



# INTRODUCTION TO 'NEXT GENERATION SEQUENCING'

**Esther Camacho<sup>1</sup>**, Mireia Ferrer<sup>1</sup>, Álex Sánchez<sup>1,2</sup>, Angel Blanco<sup>1,2</sup>, Berta Miró<sup>1</sup>

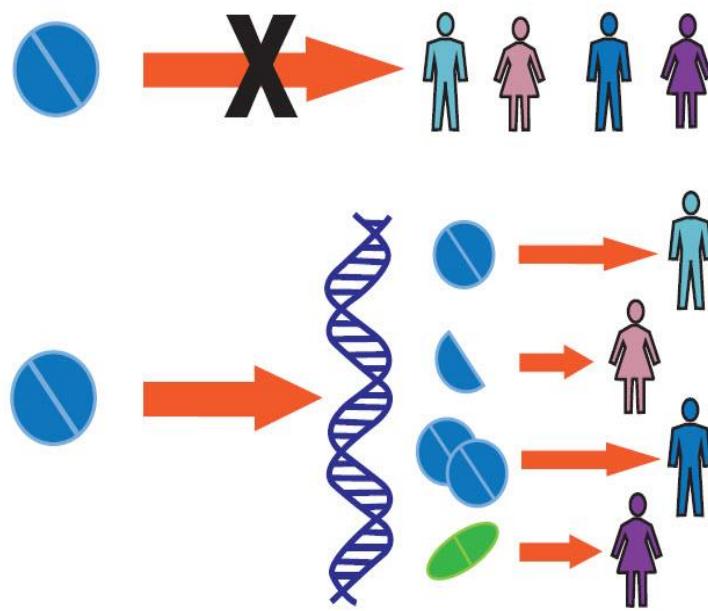
<sup>1</sup> Unitat d'Estadística i Bioinformàtica (UEB) VHIR <sup>2</sup> Departament de Genètica Microbiologia i Estadística, UB

- 1. Introduction to NGS**
- 2. First Generation Sequencing**
- 3. Second Generation Sequencing**
- 4. Third Generation Sequencing**
- 5. Sequencing generation face to face**
- 6. Applications of NGS techniques**
- 7. A (very) brief introduction to DoE**

# 1. Introduction to NGS

## Personalized medicine era

The right therapeutic strategy for the right person at the right time



<https://www.pharmgkb.org/>

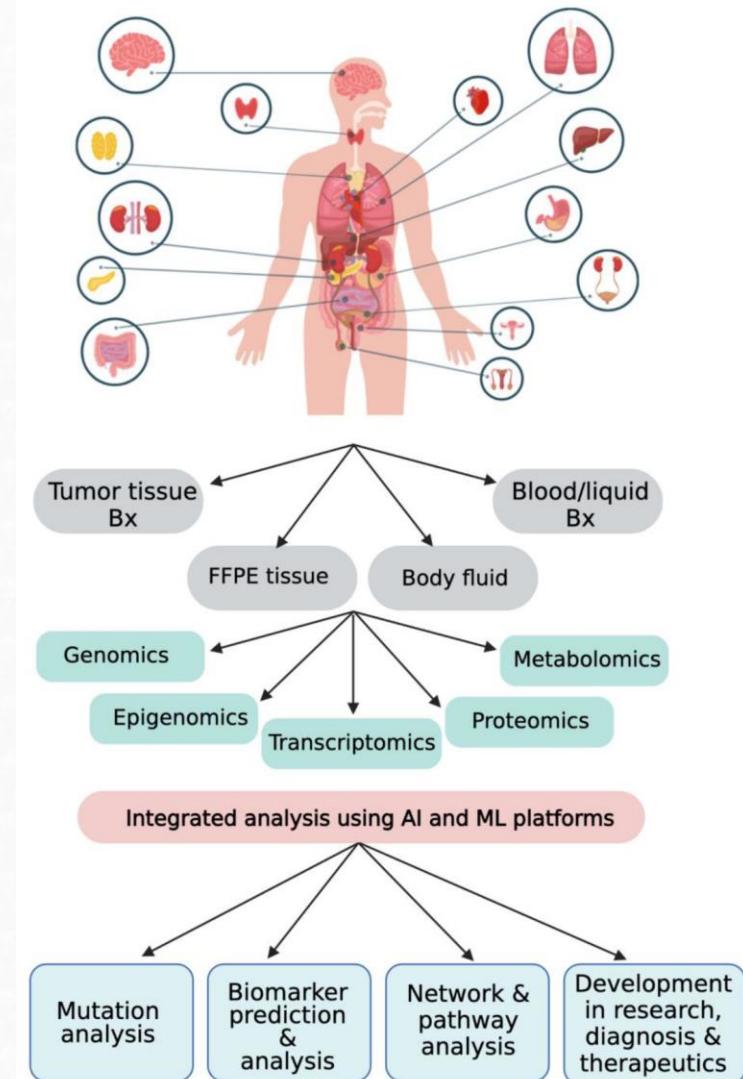
- Diagnostic
- Susceptibility/risk (prevention)
- Prognostic
- Predictive (response)

# 1. Introduction to NGS

## Personalized medicine era

- Depending on the goal of the analysis and the sample characteristics, we will choose a different omic approach and perform a different bioinformatic analysis.

Satam, H.; Joshi, K.; Mangrolia, U.; Waghoo, S.; Zaidi, G.; Rawool, S.; Thakare, R.P.; Banday, S.; Mishra, A.K.; Das, G.; et al. Next-Generation Sequencing Technology: Current Trends and Advancements. *Biology* 2023, 12, 997.  
<https://doi.org/10.3390/biology12070997>



# 1. Introduction to NGS

**Genomics** is a branch of genetics that enables the study of genomes of whole organisms.



Gregor Mendel



It differs from “classical genetics” in that it considers the hereditary material of an organism **in global** rather than **one gene or one gene product** at a time.

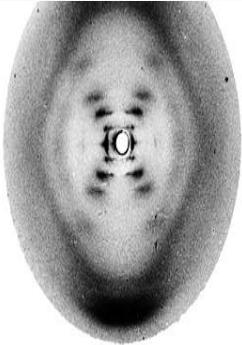
# 1. Introduction to NGS



"We wish to suggest a structure for the salt of deoxyribose nucleic acid (D.N.A.). This structure has novel features which are of considerable biological interest."

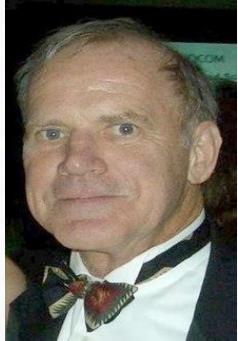


J.D. Watson & F. H. C. Crick. (1953). Molecular structure of Nucleic Acids. *Nature*. **171**: 737-738.



Franklin &  
Rosalind  
DNA  
nucleic  
acid  
Type B

Deoxyribose  
nucleic acid  
(DNA) 1953

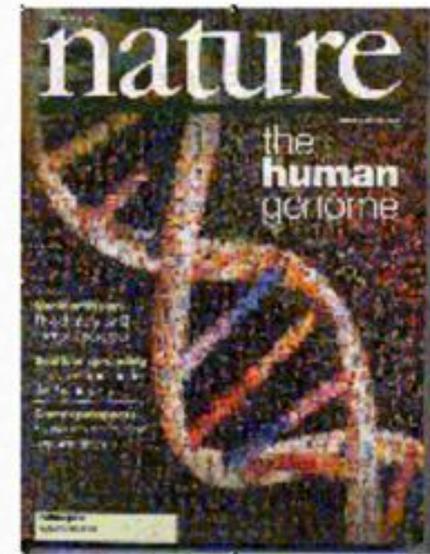


Polymerase Chain  
Reaction (PCR)  
1983

# 1. Introduction to NGS

## Human Genome Project

The Human Genome Project (HGP) goal was the complete mapping and understanding of all the genes of human beings.



- Begin in 1990
- First draft in February 2001
- full sequence April 2003
- It catalyzes the developing and improving of sequencing techniques

# Genome sequencing

Genome sequencing is figuring out the order of DNA nucleotides, or bases, in a genome—the order of As, Cs, Gs, and Ts that make up an organism's DNA.

## 1. Introduction to NGS

### Genome sequencing

Genome sequencing is figuring out the order of DNA nucleotides, or bases, in a genome—the order of As, Cs, Gs, and Ts that make up an organism's DNA.

3,054,815,472-bp (nuclear)

16,569-bp (mitochondrial)

**TOTAL: 3,054,832,041-bp**

Sergey Nurk *et al.*, The complete sequence of a human genome. *Science* 376, 44-53 (2022). DOI: [10.1126/science.abj6987](https://doi.org/10.1126/science.abj6987)

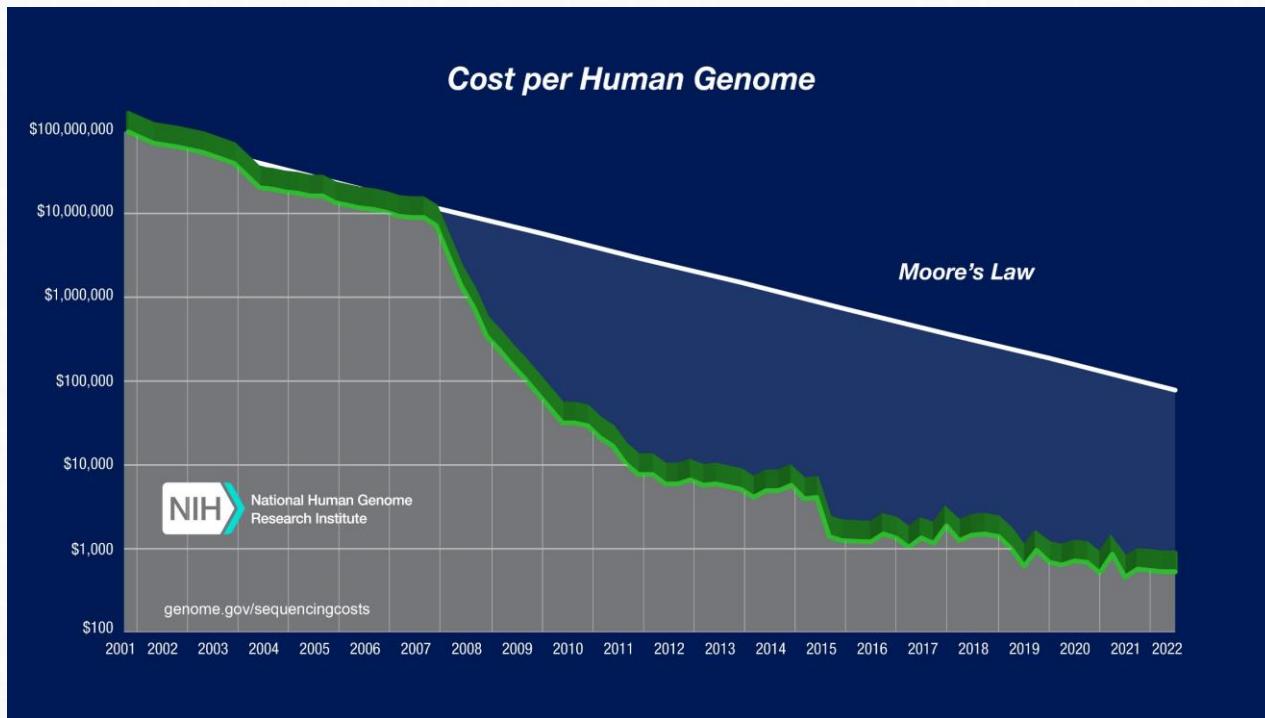
# 1. Introduction to NGS

## Why is genome sequencing so important?

- **How the genome as a whole works:** how genes work together to direct the growth, development and maintenance of an entire organism.
- **Find genes** much more easily and quickly.
- Genes account for less than 25 percent of the DNA in the genome, and so knowing the entire genome sequence will help scientists **study the parts of the genome outside the genes**.

# 1. Introduction to NGS

## Cost of sequencing



Cost of human genome sequence:

HGP:  $1 - 3 \times 10^{12}$ \$

2006:  $14 \times 10^6$ \$

2016: 1000-4000\$

2019: 200 – 500\$

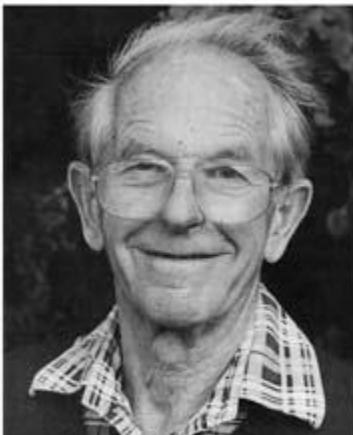
<https://www.genome.gov/about-genomics/fact-sheets/Sequencing-Human-Genome-cost>

- 1. Introduction to NGS**
- 2. First Generation Sequencing**
- 3. Second Generation Sequencing**
- 4. Third Generation Sequencing**
- 5. Sequencing generation face to face**
- 6. Applications of NGS techniques**
- 7. A (very) brief introduction to DoE**

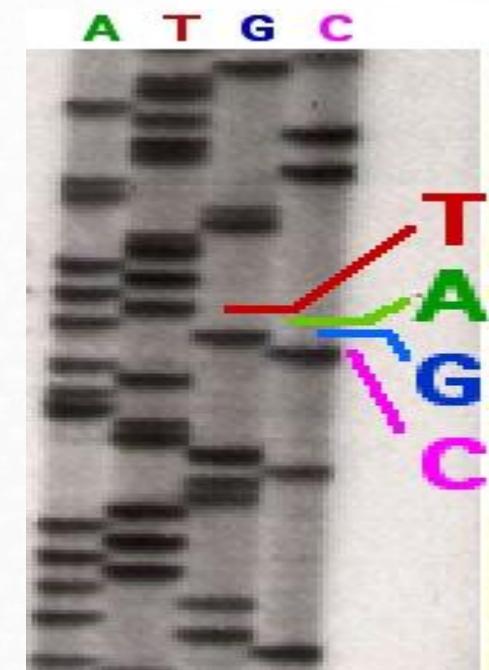
## 2. First Generation Sequencing

### Sanger sequencing

Method of **DNA sequencing** based on the selective incorporation of chain terminating dideoxynucleotides by DNA polymerase during *in vitro* DNA replication. Developed by Frederick Sanger and colleagues in 1977, it was the most widely used sequencing method for approximately 25 years.



Courtesy of Dr. F. Sanger, MRC, Cambridge.  
Noncommercial, educational use only.



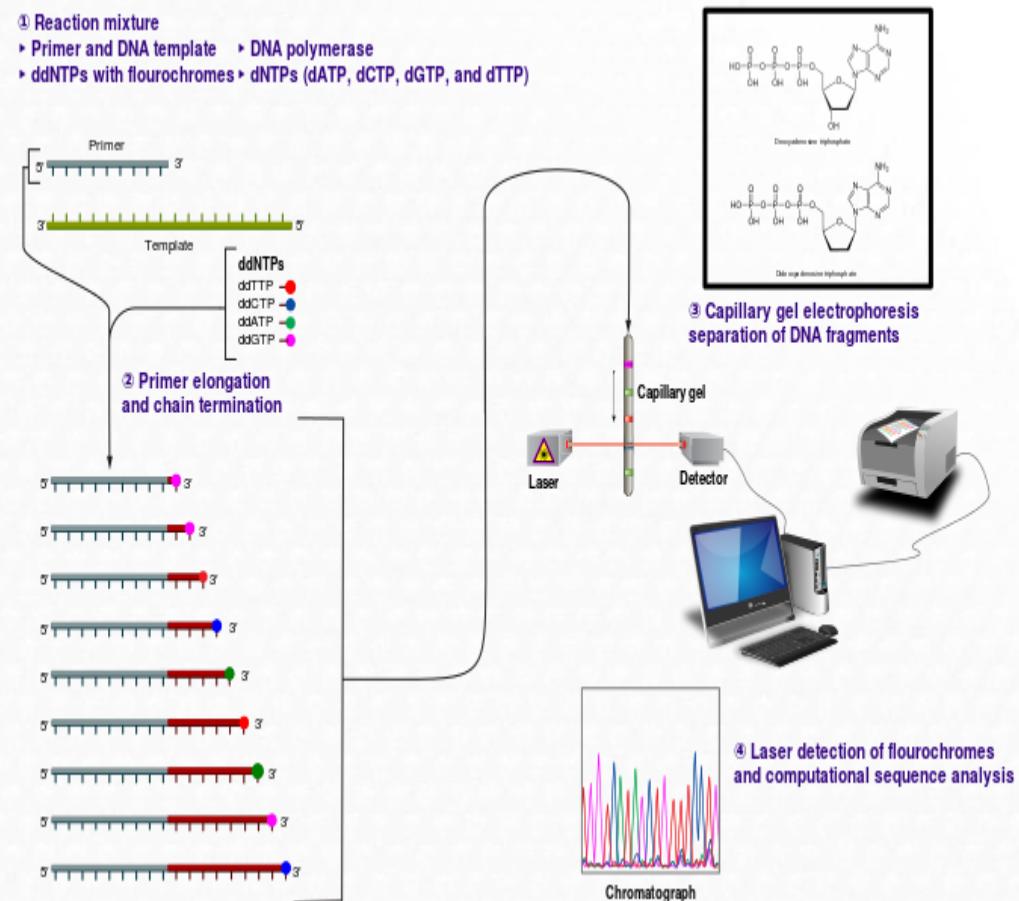
Frederick Sanger received two Nobel prizes (in the same category), for his work on protein sequencing and DNA sequencing

<http://www.yourgenome.org/stories/third-generation-sequencing>

## 2. First Generation Sequencing

### Sanger sequencing. How it works?

- A DNAP enzyme is used to **replicate a ssDNA**. In the mix reaction, there exist normal and modified nucleotides.
- Random incorporation of **modified nucleotides** stops the synthesis reaction.
- Each generated fragment will have a different length that could be distinguish by **gel electrophoresis**.



<https://www.youtube.com/watch?v=vK-HIMaitnE>

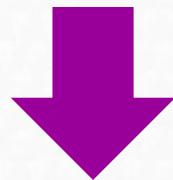
<1kb read

- 1. Introduction to NGS**
- 2. First Generation Sequencing**
- 3. Second Generation Sequencing**
- 4. Third Generation Sequencing**
- 5. Sequencing generation face to face**
- 6. Applications of NGS techniques**
- 7. A (very) brief introduction to DoE**

### 3. Second Generation Sequencing

#### Why second generation sequencing?

- Disadvantage of Sanger sequencing: **low sequence output**
  - using of gels or polymers as separation media
  - limited number of samples which could be handled in parallel
  - difficulties with automation of the sample preparation



These limitations triggered the efforts to develop new techniques

### 3. Second Generation Sequencing

#### Main characteristics of NGS:

- high speed and throughput
- **shorter reads**
- accuracy
- much higher degree of sequence coverage
- Huge data storage demands

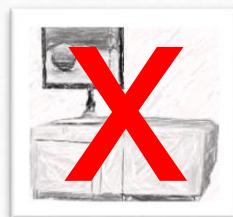


## 3. Second Generation Sequencing

### Instruments

#### High throughput

ROCHE



GS FLX+ 454

Illumina



NextSeq 550 Series



NextSeq 2000



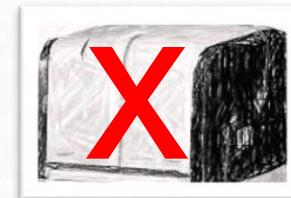
NovaSeq 6000 System

Thermofisher



Ion Gene Studio S5 PrimeSystem

#### Benchtop



GS Junior 454



iSeq 100 System



MiniSeq System



MiSeq Series



Ion Torrent Genexus



Ion PGM

### 3. Second Generation Sequencing

#### Basic NGS workflow.

##### 1. Library Preparation

It is prepared by **random fragmentation of DNA or cDNA sample**, followed by **adapter ligation**. Adapter-ligated fragments are then **PCR amplified and gel purified**

##### 2. Clonal amplification

Each DNA fragment is amplified **millions of times**.

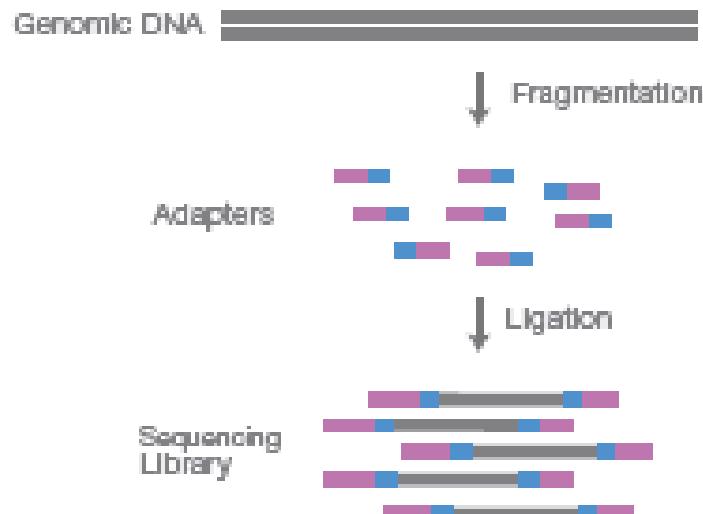
##### 3. Sequencing

The nucleotides incorporated are read by the **detector**

### 3. Second Generation Sequencing

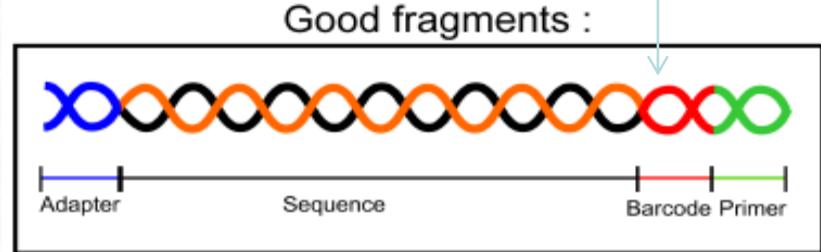
#### Library preparation:

##### A. Library Preparation



NGS library is prepared by fragmenting a gDNA sample and ligating specialized adapters to both fragment ends.

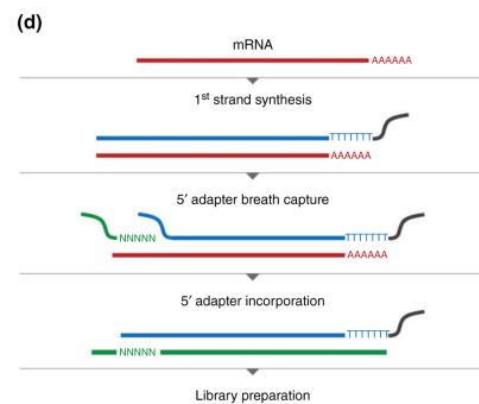
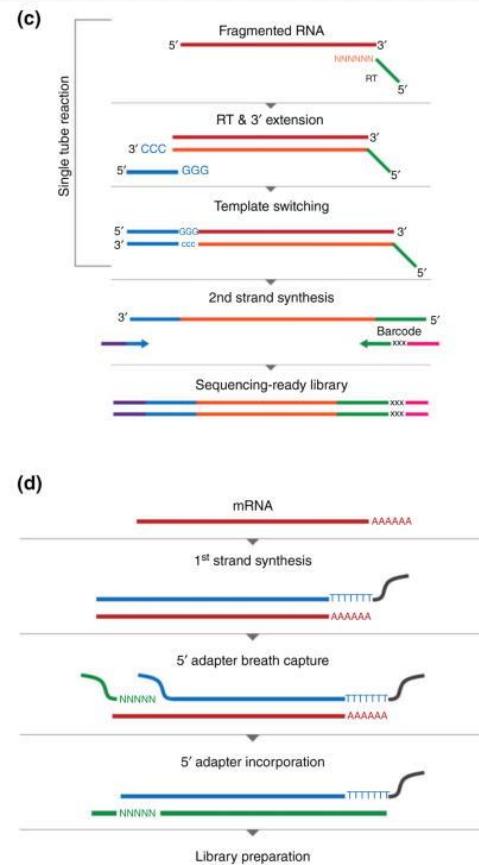
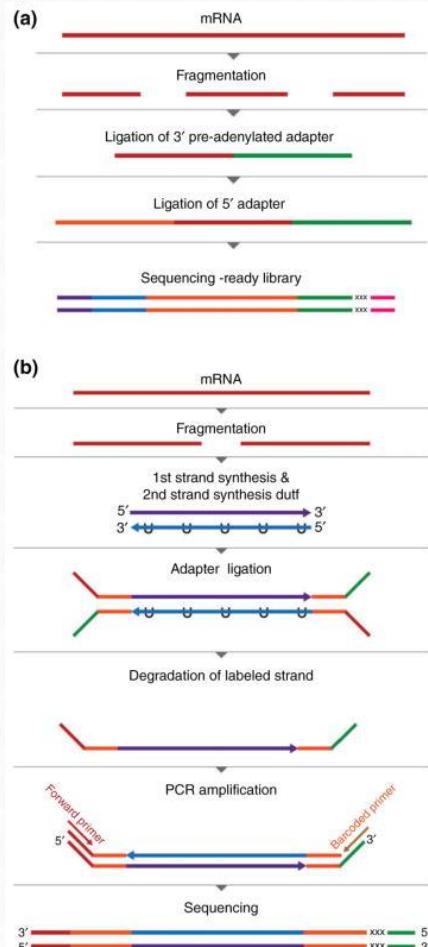
Useful for multiplexing samples



Depending on the final application and method used for sequencing, different kits are available

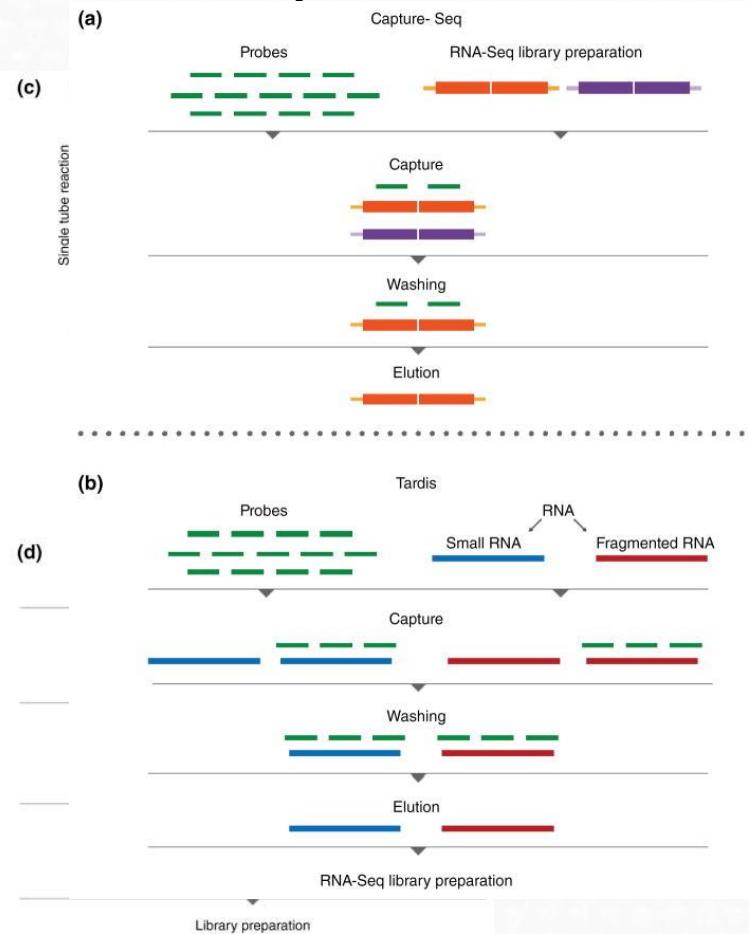
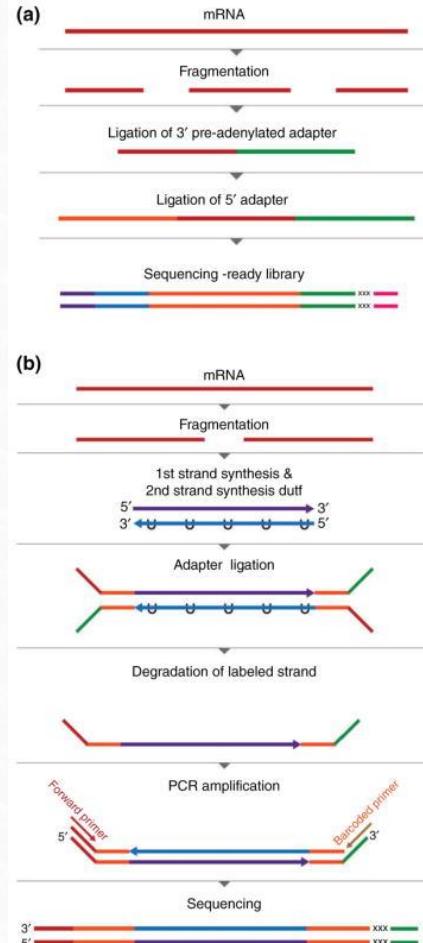
### 3. Second Generation Sequencing

**Library preparation:** Too many methods / kits available



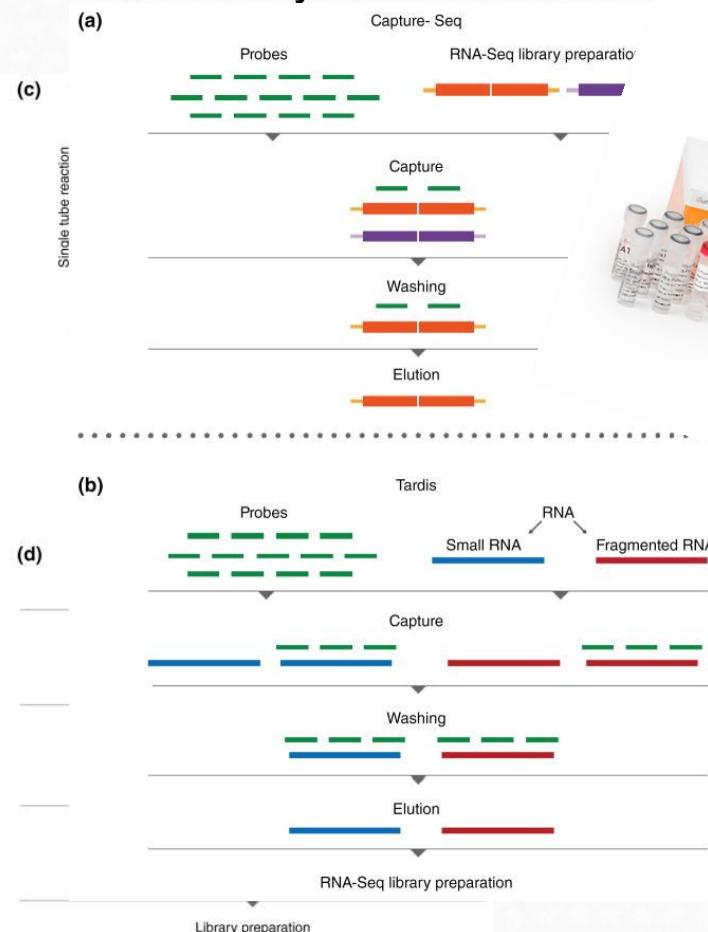
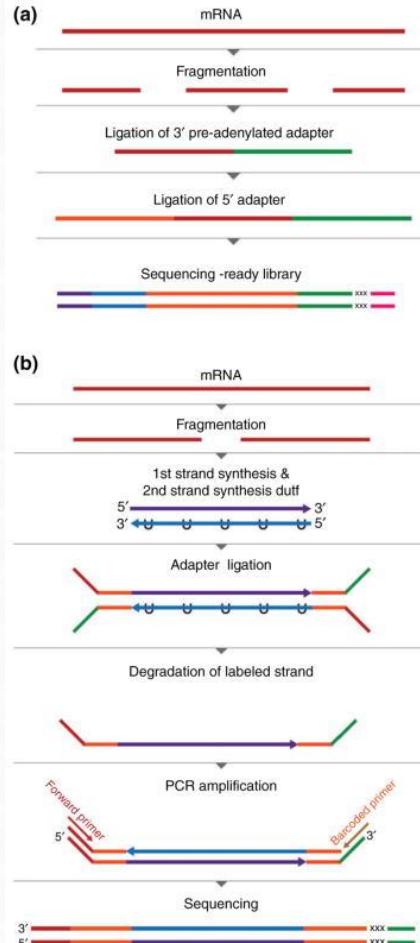
### 3. Second Generation Sequencing

**Library preparation:** Too many methods / kits available



### 3. Second Generation Sequencing

**Library preparation:** Too many methods / kits available



Hrdlickova R, Toloue M, Tian B. RNA-Seq methods for transcriptome analysis. *Wiley Interdiscip Rev RNA*. 2016;8(1):10.1002/wrna.1364.

## 3. Second Generation Sequencing

**Library preparation:** Too many methods / kits available



Hrdlickova R, Toloue M, Tian B. RNA-Seq methods for transcriptome analysis. *Wiley Interdiscip Rev RNA*. 2016;8(1):10.1002/wrna.1364.

### 3. Second Generation Sequencing

#### Library preparation: Too many methods / kits available



### 3. Second Generation Sequencing

**Library preparation:** Too many methods / kits available



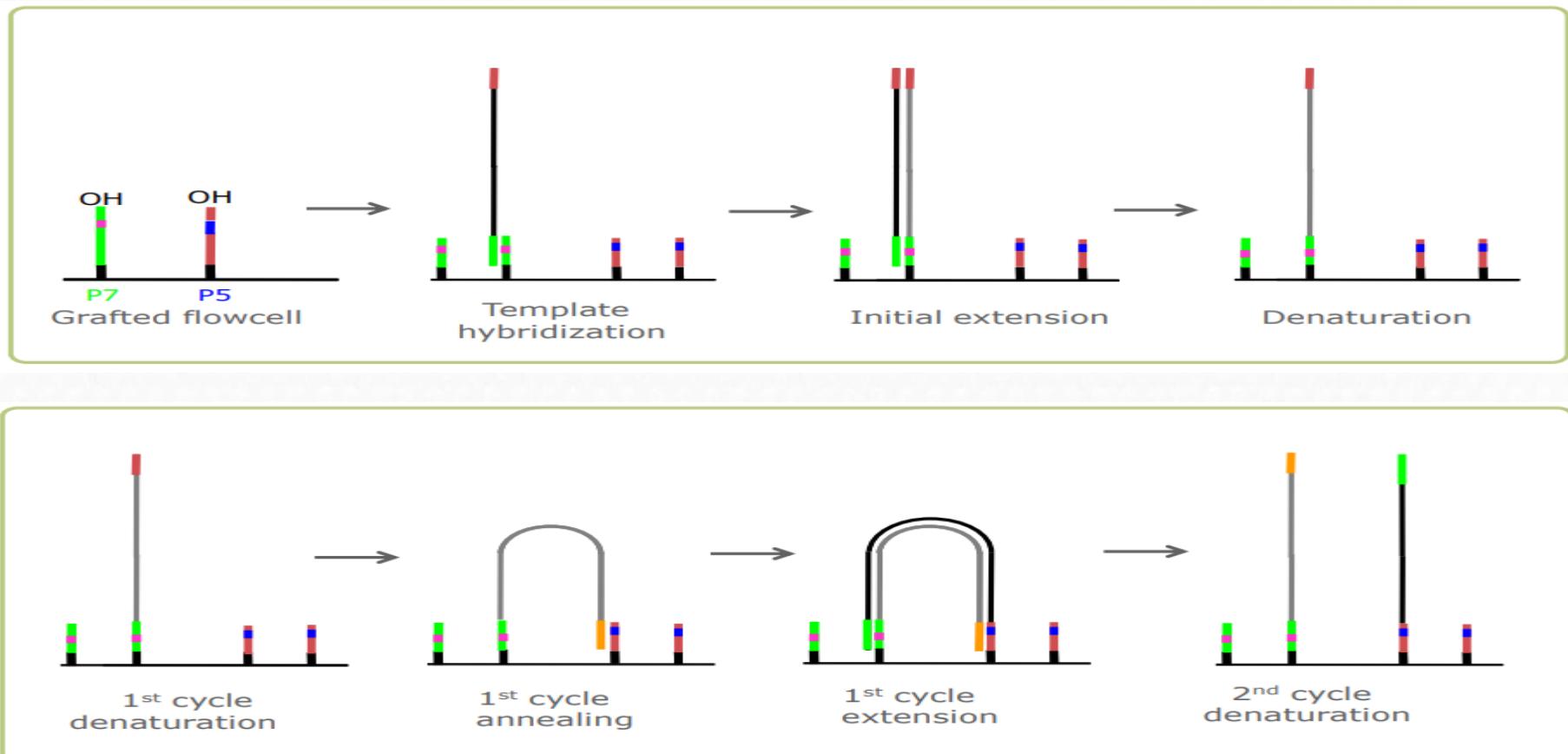
Ion Chef™ Instrument



Hrdlickova R, Toloue M, Tian B. RNA-Seq methods for transcriptomic analysis. *Wiley Interdiscip Rev RNA*. 2016;8(1):10.1002/wrna.1364.

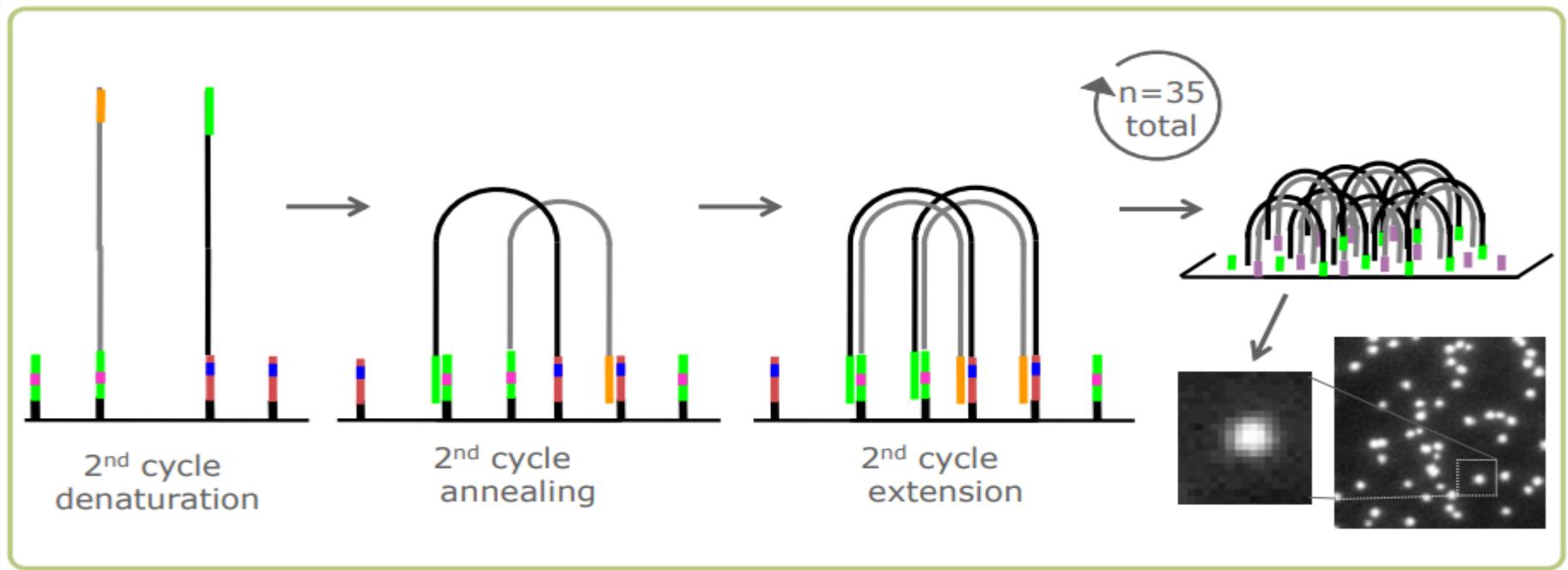
### 3. Second Generation Sequencing

#### Clonal amplification: Bridge PCR (Illumina):



### 3. Second Generation Sequencing

#### Clonal amplification: Bridge PCR (Illumina)



### 3. Second Generation Sequencing

#### Clonal amplification: Bridge PCR (Illumina):

**SYBR QC:** Ensure successful amplification before continuing

- GOAL: Visually confirm successful cluster generation and optimal density before continuing



Sparse



Good



Dense

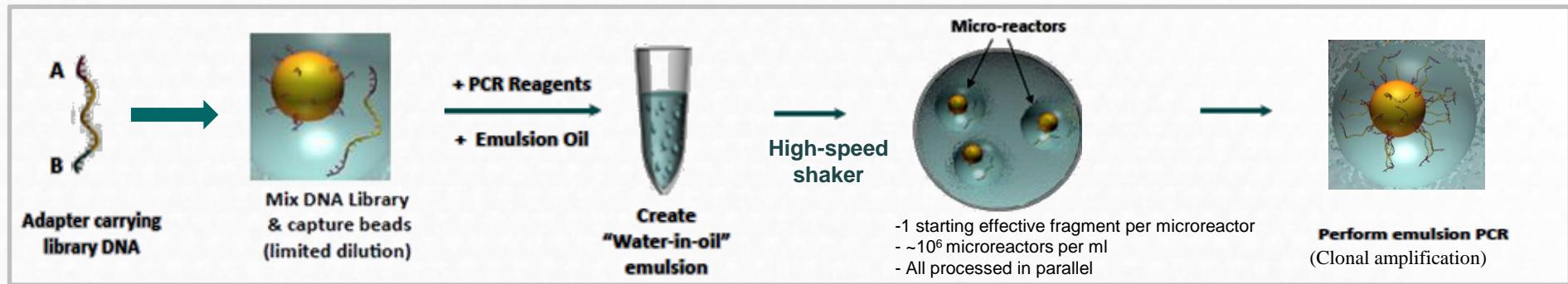
\*1.6 RTA

- Too Sparse: Loss of valuable real estate on flow cell
- Too Dense: Analysis problems

### 3. Second Generation Sequencing

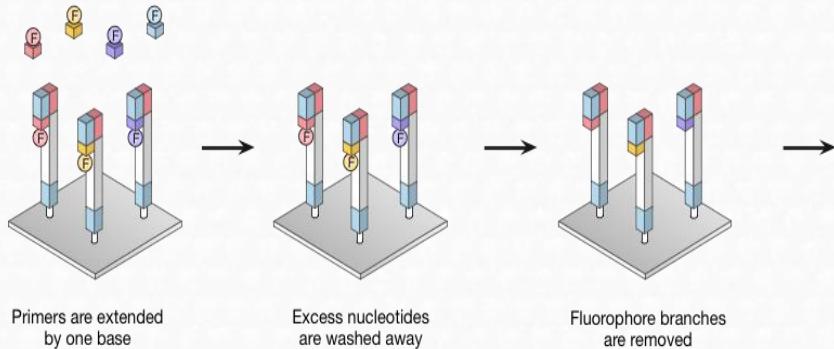
#### Clonal amplification:

For emPCR based systems (Ion/PGM, 454)

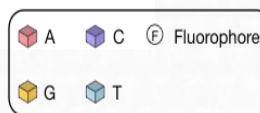
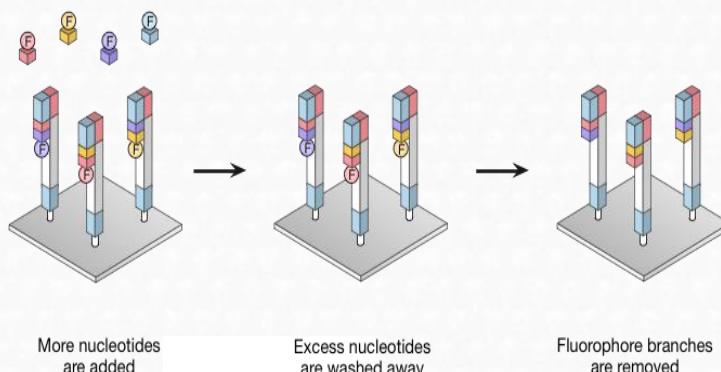


### 3. Second Generation Sequencing

#### Sequencing: Illumina (dye terminator nt, sequencing by synthesis)



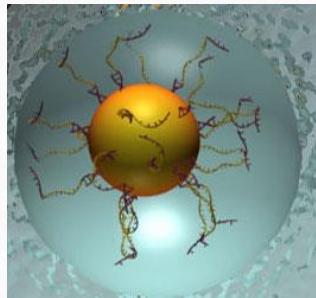
- Limited by the fragment length than can effectively “bridge”
- Labelled nucleotides are not incorporated as efficiently as native ones
- Short sequences
- Scalable set of machines suitable for nearly all the applications
- High throughput



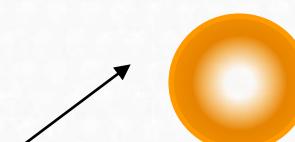
### 3. Second Generation Sequencing

#### Clonal amplification:

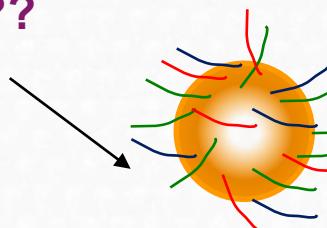
For emPCR based systems (Ion/PGM, 454)



Clonal amplification??



No empty beads



No beads containing more than one amplified fragment



- 1) Titration: constant number of beads vs. different DNA starting quantities
- 2) Optimal enrichment: one single fragment amplified millions of times in one single bead



### 3. Second Generation Sequencing

#### Illumina workflow.

[https://www.youtube.com/watch?annotation\\_id=annotation\\_1533942809&feature=iv&src\\_vid=HMyCqWhwB8E&v=fCd6B5HRaZ8](https://www.youtube.com/watch?annotation_id=annotation_1533942809&feature=iv&src_vid=HMyCqWhwB8E&v=fCd6B5HRaZ8)

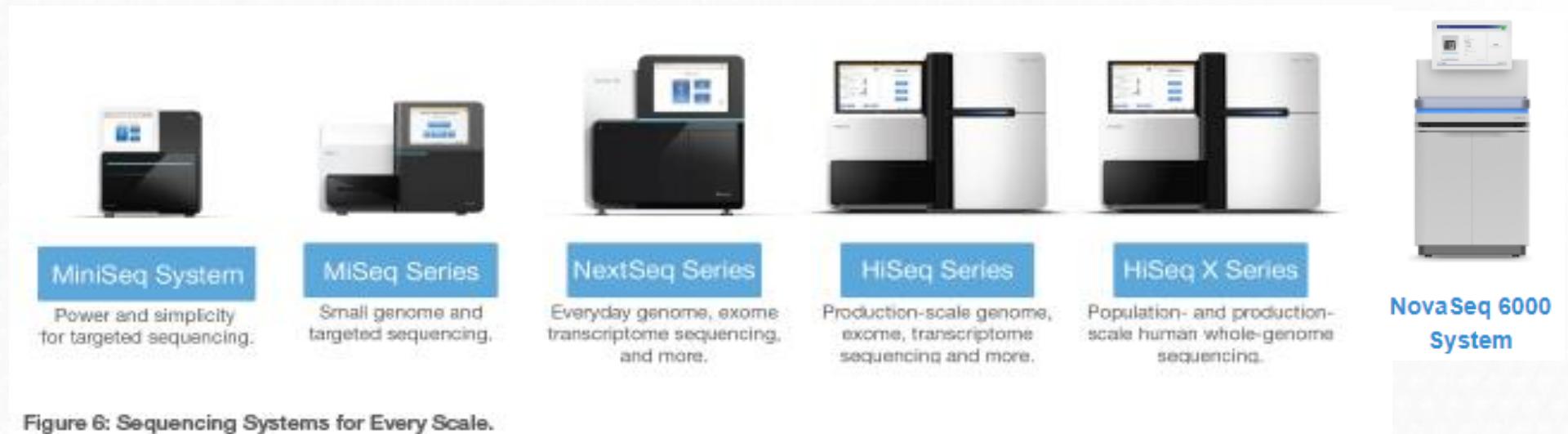
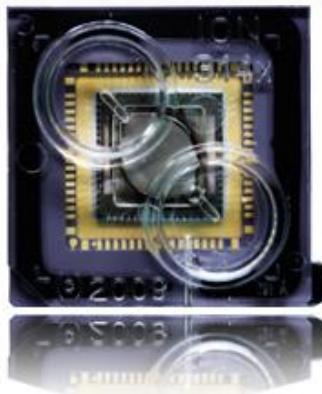


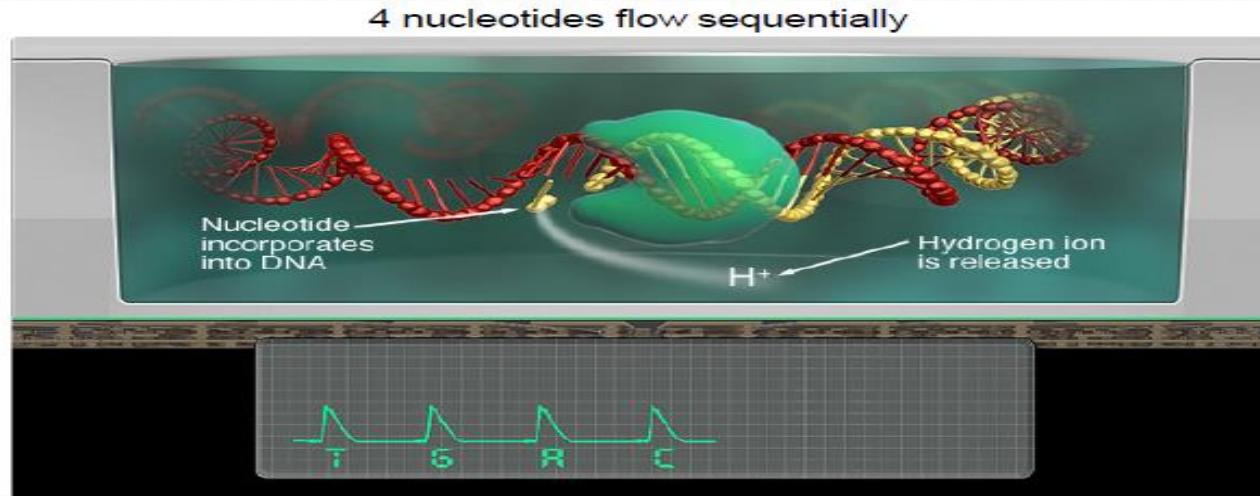
Figure 6: Sequencing Systems for Every Scale.

### 3. Second Generation Sequencing

#### Sequencing: Ion S5/PGM



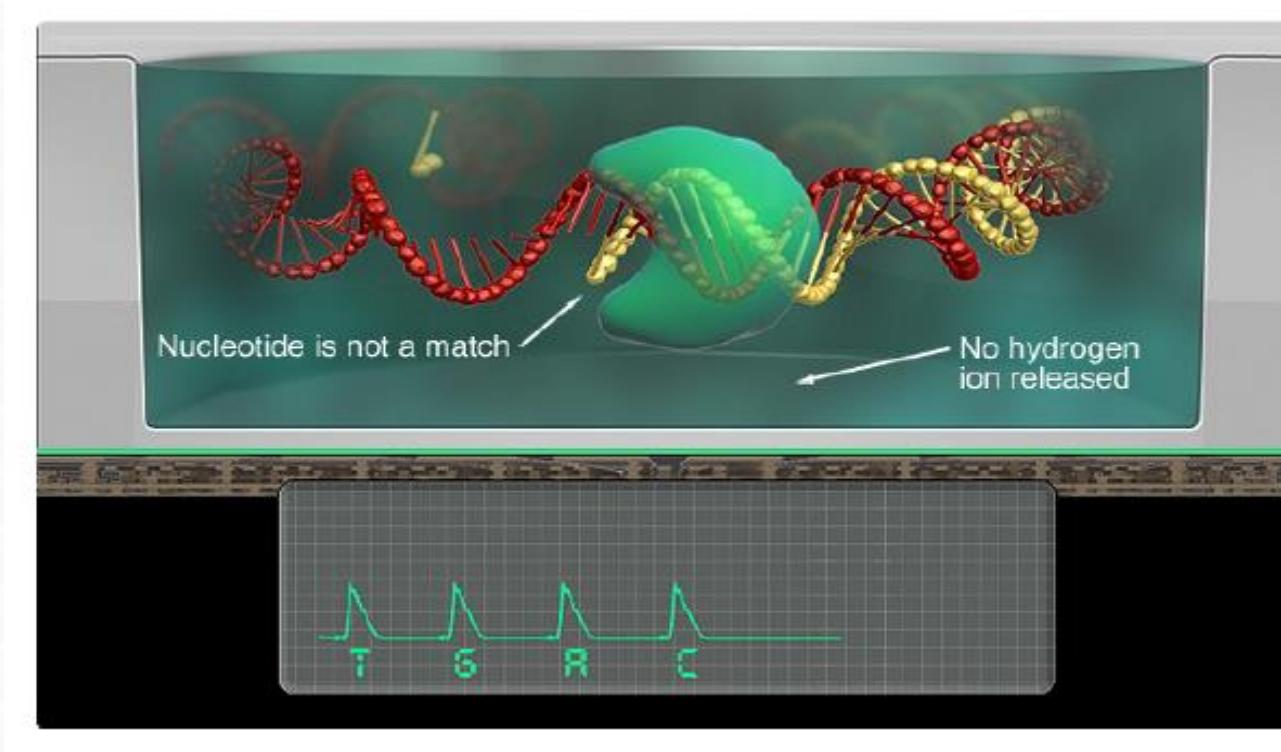
Beads containing the clonally amplified library are loaded onto the chip.  
The chip is run in the machine



No camera, just a pH sensor

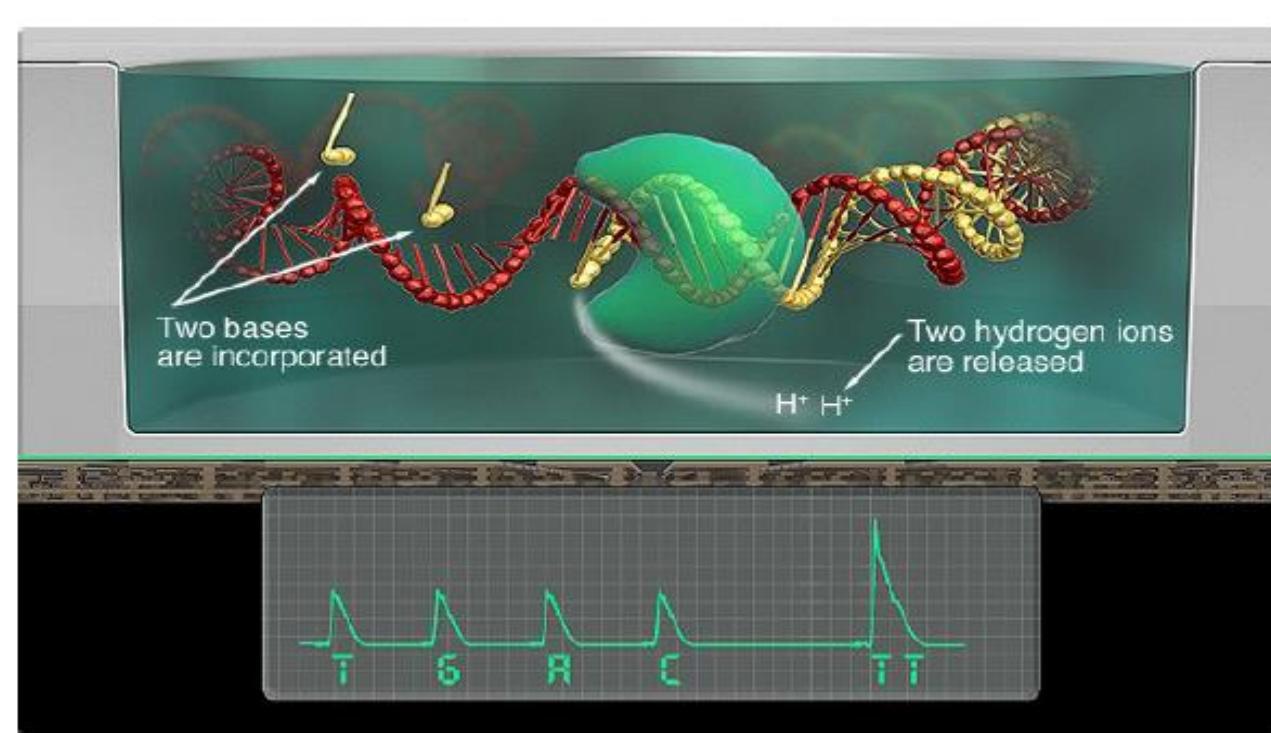
### 3. Second Generation Sequencing

#### Sequencing: Ion Torrent



### 3. Second Generation Sequencing

#### Sequencing: Ion Torrent



[https://www.youtube.com/watch?v=zBPKj0mMcDg&ab\\_channel=ThermoFisherScientific](https://www.youtube.com/watch?v=zBPKj0mMcDg&ab_channel=ThermoFisherScientific)

### 3. Second Generation Sequencing

#### Small NGS platforms



GS Junior Plus (Roche)

Read Length: **700 pb**  
Output: 70 Mb  
Running time: 18 hours



Ion Torrent PGM (LifeTechnol.)

Read length: 200 to 400 bp  
Output: 30Mb – 2Gb  
Running time: **2,3 - 4,4 hours**



MiniSeq (Illumina)

Read Length: 2x150 bp  
Output: **0.6 - 7.5 Gb**  
Running time: 4 - 24 hours

#### Applications:

- Amplicon sequencing
- Targeted sequencing (DNA / RNA)
- Metagenomics (16S)

### 3. Second Generation Sequencing

#### High throughput NGS Platforms



GS FLX+

Read length: **700 bp**  
Output: 1 Mb  
Running time: 23 hours



Ion Gene Studio S5 Prime System

Read length: Up to 200 bp  
Output: 50Gb/day (2 chips)  
Run time: **8,5 hours**



**NovaSeq 6000  
System**

Read length: 2x150 bp  
Output: **6.000 Gb**  
Run time: 44 hours

#### Applications:

- Amplicon sequencing
- Targeted sequencing
- Metagenomics (16S)
- Genomes
- Exomes, transcriptome

### 3. Second Generation Sequencing

## High throughput NGS Platforms

	iSeq 100 System	MiniSeq System	MiSeq Series +	NextSeq Series +
Popular Applications & Methods	Key Application	Key Application	Key Application	Key Application
Large Whole-Genome Sequencing (human, plant, animal)				●
Small Whole-Genome Sequencing (microbe, virus)	●	●	●	●
Exome Sequencing				●
Targeted Gene Sequencing (amplicon, gene panel)	●	●	●	●
Whole-Transcriptome Sequencing				●
Gene Expression Profiling with mRNA-Seq				●
Targeted Gene Expression Profiling	●	●	●	
Long-Range Amplicon Sequencing*	●	●	●	
miRNA & Small RNA Analysis	●	●	●	●
DNA-Protein Interaction Analysis			●	●
Methylation Sequencing				●
16S Metagenomic Sequencing		●	●	●

### 3. Second Generation Sequencing

## High throughput NGS Platforms

	 NextSeq Series <small>⊕</small>	 HiSeq 4000 System	 HiSeq X Series <sup>‡</sup>	 NovaSeq 6000 System
Popular Applications & Methods	Key Application 	Key Application 	Key Application 	Key Application 
Large Whole-Genome Sequencing (human, plant, animal)	●	●	●	●
Small Whole-Genome Sequencing (microbe, virus)	●	●		●
Exome Sequencing	●	●		●
Targeted Gene Sequencing (amplicon, gene panel)	●	●		●
Whole-Transcriptome Sequencing	●	●		●
Gene Expression Profiling with mRNA-Seq	●	●		●
miRNA & Small RNA Analysis	●	●		●
DNA-Protein Interaction Analysis	●	●		●
Methylation Sequencing	●	●		●
Shotgun Metagenomics	●	●		●

### 3. Second Generation Sequencing

## High throughput NGS Platforms



**Ion GeneStudio S5 Prime System**

Turnaround time: 6.5 hr\*

	Ion 510™ Chip	Ion 520™ Chip	Ion 530™ Chip	Ion 540™ Chip	Ion 550™ Chip
Max. output (reads)	3 M	6 M	20 M	80 M	130 M
Targeted DNA sequencing ** e.g., Ion Torrent™ Oncomine™ Focus Assay	•	•	•	•	•
Small genome sequencing† e.g., Bacterial typing using Ion Xpress™ Plus Fragment Library Kit		•	•		
16S metagenomics sequencing†† e.g., Ion 16S™ Metagenomics Kit		•	•		
Exome sequencing e.g., Ion AmpliSeq™ Exome Panel				•	•
Targeted RNA sequencing e.g., Ion AmpliSeq™ made-to-order RNA panels	•	•	•	•	•
miRNA/small RNA profiling e.g., Ion Total RNA-Seq v2 Kit	•	•	•		
Targeted transcriptome sequencing e.g., Ion AmpliSeq™ Transcriptome Human Gene Expression Kit				•	•
Whole transcriptome sequencing e.g., Ion Total RNA-Seq v2 Kit				•	•
Low-pass whole genome sequencing (PGS) e.g., Ion ReproSeq™ PGS Kit	•	•	•		

Five Ion Torrent™ sequencing chips achieve 2–130 M reads per run (or 2–260 M reads per day) to enable a broad range of sequencing applications.



Targeted DNA sequencing



Targeted RNA sequencing



Microbial sequencing

#### Simplest & fastest\* workflow

Single day workflow from sample to annotated variants for gene panel sequencing featuring Ion AmpliSeq™ target technology, Ion PGM™ System, and the automated Ion Chef™ System\*\*.

#### Most accurate for multiple gene panels

Up to 100% sensitivity for multiple gene panels, with Torrent Suite™ software and an improved variant calling algorithm that provides high-quality consensus accuracy for SNP detection.

#### Cost to buy & run

Affordable sequencing with Ion PGM™ v2 chips that dramatically reduce cost per sample and the Ion PGM™ System that is a fraction of the cost of the alternative.

- 1. Introduction to NGS**
- 2. First Generation Sequencing**
- 3. Second Generation Sequencing**
- 4. Third Generation Sequencing**
- 5. Sequencing generation face to face**
- 6. Applications of NGS techniques**
- 7. A (very) brief introduction to DoE**

## 4. Third Generation Sequencing

### Single molecule sequencing

#### Advantages:

- Less sample preparation (no PCR)
- No amplification
  - ✓ No PCR errors
  - ✓ Fewer contamination issues
  - ✓ No GC-bias
  - ✓ Analyze every sample (unPCRable, unclonable)
  - ✓ Analyze low quality DNA (forensics samples, archeological)
- Absolute quantification
- Sequence RNA directly

## 4. Third Generation Sequencing

### Helicos Genetic Analysis system



Workflow similar to Illumina, but  
**without amplification:**

- relative slow and expensive
- short reads
- **single molecule sequencing**

	<b>Helicos</b>
<b>Read Length</b>	35 bp
<b>Throughput</b>	35 Gb
<b>Reads per run</b>	600,000,000 - 1,000,000,000
<b>Accuracy</b>	97 %
<b>Run Time</b>	8 days

## 4. Third Generation Sequencing

### Helicos Genetic Analysis system



Workflow similar to Illumina, but  
**without amplification:**

- relative slow and expensive
- short reads
- **single molecule sequencing**

	<b>Helicos</b>
<b>Read Length</b>	35 bp
<b>Throughput</b>	35 Gb
<b>Reads per run</b>	600,000,000 - 1,000,000,000
<b>Accuracy</b>	97 %
<b>Run Time</b>	8 days

## 4. Third Generation Sequencing

### Sequel System (Pacific bioscience)



- Quick library construction (< 3 hours)
- Run time: from 30 min to 30 hours
- Sequencing by synthesis
- No library amplification
- **Long reads:** 5 Kb – 20 Kb

## 4. Third Generation Sequencing

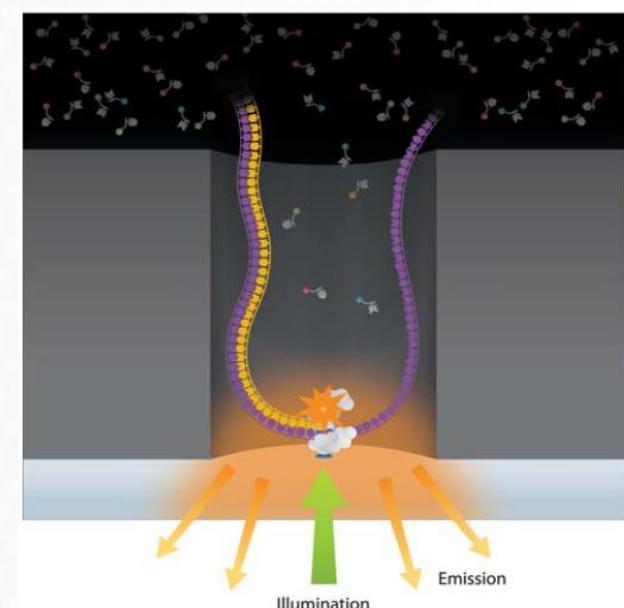
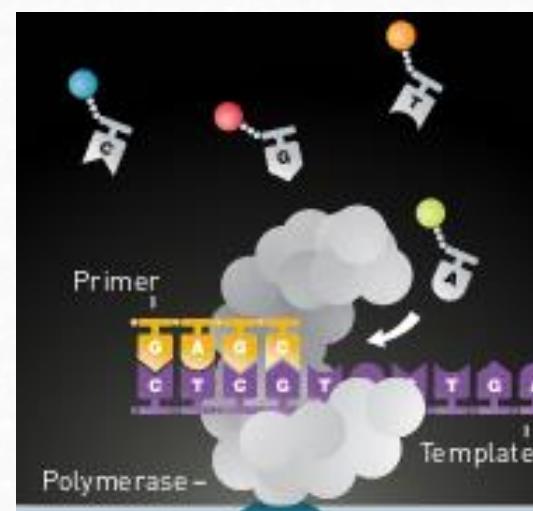
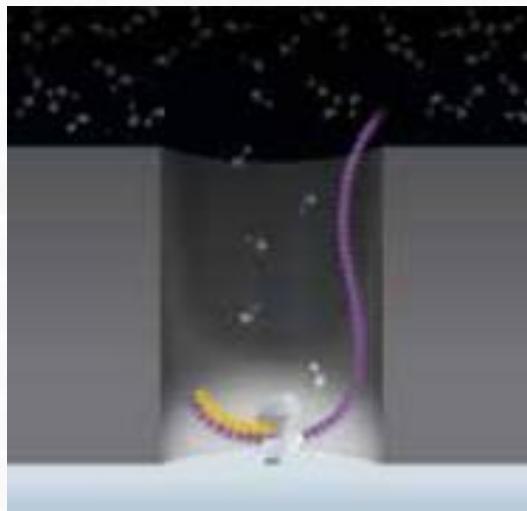
### Sequel System (Pacific bioscience)



- Quick library construction (< 3 hours)
- Run time: from 30 min to 30 hours
- Sequencing by synthesis
- No library amplification
- **Long reads: 5 Kb – 20 Kb**

## 4. Third Generation Sequencing

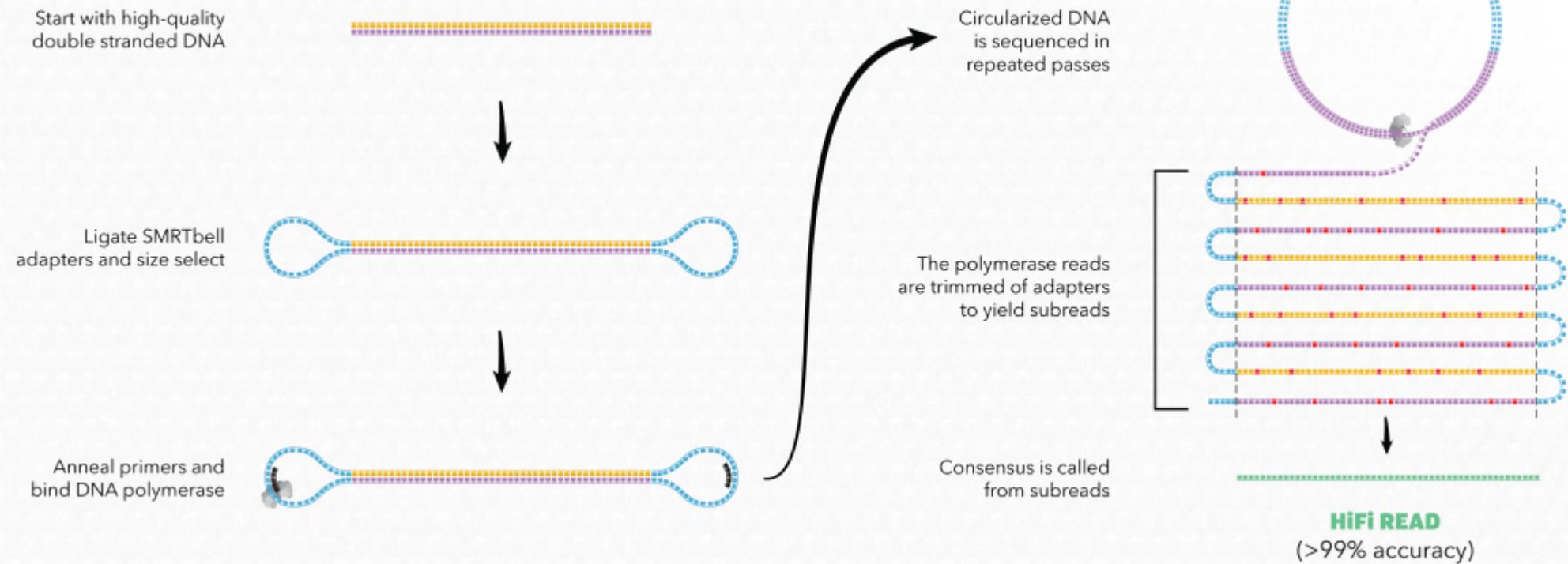
### Sequel System (Pacific bioscience)



[https://www.youtube.com/watch?v=\\_ID8JyAbwEo&feature=emb\\_rel\\_end&ab\\_channel=PacBio](https://www.youtube.com/watch?v=_ID8JyAbwEo&feature=emb_rel_end&ab_channel=PacBio)

## 4. Third Generation Sequencing

### Sequel System (Pacific bioscience)



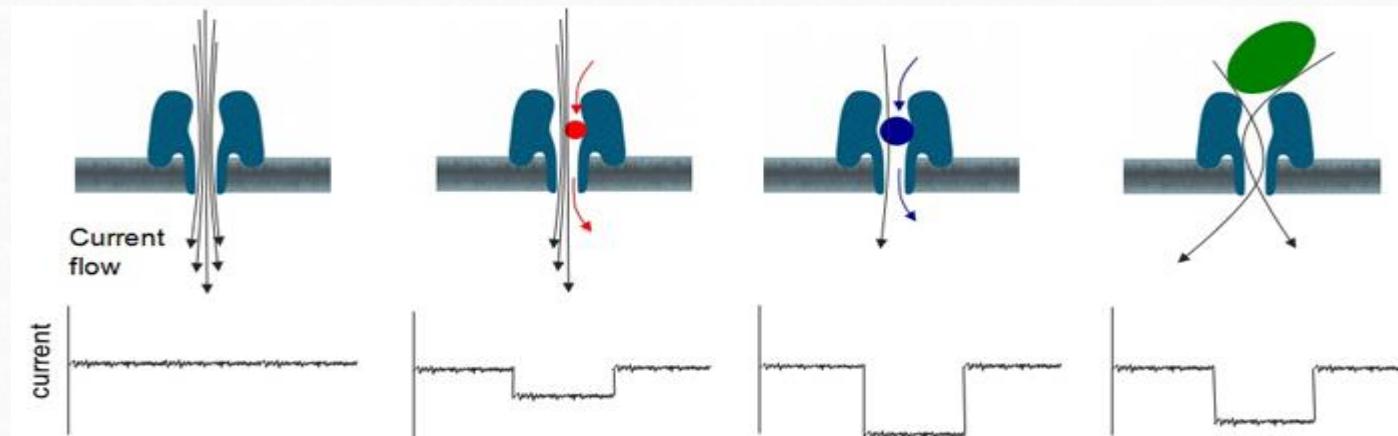
<https://ngisweden.scilifelab.se/technologies/pacific-biosciences/pacbio-sequel-ii/>

## 4. Third Generation Sequencing

### Oxford nanopore



- alpha-hemolysin
- Heptameric protein with a pore of inner diameter 1nm
- Pore diameter same scale of DNA
- Protein nanopores can be adapted.
- The company has optimised its large-scale production.
- DNA, RNA, miRNA and protein analysis
- Changes in membrane voltage are measured



## 4. Third Generation Sequencing

### Oxford nanopore

MinION



GridION



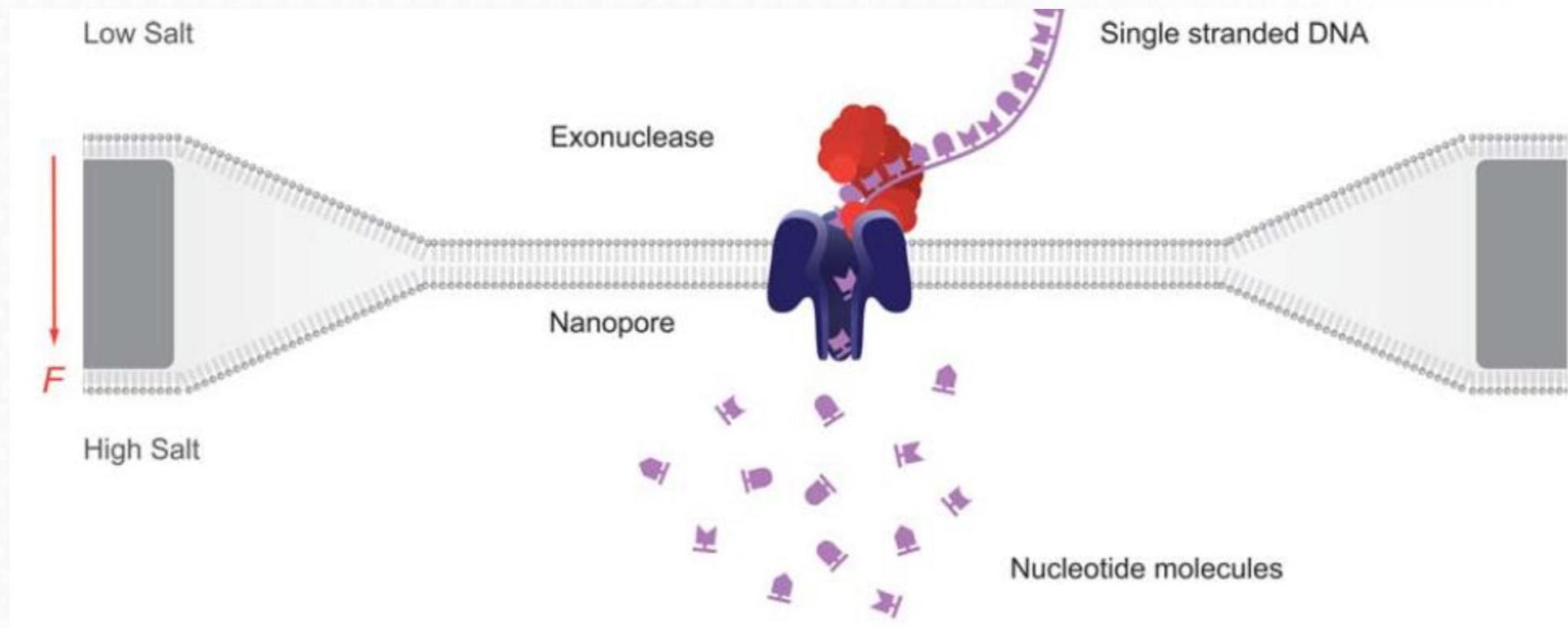
PromethION



- **Simple** 10 minute sample prep available
- Read length: **Ultra long** reads (2 Mb)
- Very fast, but **high error rates**.
- **Easy** to work in the field (Ebola viruses sequenced in Guinea 2 days after sample collection, Quick J, 2016)

## 4. Third Generation Sequencing

### Oxford nanopore



*Human Molecular Genetics, 2010, Vol. 19, Review Issue 2*

<https://www.youtube.com/watch?v=GUb1TZvMWsw>

## 4. Third Generation Sequencing

### 3rd generation instruments

Pacific  
Bioscience

High throughput



Sequel system

Oxford  
Nanopore  
Technologies



PromethION



GridION

Benchtop

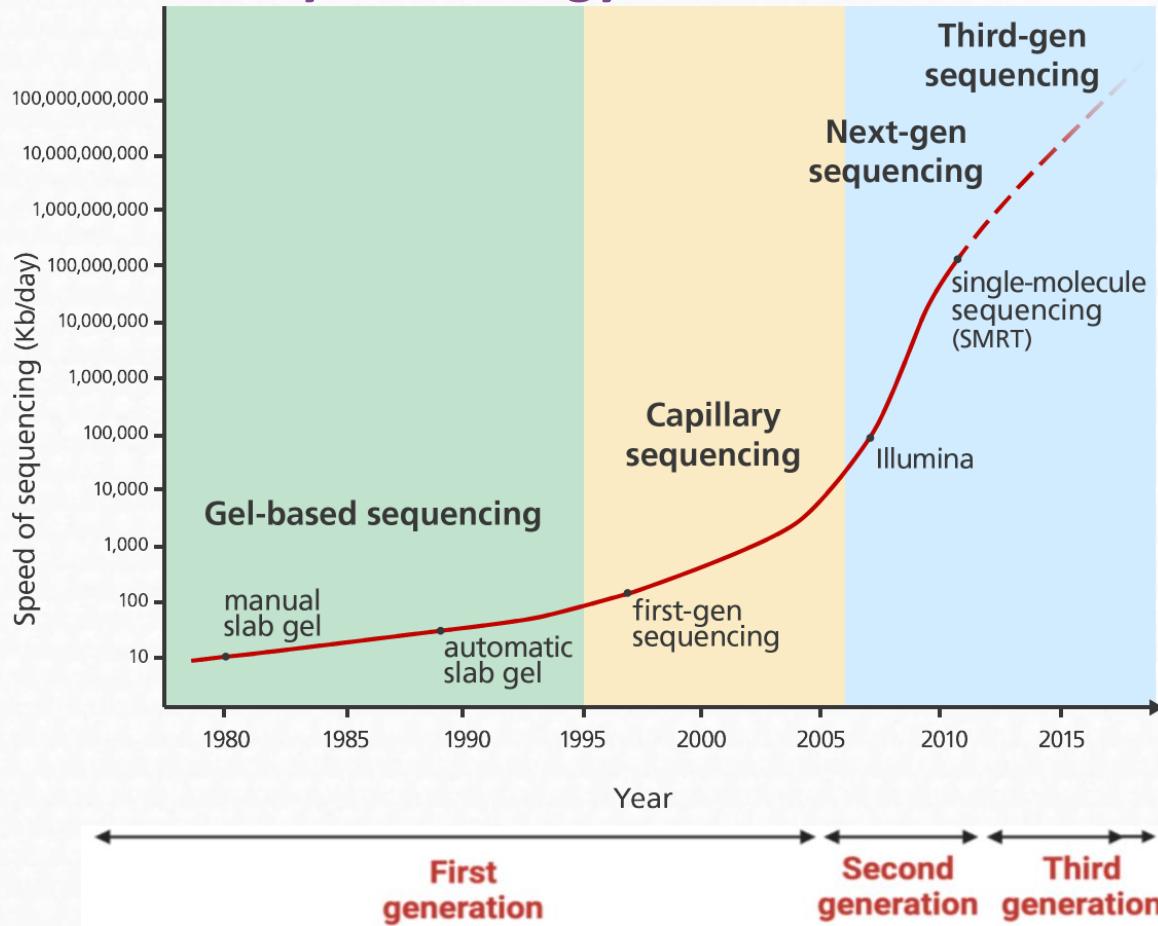


minION

- 1. Introduction to NGS**
- 2. First Generation Sequencing**
- 3. Second Generation Sequencing**
- 4. Third Generation Sequencing**
- 5. Sequencing generation face to face**
- 6. Applications of NGS techniques**
- 7. A (very) brief introduction to DoE**

## 5. Sequencing Generation face to face

### Speed variation by technology



<http://www.yourgenome.org/stories/third-generation-sequencing>  
<https://www.mdpi.com/2079-7737/12/7/997>

## 5. Sequencing Generation face to face

Table 1. Comparison of first-generation sequencing, SGS and TGS

	First generation	Second generation <sup>a</sup>	Third generation <sup>a</sup>
Fundamental technology	Size-separation of specifically end-labeled DNA fragments, produced by SBS or degradation	Wash-and-scan SBS	SBS, by degradation, or direct physical inspection of the DNA molecule
Resolution	Averaged across many copies of the DNA molecule being sequenced	Averaged across many copies of the DNA molecule being sequenced	Single-molecule resolution
Current raw read accuracy	High	High	Moderate
Current read length	Moderate (800–1000 bp)	Short, generally much shorter than Sanger sequencing	Long, 1000 bp and longer in commercial systems
Current throughput	Low	High	Moderate
Current cost	High cost per base Low cost per run	Low cost per base High cost per run	Low-to-moderate cost per base Low cost per run
RNA-sequencing method	cDNA sequencing	cDNA sequencing	Direct RNA sequencing and cDNA sequencing
Time from start of sequencing reaction to result	Hours	Days	Hours
Sample preparation	Moderately complex, PCR amplification not required	Complex, PCR amplification required	Ranges from complex to very simple depending on technology
Data analysis	Routine	Complex because of large data volumes and because short reads complicate assembly and alignment algorithms	Complex because of large data volumes and because technologies yield new types of information and new signal processing challenges
Primary results	Base calls with quality values	Base calls with quality values	Base calls with quality values, potentially other base information such as kinetics

## 5. Sequencing Generation face to face

Table 1. Comparison of first-generation sequencing, SGS and TGS

	First generation	Second generation <sup>a</sup>	Third generation <sup>a</sup>
Fundamental technology	Size-separation of specifically end-labeled DNA fragments, produced by SBS or degradation	Wash-and-scan SBS	SBS, by degradation, or direct physical inspection of the DNA molecule
Resolution	Averaged across many copies of the DNA molecule being sequenced	Averaged across many copies of the DNA molecule being sequenced	Single-molecule resolution
Current raw read accuracy	High	High	Moderate
Current read length	Moderate (800–1000 bp)	Short, generally much shorter than Sanger sequencing	Long, 1000 bp and longer in commercial systems
Current throughput	Low	High	Moderate
Current cost	High cost per base Low cost per run	Low cost per base High cost per run	Low-to-moderate cost per base Low cost per run
RNA-sequencing method	cDNA sequencing	cDNA sequencing	Direct RNA sequencing and cDNA sequencing
Time from start of sequencing reaction to result	Hours	Days	Hours
Sample preparation	Moderately complex, PCR amplification not required	Complex, PCR amplification required	Ranges from complex to very simple depending on technology
Data analysis	Routine	Complex because of large data volumes and because short reads complicate assembly and alignment algorithms	Complex because of large data volumes and because technologies yield new types of information and new signal processing challenges
Primary results	Base calls with quality values	Base calls with quality values	Base calls with quality values, potentially other base information such as kinetics

## 5. Sequencing Generation face to face

Table 1. Comparison of first-generation sequencing, SGS and TGS

	First generation	Second generation <sup>a</sup>	Third generation <sup>a</sup>
Fundamental technology	Size-separation of specifically end-labeled DNA fragments, produced by SBS or degradation	Wash-and-scan SBS	SBS, by degradation, or direct physical inspection of the DNA molecule
Resolution	Averaged across many copies of the DNA molecule being sequenced	Averaged across many copies of the DNA molecule being sequenced	Single-molecule resolution
Current raw read accuracy	High	High	Moderate
Current read length	Moderate (800–1000 bp)	Short, generally much shorter than Sanger sequencing	Long, 1000 bp and longer in commercial systems
Current throughput	Low	High	Moderate
Current cost	High cost per base Low cost per run	Low cost per base High cost per run	Low-to-moderate cost per base Low cost per run
RNA-sequencing method	cDNA sequencing	cDNA sequencing	Direct RNA sequencing and cDNA sequencing
Time from start of sequencing reaction to result	Hours	Days	Hours
Sample preparation	Moderately complex, PCR amplification not required	Complex, PCR amplification required	Ranges from complex to very simple depending on technology
Data analysis	Routine	Complex because of large data volumes and because short reads complicate assembly and alignment algorithms	Complex because of large data volumes and because technologies yield new types of information and new signal processing challenges
Primary results	Base calls with quality values	Base calls with quality values	Base calls with quality values, potentially other base information such as kinetics

## 5. Sequencing Generation face to face

Table 1. Comparison of first-generation sequencing, SGS and TGS

	First generation	Second generation <sup>a</sup>	Third generation <sup>a</sup>
Fundamental technology	Size-separation of specifically end-labeled DNA fragments, produced by SBS or degradation	Wash-and-scan SBS	SBS, by degradation, or direct physical inspection of the DNA molecule
Resolution	Averaged across many copies of the DNA molecule being sequenced	Averaged across many copies of the DNA molecule being sequenced	Single-molecule resolution
Current raw read accuracy	High	High	Moderate
Current read length	Moderate (800–1000 bp)	Short, generally much shorter than Sanger sequencing	Long, 1000 bp and longer in commercial systems
Current throughput	Low	High	Moderate
Current cost	High cost per base Low cost per run	Low cost per base High cost per run	Low-to-moderate cost per base Low cost per run
RNA-sequencing method	cDNA sequencing	cDNA sequencing	Direct RNA sequencing and cDNA sequencing
Time from start of sequencing reaction to result	Hours	Days	Hours
Sample preparation	Moderately complex, PCR amplification not required	Complex, PCR amplification required	Ranges from complex to very simple depending on technology
Data analysis	Routine	Complex because of large data volumes and because short reads complicate assembly and alignment algorithms	Complex because of large data volumes and because technologies yield new types of information and new signal processing challenges
Primary results	Base calls with quality values	Base calls with quality values	Base calls with quality values, potentially other base information such as kinetics

## 5. Sequencing Generation face to face

Table 1. Comparison of first-generation sequencing, SGS and TGS

	First generation	Second generation <sup>a</sup>	Third generation <sup>a</sup>
Fundamental technology	Size-separation of specifically end-labeled DNA fragments, produced by SBS or degradation	Wash-and-scan SBS	SBS, by degradation, or direct physical inspection of the DNA molecule
Resolution	Averaged across many copies of the DNA molecule being sequenced	Averaged across many copies of the DNA molecule being sequenced	Single-molecule resolution
Current raw read accuracy	High	High	Moderate
Current read length	Moderate (800–1000 bp)	Short, generally much shorter than Sanger sequencing	Long, 1000 bp and longer in commercial systems
Current throughput	Low	High	Moderate
Current cost	High cost per base Low cost per run	Low cost per base High cost per run	Low-to-moderate cost per base Low cost per run
RNA-sequencing method	cDNA sequencing	cDNA sequencing	Direct RNA sequencing and cDNA sequencing
Time from start of sequencing reaction to result	Hours	Days	Hours
Sample preparation	Moderately complex, PCR amplification not required	Complex, PCR amplification required	Ranges from complex to very simple depending on technology
Data analysis	Routine	Complex because of large data volumes and because short reads complicate assembly and alignment algorithms	Complex because of large data volumes and because technologies yield new types of information and new signal processing challenges
Primary results	Base calls with quality values	Base calls with quality values	Base calls with quality values, potentially other base information such as kinetics

# 5. Sequencing Generation face to face

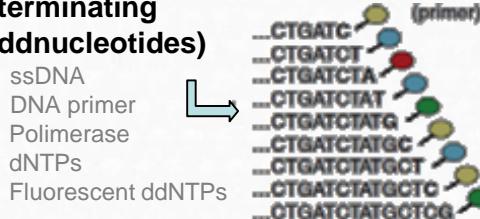
## 1. DNA fragmentation.



## 2. Vector cloning, bacterial transformation and growth, DNA isolation and purification

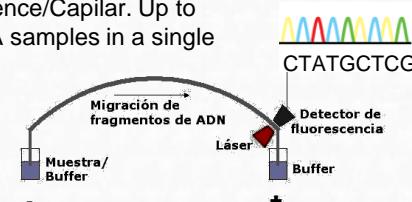


## 3. Sequencing (chain-terminating ddNucleotides)



## 4. Image processing

Capillary electrophoresis  
(1 Sequence/Capilar. Up to 384 DNA samples in a single run)



**FGS**

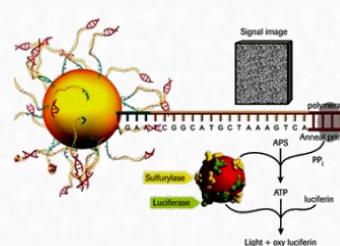
## 1. DNA fragmentation



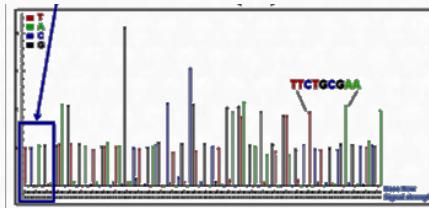
## 2. In vitro adaptor ligation + clonal amplification



## 3. Massive parallel sequencing



## 4. Image processing and data analysis

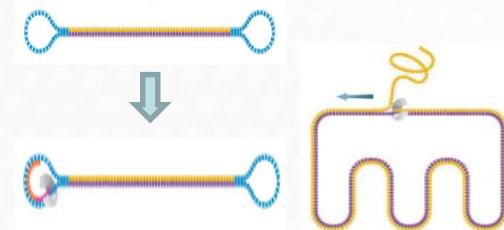


**SGS**

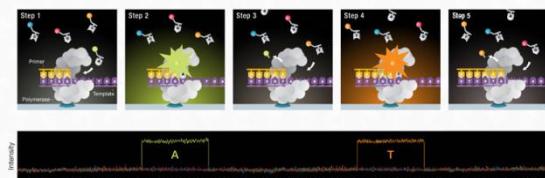
## 1. DNA fragmentation



## 2. y 3. in vitro adaptor ligation. NO AMPLIFICATION. Massive parallel sequencing.



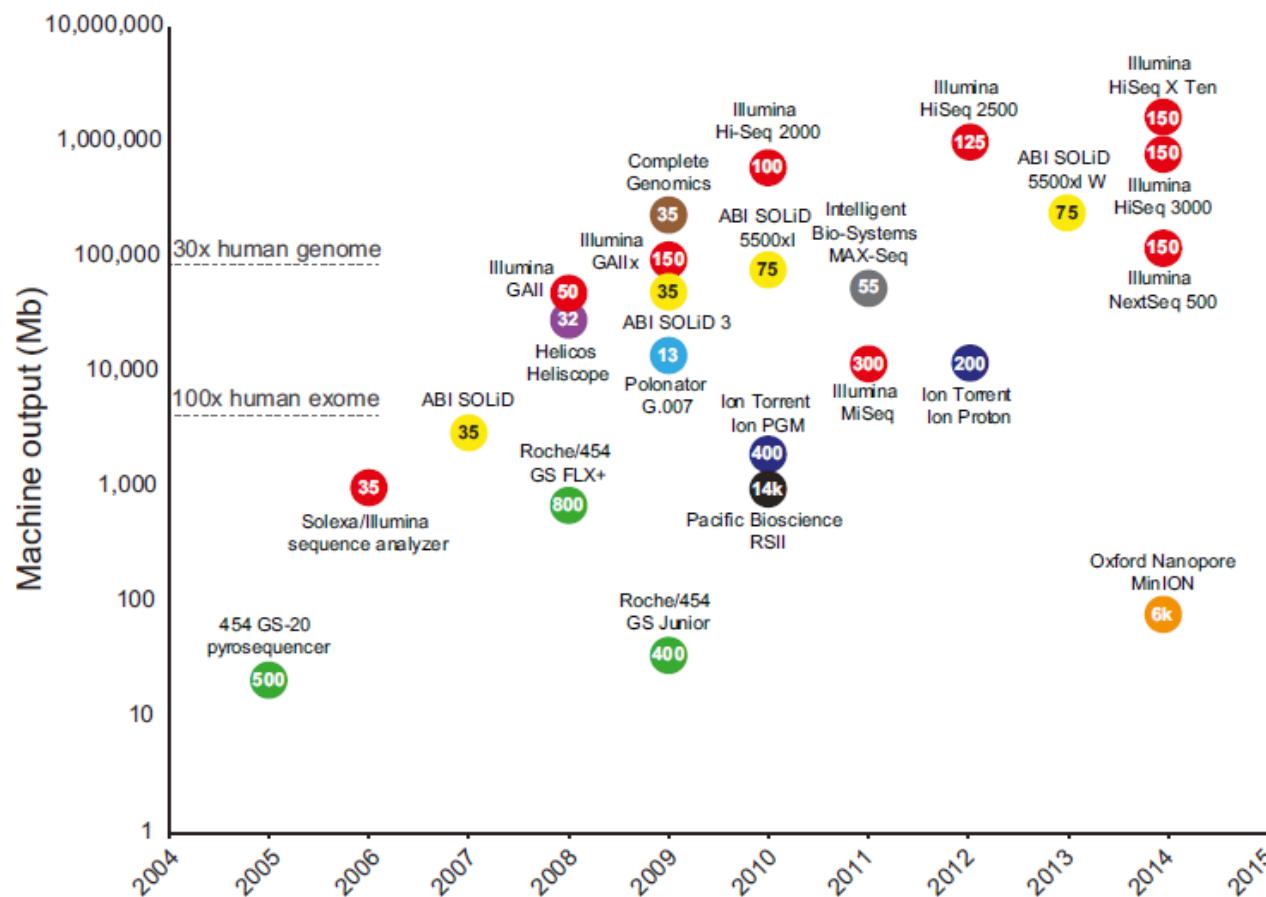
## 4. Image processing and data analysis.



**TGS**

## 5. Sequencing Generation face to face

Release dates vs Machine outputs per run



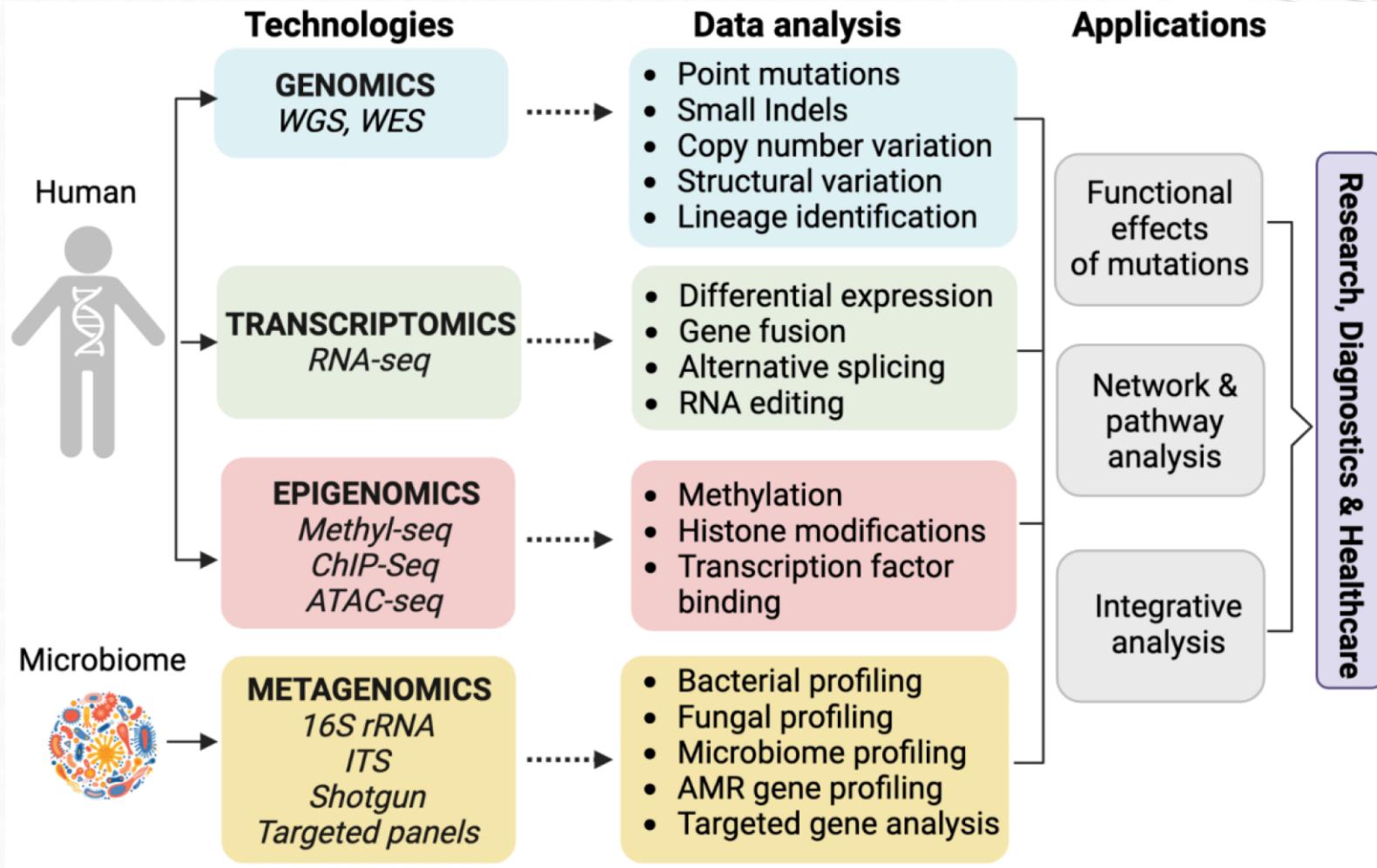
Molecular Cell 58, May 21, 2015 ©2015 Elsevier Inc.

- 1. Introduction to NGS**
- 2. First Generation Sequencing**
- 3. Second Generation Sequencing**
- 4. Third Generation Sequencing**
- 5. Sequencing generation face to face**
- 6. Applications of NGS techniques**
- 7. A (very) brief introduction to DoE**

## 6. Applications of NGS techniques



## 6. Applications of NGS techniques



## 6. Applications of NGS techniques

**Table 1. Selected HTS Methods**

Method	Purpose	Reference
RNA-seq	Transcript analysis	Nagalakshmi et al., 2008
Global run-on sequencing (GRO-seq)	Transcription	Core et al., 2008
Nascent-seq	Transcription	Khodor et al., 2011
Native elongating transcript sequencing (NET-seq)	Transcription	Churchman and Weissman, 2011
Ribo-seq	Translation	Ingolia et al., 2009
Replication sequencing (Repli-seq)	Replication	Hansen et al., 2010
Hi-C	Chromatin conformation	Lieberman-Aiden et al., 2009
Chromatin interaction analysis by paired-end tag sequencing (ChIA-PET)	Chromatin conformation	Fullwood et al., 2009
Chromosome conformation capture carbon copy (5-C)	Chromatin conformation	Dostie et al., 2006
Chromatin isolation by RNA purification sequencing (ChIRP-seq)	Genome localization	Chu et al., 2011
Reduced representation bisulphite sequencing (RRBS-seq)	Genome methylation	Meissner et al., 2008
Bisulfite sequencing (BS-seq)	Genome methylation	Cokus et al., 2008
DNase-seq	Open chromatin	Crawford et al., 2006
Assay for transposase-accessible chromatin using sequencing (ATAC-seq)	Open chromatin	Buenrostro et al., 2013
Parallel Analysis of RNA structure (PARS)	RNA structure	Kertesz et al., 2010
Structure-seq	RNA structure	Ding et al., 2014
RNA on a massively parallel array (RNA-MaP)	RNA-protein interactions	Buenrostro et al., 2014
RNA immunoprecipitation sequencing (RIP-seq)	RNA-protein interactions	Sephton et al., 2011
Parallel analysis of RNA ends sequencing (PARE-seq)	microRNA target discovery	German et al., 2008
Massively parallel functional dissection sequencing (MPFD)	Enhancer assay	Patwardhan et al., 2012

## 6. Applications of NGS techniques

### Depth of Coverage (DNA)

#### Estimate of Coverage Requirements by Application Type

Application Type	Coverage
DNA-Seq (Re-Sequencing)	30 - 80X
DNA-Seq (De novo assembly)	100X
SNP Analysis / Rearrangement Detection	10 - 30X
Exome	100 - 200X
ChIP-Seq	10 - 40X

### Depth of Coverage (RNA)

Sample Type	Reads Needed for Differential Expression (millions)	Reads Needed for Rare Transcript or De Novo Assembly (millions)	Read Length
Small Genomes (i.e. Bacteria / Fungi)	5	30 - 65	50 SR or PE for positional info
Intermediate Genomes (i.e. Drosophila / C. Elegans)	10	70 - 130	50 – 100 SR or PE for positional info
Large Genomes (i.e. Human / Mouse)	15 - 25	100 - 200	>100 SR or PE for positional info

## 6. Applications of NGS techniques

### Depth of Coverage (DNA)

#### Estimate of Coverage Requirements by Application Type

Application Type	Coverage
DNA-Seq (Re-Sequencing)	30 - 80X
DNA-Seq (De novo assembly)	10X
SNP Analysis / Rearrangement detection	200X
Exome	200X
ChIP-Seq	10X

**Depth of Coverage:** average number of reads that align to, or "cover," known reference bases.

**30x** = each base has been covered by 30 sequences (in average)

### Depth of Coverage (RNA)

Sample Type	Reads Needed for Differential Expression (millions)	Reads Needed for Rare Transcript or De Novo Assembly (millions)	Read Length
Small Genomes (i.e. Bacteria / Fungi)	5	30 - 65	50 SR or PE for positional info
Intermediate Genomes (i.e. Drosophila / C. Elegans)	10	70 - 130	50 – 100 SR or PE for positional info
Large Genomes (i.e. Human / Mouse)	15 - 25	100 - 200	>100 SR or PE for positional info

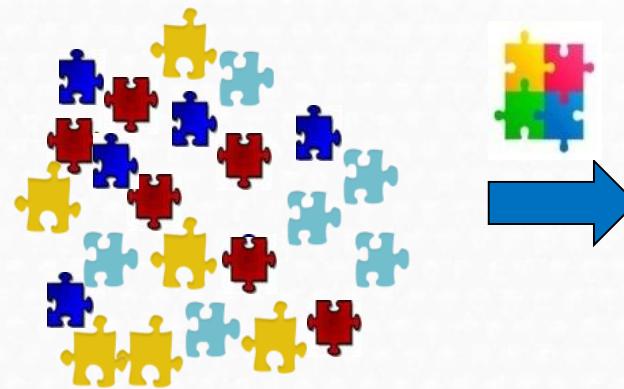
# 6. Applications of NGS techniques

Category	Detection or Application	Recommended Coverage (x) or Reads (millions)	References
Whole genome sequencing	<a href="#">Homozygous SNVs</a>	15x	Bentley et al., 2008
	<a href="#">Heterozygous SNVs</a>	33x	Bentley et al., 2008
	<a href="#">INDELS</a>	60x	Feng et al., 2014
	<a href="#">Genotype calls</a>	35x	Ajay et al., 2011
	<a href="#">CNV</a>	1-8x	Xie et al., 2009; Medvedev et al., 2010
Whole exome sequencing	<a href="#">Homozygous SNVs</a>	100x (3x local depth)	Clark et al., 2011; Meynert et al., 2013
	<a href="#">Heterozygous SNVs</a>	100x (13x local depth)	Clark et al., 2011; Meynert et al., 2013
	<a href="#">INDELS</a>	not recommended	Feng et al., 2014
Transcriptome Sequencing	<a href="#">Differential expression profiling</a>	10-25M	Liu Y. et al., 2014; ENCODE 2011 RNA-Seq
	<a href="#">Alternative splicing</a>	50-100M	Liu Y. et al., 2013; ENCODE 2011 RNA-Seq
	<a href="#">Allele specific expression</a>	50-100M	Liu Y. et al., 2013; ENCODE 2011 RNA-Seq
	<a href="#">De novo assembly</a>	>100M	Liu Y. et al., 2013; ENCODE 2011 RNA-Seq
DNA Target-Based Sequencing	<a href="#">ChIP-Seq</a>	10-14M (sharp peaks); 20-40M (broad marks)	Rozowsky et al., 2009; ENCODE 2011 Genome; Landt et al., 2012
	<a href="#">Hi-C</a>	100M	Belton, J.M et al., 2012
	<a href="#">4C (Circularized Chromosome Confirmation Capture)</a>	1-5M	van de Weken, H.J.G. et al., 2012
	<a href="#">5C (Chromosome Carbon Capture Carbon Copy)</a>	15-25M	Sanyal A. et al., 2012
	<a href="#">ChIA-PET (Chromatin Interaction Analysis by Paired-End Tag Sequencing)</a>	15-20M	Zhang, J. et al., 2012
	<a href="#">FAIRE-Seq</a>	25-55M	ENCODE 2011 Genome; Landt et al., 2012
DNA Methylation Sequencing	<a href="#">DNAse 1-Seq</a>	25-55M	Landt et al., 2012
	<a href="#">CAP-Seq</a>	>20M	Long, H.K. et al., 2013
	<a href="#">MeDIP-Seq</a>	60M	Taiwo, O. et al., 2012
	<a href="#">RRBS (Reduced Representation Bisulfite Sequencing)</a>	10X	ENCODE 2011 Genome
	<a href="#">Bisulfite-Seq</a>	5-15X; 30X	Ziller, M.J et al., 2015; Epigenomics Road Map
RNA-Target-Based Sequencing	<a href="#">CLIP-Seq</a>	10-40M	Cho J. et al., 2012; Eom T. et al., 2013; Sugimoto Y. et al., 2012
	<a href="#">iCLIP</a>	5-15M	Sugimoto Y. et al., 2012; Rogelj B. et al., 2012
	<a href="#">PAR-CLIP</a>	5-15M	Rogelj B. et al., 2012
	<a href="#">RIP-Seq</a>	5-20M	Lu Z. et al., 2014
Small RNA (microRNA) Sequencing	<a href="#">Differential Expression</a>	~1-2M	Metpally RPR et al., 2013; Campbell et al., 2015
	<a href="#">Discovery</a>	~5-8M	Metpally RPR et al., 2013; Campbell et al., 2015

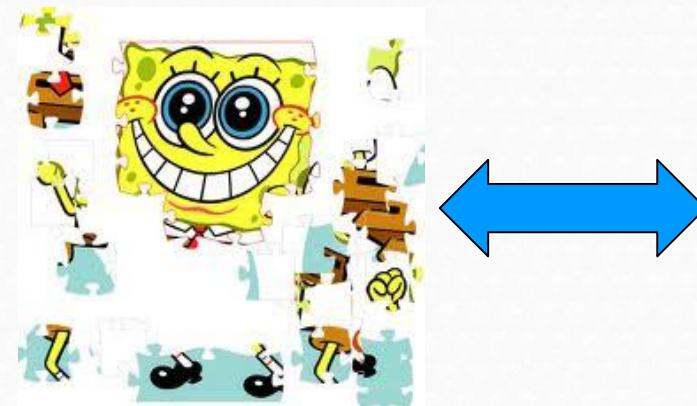
## 6. Applications of NGS techniques

### Whole Genome sequencing

*De novo* sequencing



Resequencing



## 6. Applications of NGS techniques

### Whole Genome sequencing

- Complete characterization of the **entire genome**.
- The rapid **drop in sequencing costs** allow researchers to sequence a genome **quickly**.

## 6. Applications of NGS techniques

### Whole Genome sequencing

#### PROS

- Global genome picture, no systematic missing of information
- Useful for diseases that involve multiple genetic phenomena

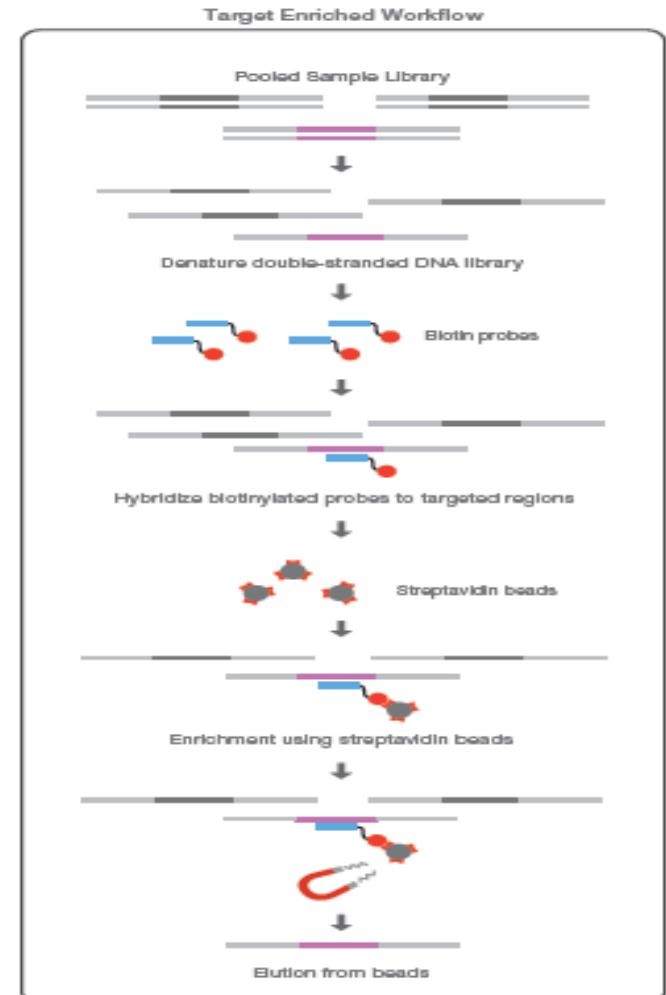
#### CONS:

- Can miss variants in the exonic regions due to **lower coverage**
- Some regions cannot be sequenced/assembled (repetitive and GC rich regions)
- More expensive and time consuming

## 6. Applications of NGS techniques

### Targeted sequencing

- Only a **subset of genes** or regions of the genome are isolated and sequenced.
- It allows to focus times, expenses and data analysis on specific areas of interest.
- Enables sequencing at **much higher coverage** levels.
- Target sequencing panels can be purchased with preselected content or can be custom designed.

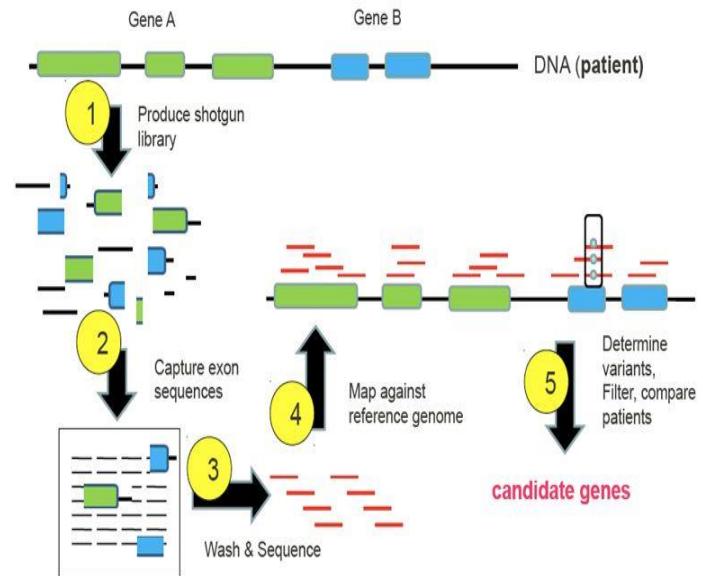


## 6. Applications of NGS techniques

### Exome sequencing

- Identifies **variants** across a wide range of applications
- Achieves comprehensive coverage of **coding regions**
- Provides a cost-effective alternative to whole-genome sequencing (4–5 Gb of sequencing per exome compared to ~90 Gb per whole human genome)
- Produces a smaller, more manageable data set for faster, easier analysis compared to whole-genome approaches

### Exome sequencing procedure



## 6. Applications of NGS techniques

### RNA-seq Expression Analysis

- Sequencing every **RNA** molecule and profiling the expression of a particular gene by counting the number of time its transcripts have been sequenced.
- Sensitivity of sequence based studies is limited by the **depth of sequencing**
- Applications:
  - **Differential gene expression** analysis (DGE)
  - **Splice variants** (resolution at base-level)
  - **Detection of novel transcripts** and **isoforms**
  - **Detection of allele specific expression patterns**

## 6. Applications of NGS techniques

### RNA-seq Expression Analysis

- Sequencing every **RNA** molecule and profiling the expression of a particular gene by counting the number of time its transcripts have been sequenced.
- Sensitivity of sequence based studies is limited by the **depth of sequencing**
- Applications:
  - Differential gene expression analysis (DGE)
  - Splice variants (resolution at base-level)
  - Detection of novel transcripts and isoforms
  - Detection of allele specific expression patterns

TGS

## 6. Applications of NGS techniques

### Classes of RNA Molecules in Human Cells

#### Ribosomal RNA – rRNA

~80% of total RNA

- 28 S
- 18 S
- 5S and 5.8 S

#### Noncoding RNA - ncRNA

- tRNA
- snoRNA
- lincRNA
- miRNA
- Many, many others...

#### Mitochondrial RNA - mtRNA

#### Messenger RNA – mRNA

1-3% of Total RNA

- Highly expressed transcripts (>10,000 copies per cell)
- Rarely expressed transcripts (~1 copy per cell)

Very high dynamic range ( $10^5$  to  $10^7$ )

## 6. Applications of NGS techniques

### ChIP-seq

- analyze protein **interactions with DNA**: Transcription factors
- ChIP-seq combines **chromatin immunoprecipitation** (ChIP) with massively parallel **DNA sequencing** to identify the **binding sites** of DNA-associated proteins
- used to map **global binding sites** precisely for any protein of interest

## 6. Applications of NGS techniques

### Single cell RNA-seq (scRNA-seq)

- Allows **comparison of the transcriptomes of individual cells**
- Sequence the transcriptomes of **up to tens of thousands of individual cells** for a single project
- Results depend of protocol used
- Special attention to **single cell purification**
- **Low signal of weak expressed genes**
- Required **number of cells increases with the complexity** of the sample under investigation
- Requires specific **bioinformatic approaches**.

## 6. Applications of NGS techniques

### Single cell RNA-seq (scRNA-seq)

Different methods for isolating single cells from a cell suspension:

- **Fluorescence-activated cell sorting (FACS)**
- **Microfluidics**: allows separation and cDNA synthesis and transcriptome amplification
- **Mechanical micromanipulation/micropipetting**
- **Laser capture microdissection**

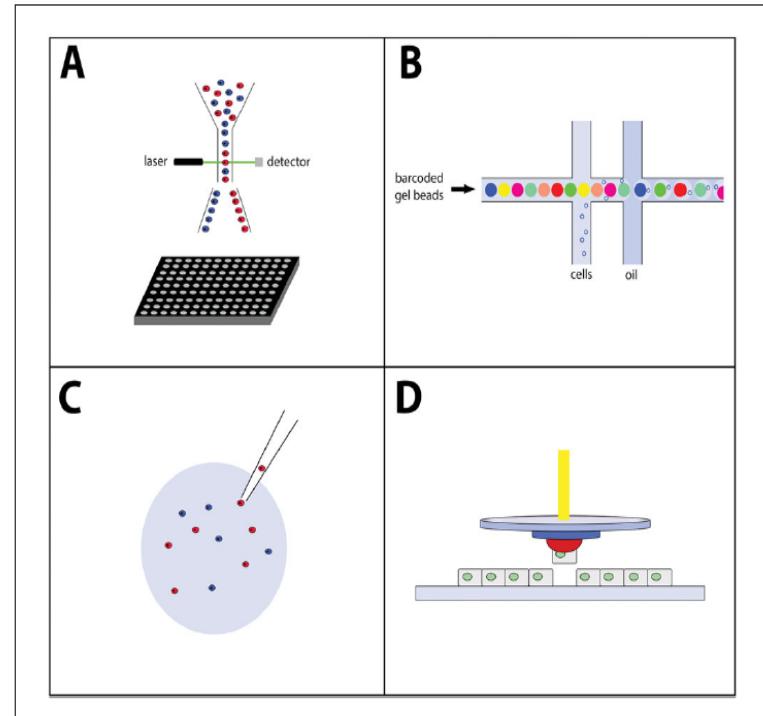


Figure 33.2.1 Single-cell isolation methods. (A) Fluorescence-activated cell sorting (FACS). Fluorescently labeled cells are exposed to a laser beam. Detectors identify cells with the desired fluorescence pattern, and the cells are sorted into plates, with one cell in each well. (B) Microdroplets. Cells and beads containing primers and reagents are enclosed in microdroplets. Downstream biochemical reactions occur within these droplets. (C) Micropipetting. Cells of interest are manually picked with a glass pipet under a microscope. (D) Laser capture microdissection (LCM). Cells on a glass slide are attached to a polymer by means of a laser beam. The polymer and cells of interest are then lifted from the slide and transferred to a microcentrifuge tube.

*Current Protocols in Molecular Biology e57*, April 2018

Published online April 2018 in Wiley Online Library ([wileyonlinelibrary.com](http://wileyonlinelibrary.com)).

doi: 10.1002/cpmb.57

Copyright © 2018 John Wiley & Sons, Inc.

## 6. Applications of NGS techniques

### Single cell RNA-seq (scRNA-seq)

Also different methods for cDNA transcription....

**TABLE 1 |** Summary of widely used scRNA-seq technologies.

Methods	Transcript coverage	UMI possibility	Strand specific	References
Tang method	Nearly full-length	No	No	Tang et al., 2009
Quartz-Seq	Full-length	No	No	Sasagawa et al., 2013
SUPeR-seq	Full-length	No	No	Fan X. et al., 2015
Smart-seq	Full-length	No	No	Ramskold et al., 2012
Smart-seq2	Full-length	No	No	Picelli et al., 2013
MATQ-seq	Full-length	Yes	Yes	Sheng et al., 2017
STRT-seq and STRT/C1	5'-only	Yes	Yes	Islam et al., 2011, 2012
CEL-seq	3'-only	Yes	Yes	Hashimshony et al., 2012
CEL-seq2	3'-only	Yes	Yes	Hashimshony et al., 2016
MARS-seq	3'-only	Yes	Yes	Jaitin et al., 2014
CytoSeq	3'-only	Yes	Yes	Fan H.C. et al., 2015
Drop-seq	3'-only	Yes	Yes	Macosko et al., 2015
InDrop	3'-only	Yes	Yes	Klein et al., 2015
Chromium	3'-only	Yes	Yes	Zheng et al., 2017
SPLiT-seq	3'-only	Yes	Yes	Rosenberg et al., 2018
sci-RNA-seq	3'-only	Yes	Yes	Cao et al., 2017
Seq-Well	3'-only	Yes	Yes	Gierahn et al., 2017
DroNC-seq	3'-only	Yes	Yes	Habib et al., 2017
Quartz-Seq2	3'-only	Yes	Yes	Sasagawa et al., 2018

## 6. Applications of NGS techniques

### Single cell RNA-seq (scRNA-seq)

Also different methods for reads mapping....

**TABLE 2 |** Tools for read mapping and expression quantification of scRNA-seq data.

Tools	Category	URL	References
TopHat2	Read mapping	<a href="https://ccb.jhu.edu/software/tophat/index.shtml">https://ccb.jhu.edu/software/tophat/index.shtml</a>	Kim et al., 2013
STAR	Read mapping	<a href="https://github.com/alexdobin/STAR">https://github.com/alexdobin/STAR</a>	Dobin and Gingeras, 2015
HISAT2	Read mapping	<a href="https://ccb.jhu.edu/software/hisat2/index.shtml">https://ccb.jhu.edu/software/hisat2/index.shtml</a>	Kim et al., 2015
Cufflinks	Expression quantification	<a href="https://github.com/cole-trapnell-lab/cufflinks">https://github.com/cole-trapnell-lab/cufflinks</a>	Trapnell et al., 2010
RSEM	Expression quantification	<a href="https://github.com/deweylab/RSEM">https://github.com/deweylab/RSEM</a>	Li and Dewey, 2011
StringTie	Expression quantification	<a href="https://github.com/gperteal/stringtie">https://github.com/gperteal/stringtie</a>	Pertea et al., 2015

## 6. Applications of NGS techniques

### Single cell RNA-seq (

Also different methods

**TABLE 2 |** Tools for read mapping and expression quantification of scRNA-seq data.

Tools	Category	URL
TopHat2	Read mapping	<a href="https://ccb.jhu.edu/software/tophat/index.shtml">https://ccb.jhu.edu/software/tophat/index.shtml</a>
STAR	Read mapping	<a href="https://github.com/alexdobin/STAR">https://github.com/alexdobin/STAR</a>
HISAT2	Read mapping	<a href="https://ccb.jhu.edu/software/hisat2/index.shtml">https://ccb.jhu.edu/software/hisat2/index.shtml</a>
Cufflinks	Expression quantification	<a href="https://github.com/cole-trapnell-lab/cufflinks">https://github.com/cole-trapnell-lab/cufflinks</a>
RSEM	Expression quantification	<a href="https://github.com/deweylab/RSEM">https://github.com/deweylab/RSEM</a>
StringTie	Expression quantification	<a href="https://github.com/gperteal/stringtie">https://github.com/gperteal/stringtie</a>

**TABLE 4 |** Differential expression analysis tools for RNA-seq data.

Methods	Category	URL	References
ROTS	Single cell	<a href="https://bioconductor.org/packages/release/bioc/html/ROTS.html">https://bioconductor.org/packages/release/bioc/html/ROTS.html</a>	Seyednasrollah et al., 2016
MAST	Single cell	<a href="https://github.com/RGLab/MAST">https://github.com/RGLab/MAST</a>	Finak et al., 2015
BCseq	Single cell	<a href="https://bioconductor.org/packages/devel/bioc/html/bcSeq.html">https://bioconductor.org/packages/devel/bioc/html/bcSeq.html</a>	Chen and Zheng, 2018
SCDE	Single cell	<a href="http://hms-dbm.github.io/scde/">http://hms-dbm.github.io/scde/</a>	Kharchenko et al., 2014
DEsingle	Single cell	<a href="https://bioconductor.org/packages/DEsingle">https://bioconductor.org/packages/DEsingle</a>	Miao et al., 2018
Census	Single cell	<a href="http://cole-trapnell-lab.github.io/monocle-release/">http://cole-trapnell-lab.github.io/monocle-release/</a>	Qiu et al., 2017
D3E	Single cell	<a href="https://github.com/hemberg-lab/D3E">https://github.com/hemberg-lab/D3E</a>	Delmans and Hemberg, 2016
BPSC	Single cell	<a href="https://github.com/ngliaivtr/BPSC">https://github.com/ngliaivtr/BPSC</a>	Vu et al., 2016
DESeq2	Bulk	<a href="https://bioconductor.org/packages/release/bioc/html/DESeq2.html">https://bioconductor.org/packages/release/bioc/html/DESeq2.html</a>	Love et al., 2014
edgeR	Bulk	<a href="https://bioconductor.org/packages/release/bioc/html/edgeR.html">https://bioconductor.org/packages/release/bioc/html/edgeR.html</a>	Robinson et al., 2010
Limma	Bulk	<a href="http://bioconductor.org/packages/release/bioc/html/limma.html">http://bioconductor.org/packages/release/bioc/html/limma.html</a>	Ritchie et al., 2015
Ballgown	Bulk	<a href="http://www.bioconductor.org/packages/release/bioc/html/ballgown.html">http://www.bioconductor.org/packages/release/bioc/html/ballgown.html</a>	Frazee et al., 2015

For differential expression analysis.....

## 6. Applications of NGS techniques

### Single cell RNA-seq (scRNA-seq)



HUMAN  
CELL  
ATLAS

To create comprehensive reference maps of all human cells

<https://www.humancellatlas.org/>

Mapping the Human Body  
at the Cellular Level

Community generated, multi-omic,  
open data processed by standardized pipelines

 4.5M  
CELLS

 33  
ORGANS

 289  
DONORS

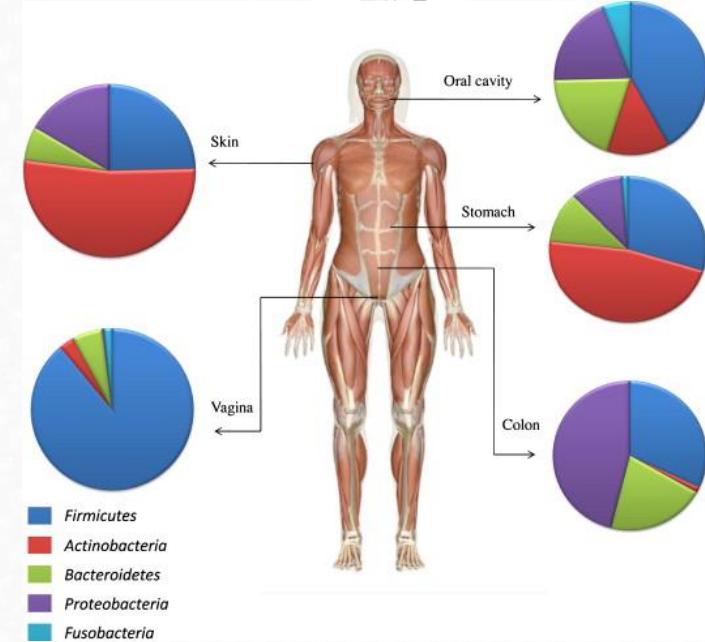
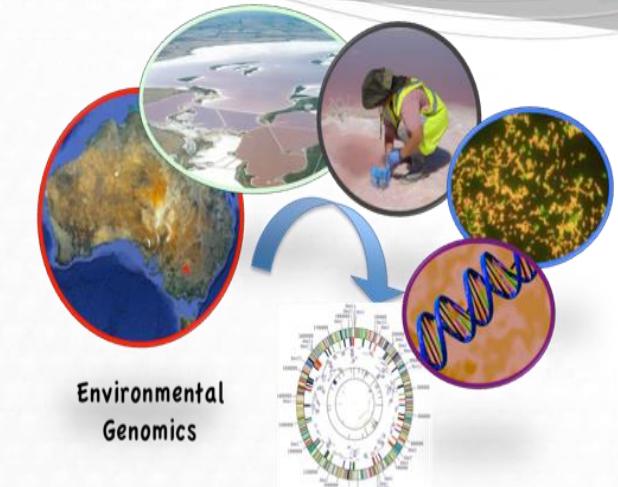
 28  
PROJECTS

 81  
LABS

## 6. Applications of NGS techniques

### Metagenomics

- Is a way to make an inventory of what (DNA) is present in a sample
- Two approaches:
  - Sequence it all
  - Focus on specific conserved sequences (ribosomal genes)
- Technology facilitates the study of the consequences of environmental changes and the causes of the changes.



- 1. Introduction to NGS**
- 2. First Generation Sequencing**
- 3. Second Generation Sequencing**
- 4. Third Generation Sequencing**
- 5. Sequencing generation face to face**
- 6. Applications of NGS techniques**
- 7. A (very) brief introduction to DoE**

## 7. A (very) brief introduction to DoE

The **(statistical) design of experiments** is an efficient procedure for planning experiments so that the data obtained can be analyzed to yield valid and objective conclusions

Why are many life scientists so adverse to thinking about design?



It is common to think that time spent designing experiments would be better spent actually doing experiments



## 7. A (very) brief introduction to DoE

### Variability types that play in an experiment:



- **Planned systematic variability:** This is the differences in response between treatments applied.



- **Noise variability:** random noise. Differences between two consecutive measures. We cannot avoid that.



- **Systematic variability not planned:** Produce a systematic variation in the results. A priori the reason is not known. It can be avoided with the *randomization* and the *local control*.

## 7. A (very) brief introduction to DoE

### Basic principles of DoE

Local control

Randomization

Replication

## 7. A (very) brief introduction to DoE

Sample	Treatment	Sex	Batch
1	A	Male	1
2	A	Male	1
3	A	Male	1
4	A	Male	1
5	B	Female	2
6	B	Female	2
7	B	Female	2
8	B	Female	2



Treatment are confounded  
between sex and batch

## 7. A (very) brief introduction to DoE

Sample	Treatment	Sex	Batch
1	A	Male	1
2	A	Male	1
3	A	Male	1
4	A	Male	1
5	B	Female	2
6	B	Female	2
7	B	Female	2
8	B	Female	2



Treatment are confounded  
between sex and batch

Sample	Treatment	Sex	Batch
1	A	Male	1
2	A	Female	2
3	A	Male	2
4	A	Female	1
5	B	Male	1
6	B	Female	2
7	B	Male	2
8	B	Female	1



Treatment is well balanced

## 7. A (very) brief introduction to DoE

### Important steps to define before begin the experiment:

- Establish the main **objectives** of the experiment. Avoid collateral problems
- Identify all the **noise** sources: Treatment, experimental errors,...
- **Allocate** each experimental unit which each treatment
- Clarify the **type of response** expected in each treatment
- Determinate the **number** of individuals in each group
- Run a **pilot study**



## 7. A (very) brief introduction to DoE

### Important steps to define before begin the experiment:

- Establish the main **objectives** of the experiment. Avoid collateral problems
- Identify all the **noise** sources: Treatment, experimental errors,...
- **Allocate** each experimental unit which each treatment
- Clarify the **type of response** expected in each treatment
- Determinate the **number** of individuals in each group
- Run a **pilot study**



## 7. A (very) brief introduction to DoE

### Important steps to define before begin the experiment:

- Establish the main **objectives** of the experiment. Avoid collateral problems
- Identify all the **noise** sources: Treatment, experimental errors,...
- Allocate each experimental unit which each treatment
- Clarify the **type of response** expected in each treatment
- Determinate the **number** of individuals in each group
- Run a **pilot study**



## 7. A (very) brief introduction to DoE

### Important steps to define before begin the experiment:

- Establish the main **objectives** of the experiment. Avoid collateral problems
- Identify all the **noise** sources: Treatment, experimental errors,...
- Allocate each experimental unit which each treatment
- Clarify the **type of response** expected in each treatment
- Determinate the **number** of individuals in each group
- Run a **pilot study**
- How the **data** will be statistically analysed.



## 7. A (very) brief introduction to DoE

**What do you need to ask before starting a NGS experiment?**

## 7. A (very) brief introduction to DoE

### What do you need to ask before starting a NGS experiment?

- What do I want to sequence? Whole genome, exome, metagenome, epigenome, RNAseq...
- How many samples?
- Length of read required?
- Quality and quantity of starting material?
- Size of nucleic acids to sequence
- Amount of sequence needed: **coverage**

- 1. Introduction to NGS**
- 2. First Generation Sequencing**
- 3. Second Generation Sequencing**
- 4. Third Generation Sequencing**
- 5. Sequencing generation face to face**
- 6. Applications of NGS techniques**
- 7. A (very) brief introduction to DoE**