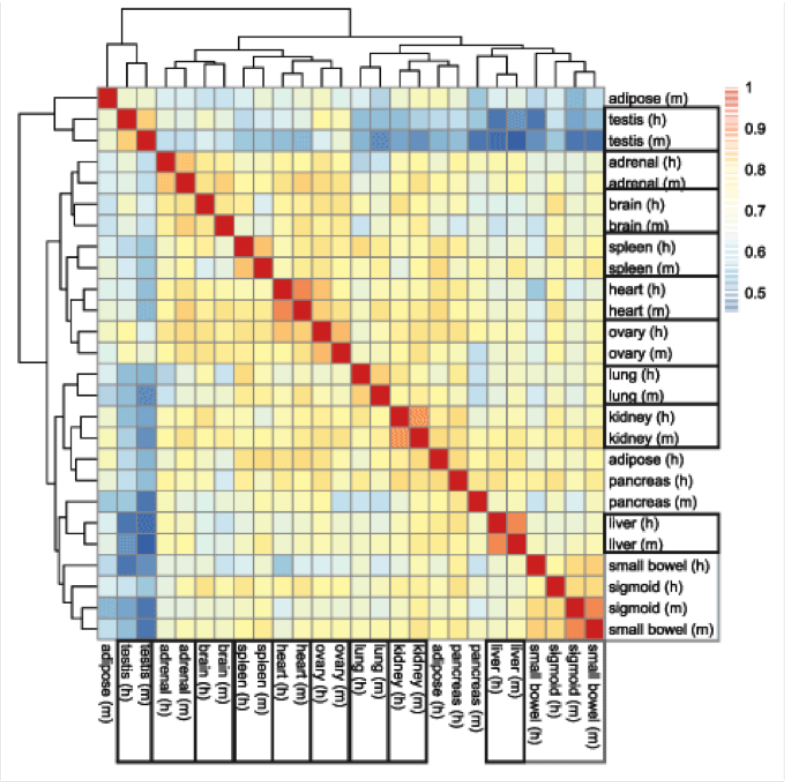


*"****Once we accounted for the batch effect*** *(...), the comparative gene expression data no longer clustered by species, and instead, we observed* ***a clear tendency for clustering by tissue****."*

Gilad & Mizrahi-Man (2015). F1000Research 4:121

Suboptimal study design

Most human samples were sequenced separately from the mouse samples:

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

Many tissues were not sex-matched

Tissue	Human	Mouse
adipose	FEMALE	MALE
adrenal	MALE	FEMALE
brain	FEMALE	MALE
heart	FEMALE	FEMALE
kidney	MALE	FEMALE
liver	MALE	FEMALE
lung	FEMALE	FEMALE
ovary	FEMALE	FEMALE
pancreas	FEMALE	FEMALE
sigmoid colo	MALE	FEMALE
small bowel	FEMALE	FEMALE
spleen	FEMALE	MALE
testis	MALE	MALE

- human data: deceased organ donors
- mouse data: 10-week-old littermates

Not all variables can be controlled for! Know the limitations of your study before making bold claims! Recommended reading: `https://f1000research.com/articles/4-121/v1`

Avoiding bias by relying on randomization

Completely randomized design

STRESS	A	B	A	A	B	A	B	A	A	B	B	B
DIET	1	2	1	2	2	1	1	2	2	1	2	1

Restricted randomized design

GENOTYPE	A	A	A	A	A	A	B	B	B	B	B	B
DIET	1	2	1	2	2	1	1	2	1	1	2	2

Blocked & randomized design

GENOTYPE	A	A	B	B	A	A	B	B	A	A	B	B
DIET	1	2	1	2	1	2	1	2	1	2	1	2
WEIGHT	•	•	•	•	●	●	●	●	●	●	●	●

Block what you can,
randomize what you cannot.

*What factors are of **interest**? Which ones might introduce noise? Which nuisance factors do you absolutely need to account for?*

Experimental design considerations

- How many replicates?
- How to avoid batch effects?
- **How many reads?**

How deep is deep enough?

lower limit should usually be whatever ENCODE says:
https://www.encodeproject.org/about/experiment-guidelines/

Application	Recommended seq. depth
differential gene expression	20 - 50 mio SR, 75 bp
variant calling	30-200x coverage
whole-genome bisulfite sequencing	30x coverage
ChIA-PET	200 mio PE

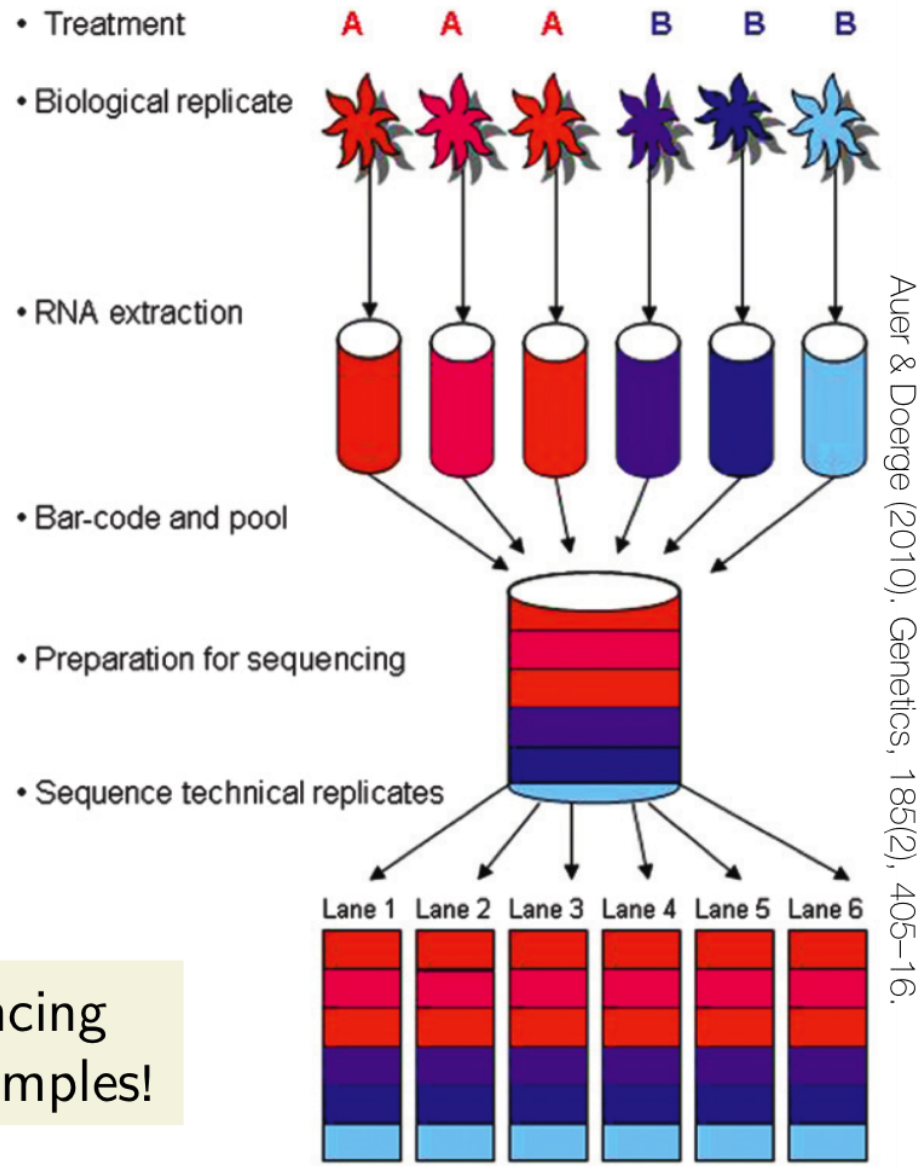
- you may need more, longer, and possibly paired-end reads
  - novel transcript identification
  - alternative splicing
  - ChIP-seq for broad histone marks
  - 3D chromatin structure assessment assays

Oftentimes the addition of replicates is more meaningful than increased sequencing depth! [Rapaport et al., 2013]

Typical experimental setup

- keep the **technical nuisance** factors (harvest date, RNA extraction kit, sequencing date...) to a minimum
- cover only as much of the **biological variation** as needed (but keep possible limitations for the final conclusions in mind)

Make sure the sequencing core **multiplexes** all samples!



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