High throughput sequencing

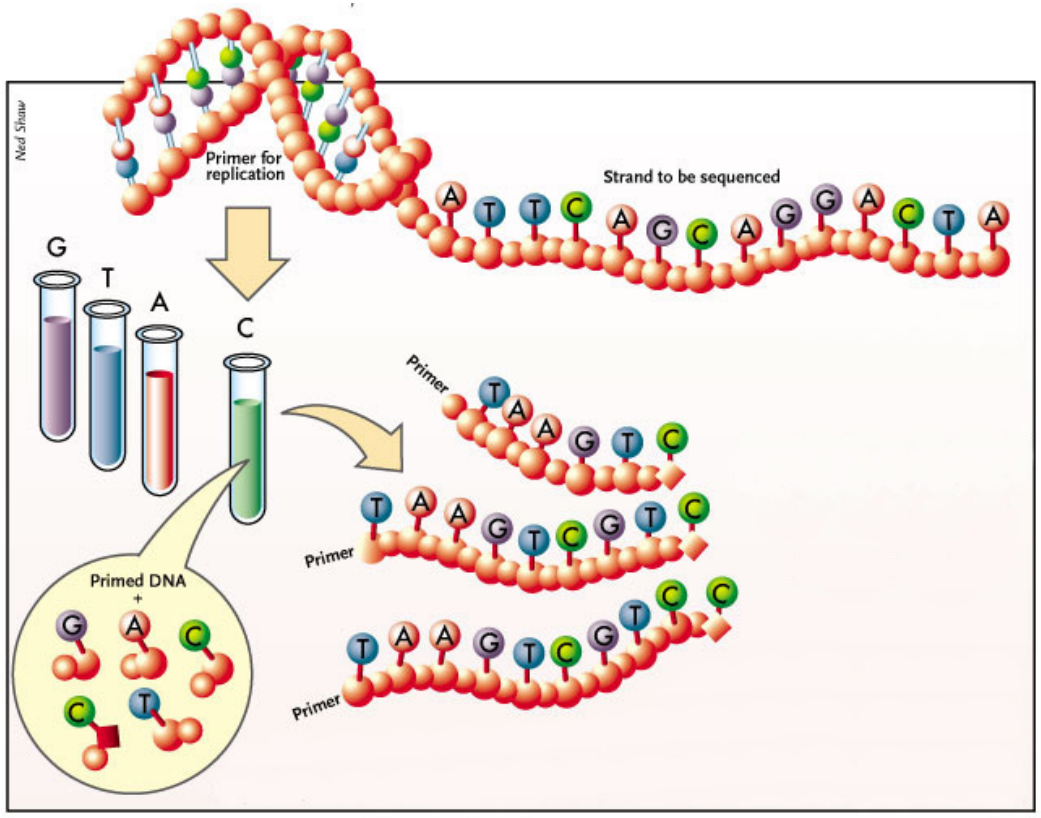
Functional genomic data analysis: transcriptomics

September 2022

First generation sequencing methods

Sanger sequencing by synthesis

- Method discovered in 1977 by Frédérick Sanger (nobel price 1980).
- DNA polymerisation using a complementary primer.
- Elongation using thermostable DNA polymerase (PCR).
- Addition of 4 **deoxynucleotides** (dATP, dCTP, dGTP, dTTP) and low concentrations of one of four dideoxynucleotides (ddATP, ddCTP, ddGTP ou ddTTP).

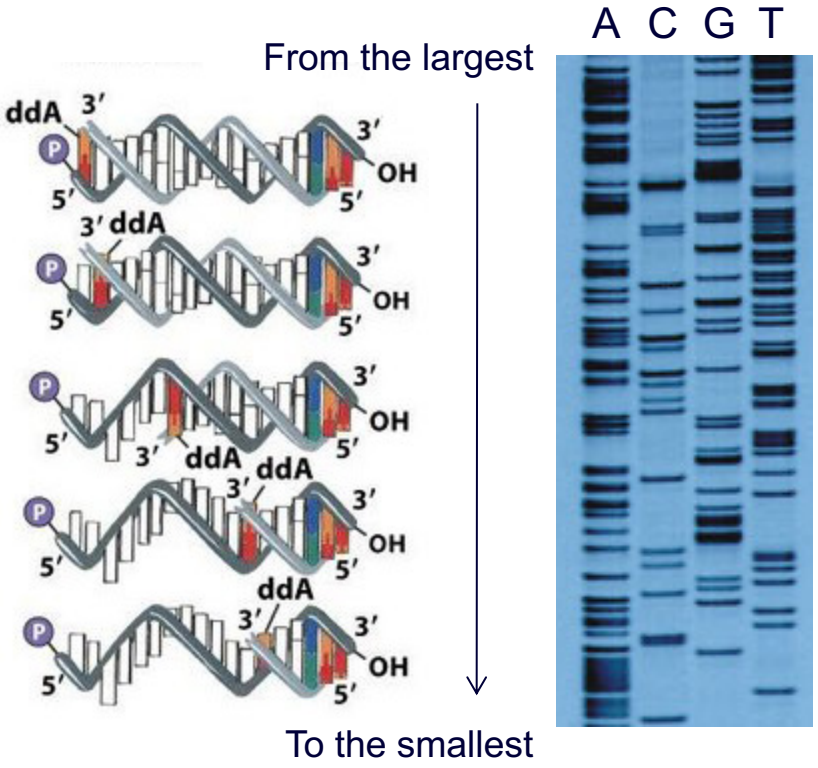


- These ddNTP once incorporated in the newly synthesized DNA strand, block elongation. Synthesis termination is done by a statistical manner on each possible positions.

From *The Scientist*

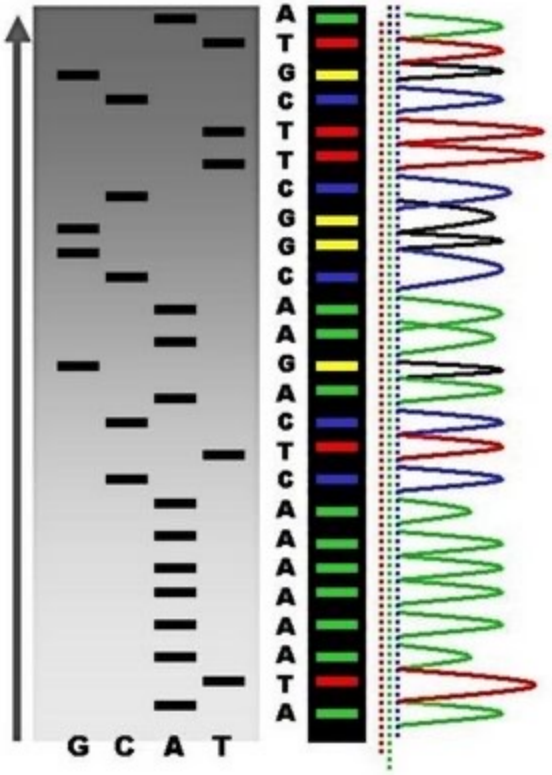
Sequence reading

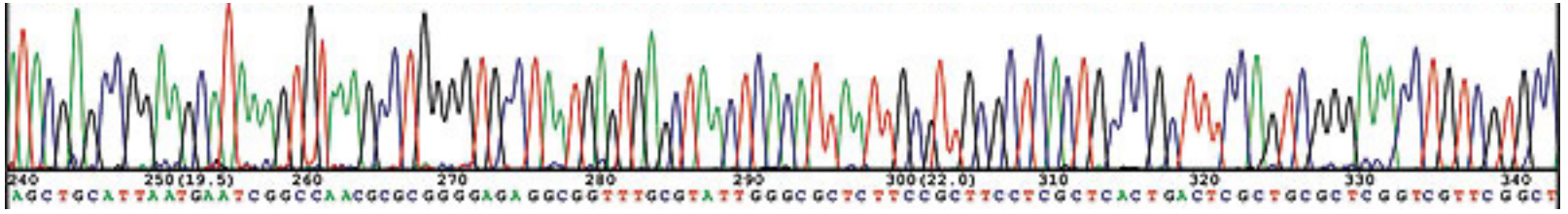
- We a get a mix of DNA fragments terminating at each position of the sequence.
- These fragments are then separated on a DNA polyacrylamide gel electrophoresis.
- Detection of synthesize fragments is done by the incorporation of labelling beacon in the DNA.
- At the origin this label was radioactive, attached either on the primer or on the dideoxynucleotide.
- Around **1 kb of DNA by run** during 6-8 hours. One read by sample.



Capillary sequencers

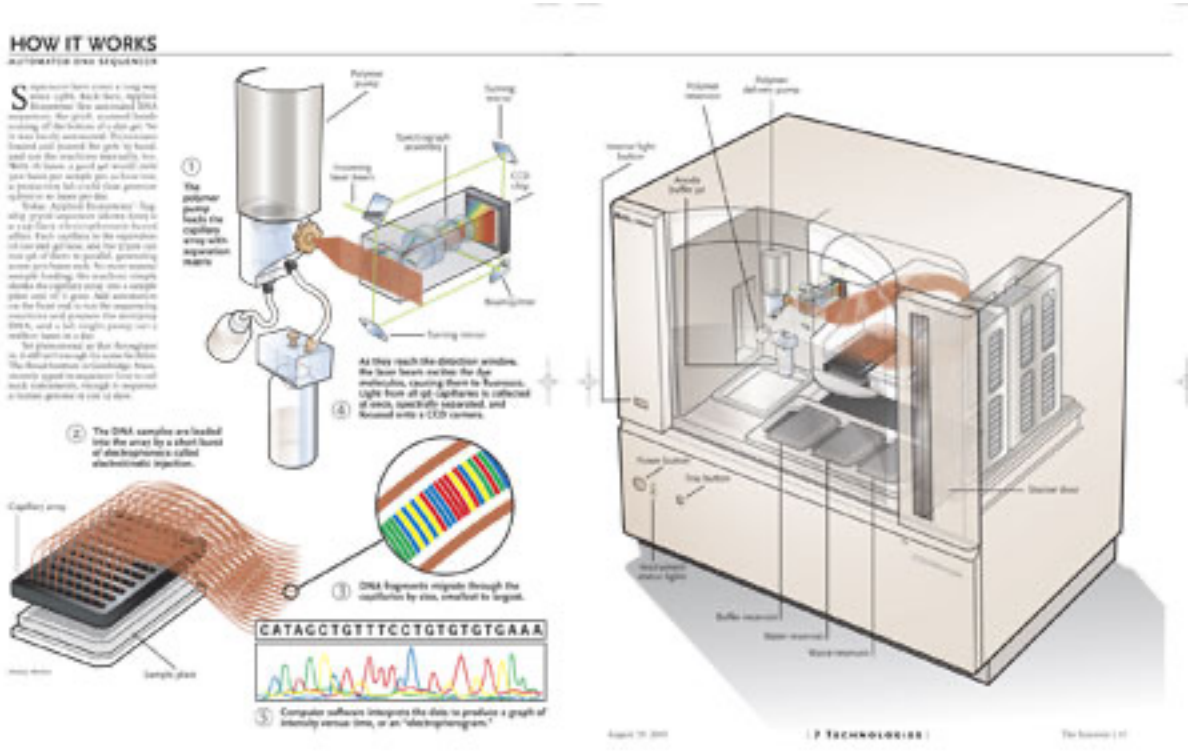
- First version in the 90's thanks to the modification of the radioactive label by a fluorescent one.
- Using glass capillary of few micron diameters, on 30 to 50 cm long.
- The four nucleotides migrate in the same tube thanks to four different fluorescent dyes.
- **300 kb of DNA** by run during 3 hours. Several hundred sample at a time.





ABI 3730xl DNA Analyzer

- 96 parallel capillary (up to 50 cm) array. **768 samples**, **690 kb** DNA, **3 hours** run.
- At the Broad Institute (Cambridge, Massachusetts) **126 devices** were able to sequence **1 human genome** in **12 days**.



From *The Scientist*

Second generation sequencing: high throughput sequencing

The first technologies

- Goal: to obtain a huge number of short reads.

since 1996

Applied Biosystems
ABI 3730XL
1 Mb / day

January '07 GS20
June '07 GS FLX

Roche / 454
Genome Sequencer FLX
100 Mb / run

January '07

Illumina / Solexa
Genome Analyzer
2,000 Mb (2 Gb) / run

November '07

Applied Biosystems
SOLiD
3,000 Mb (3 Gb) / run

Genome Analyzer

- Available: **January 2007**, provider: **Illumina**

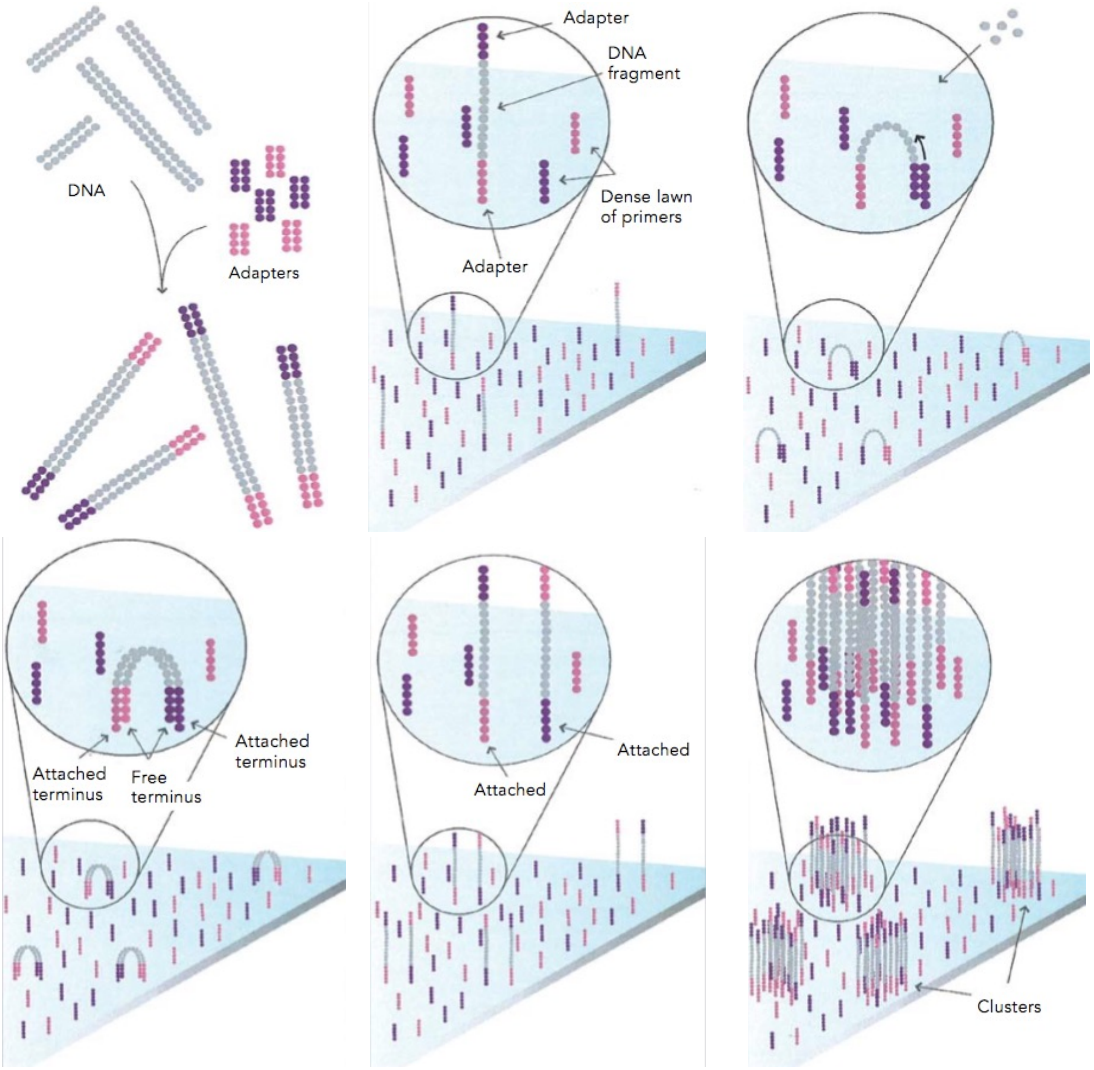
From: Clive Brown <clive.Brown@solexa.com>
Date: Sun, 20 Feb 2005 16:34:46 +0100
To: Nick McCooke <Nick.McCooke@solexa.com>, Tony Swerdlow <Harold.Swerdlow@solexa.com>, John Milt <Kevin.Hall@solexa.com>, Colin Barnes <Colin.Bar <Vincent.Smith@solexa.com>, Klaus Maisinger <Kla
Conversation: WE'VE DONE IT !!!!
Subject: WE'VE DONE IT !!!!

Tony Cox, Peta and I now agree - having looked at all of the PhiX174 data.

We have re-sequenced our first genome !!!!!!!

DNA library preparation

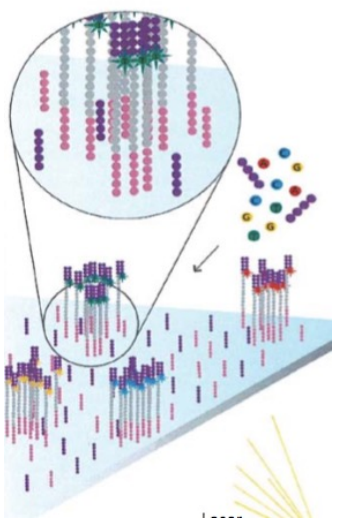
- Random DNA fragmentation and size selection.
- Ligation of adaptors.
- DNA denaturation.
- Hybridization of fragments onto the "**flowcell**" surface.
- Solid phase bridge PCR.

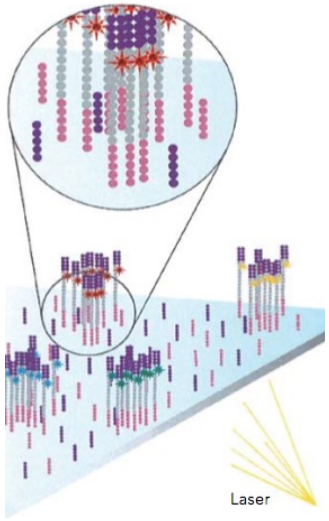


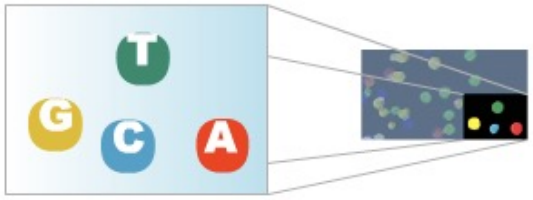
http://www.illumina.com/

Reversible terminator sequencing

- The four **reversible terminators** are added simultaneously.
- Laser scanning of the flowcell surface.
- Release of the blocking terminator.
- Sequencing cycles are repeated one base at a time.



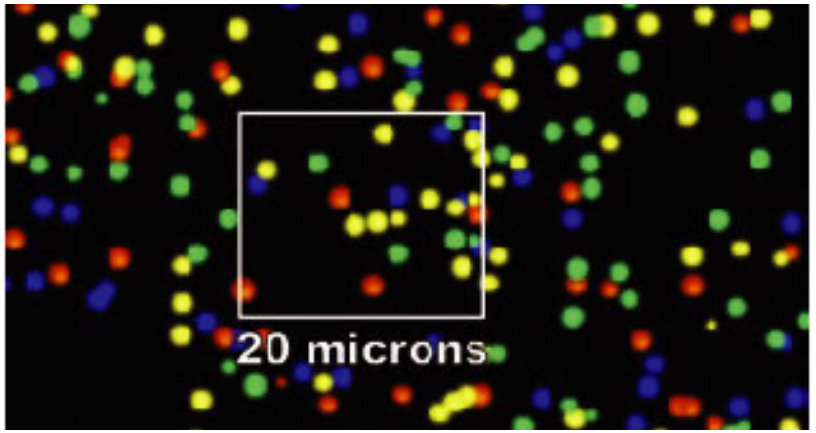


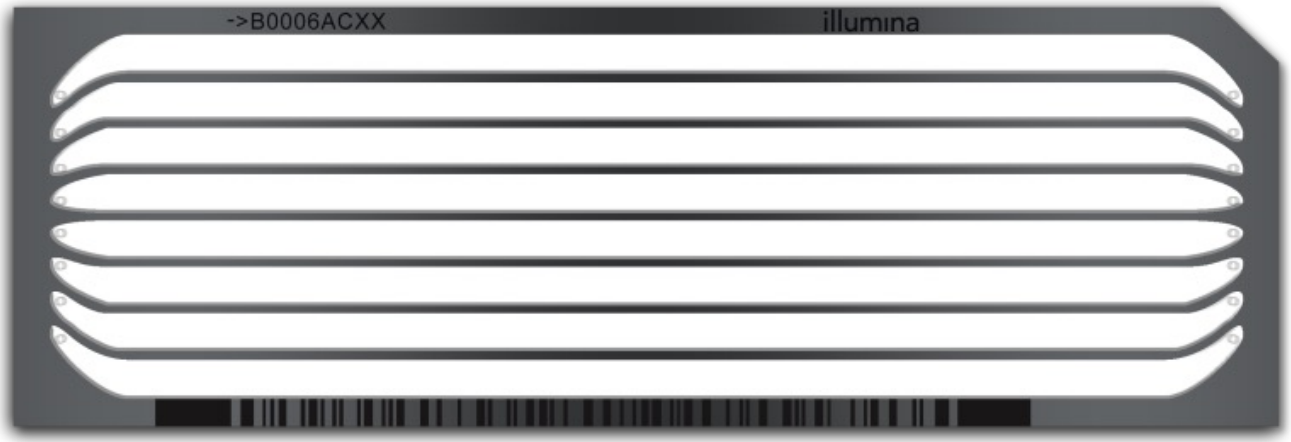


http://www.illumina.com/

Sequence analysis

- Scanning at each position for all sequences (reads) in parallel.
- Most of the errors (99%) are sequencing errors.





http://www.illumina.com/

Specifications of the latest Illumina sequencers



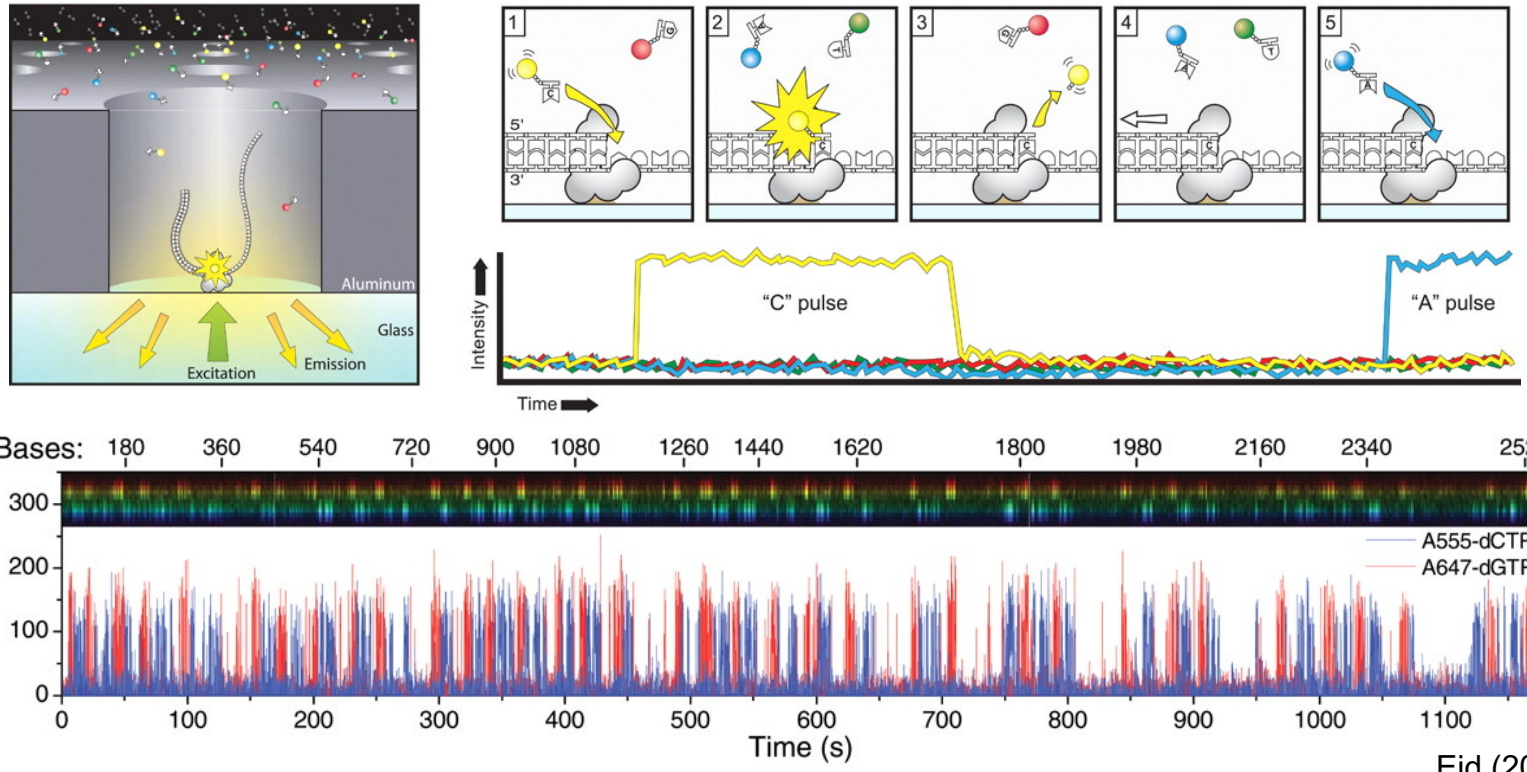


	MiniSeq	MiSeq	NextSeq 550	NextSeq 2000	NovaSeq
Run Time	24 hours	56 hours	29 hours	2 days	44 hours
Read length (bp)	2x 150	2x 300	2x 150	2x 150	2x 150
Read number	50 10^6	50 10^6	800 10^6	1 10^9	10 10^9
Ouput	7.5 Gb	15 Gb	120 Gb	300 Gb	3,000 Gb
Throughput	7 Gb/day	7 Gb/day	100 Gb/day	150 Gb/day	1,500 Gb/day

The third generation

Real time sequencing

- **Real time sequencing** on **single molecule** thanks to RNA polymerase immobilisation in wells.
- Each base incorporation is measure in real time with a CCD camera under the bottom of the plate.



Eid (2009) *Science*

Sequel specifications

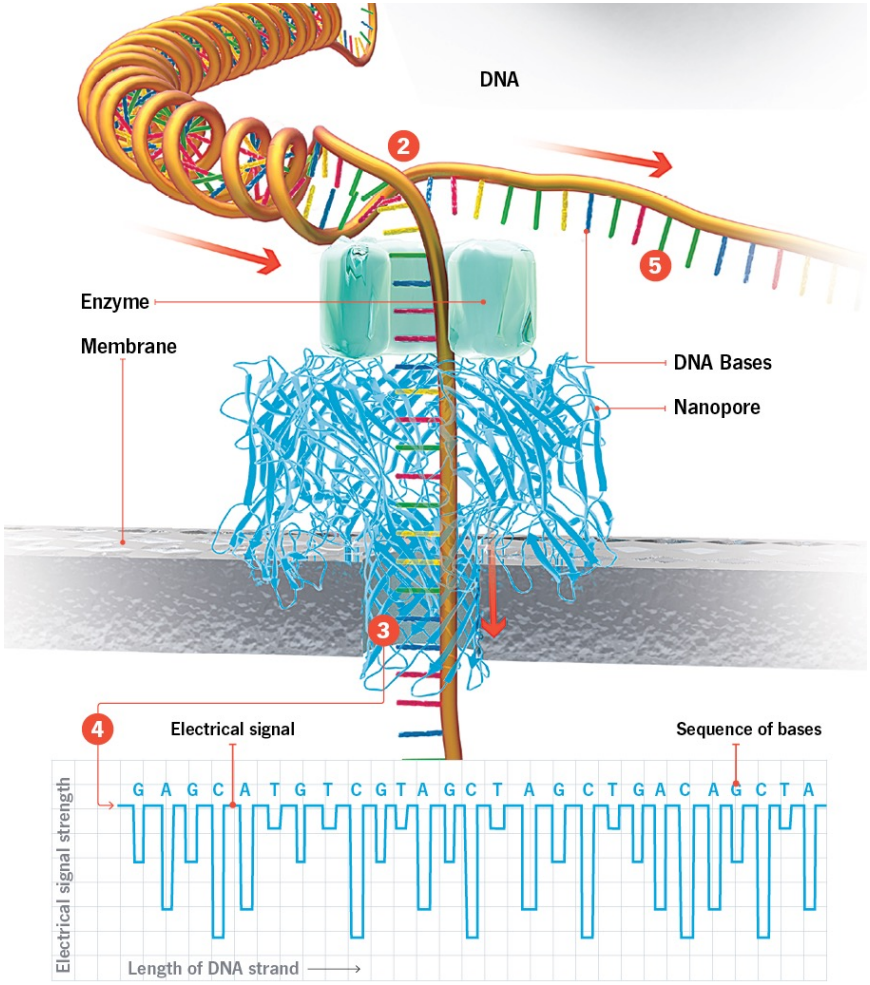
- **8 000 000 reads**/ SMRT cell;
- From **1 to 52 kb (Avg 20 kb)**;
- 450 Gb by SMRT cell;
- Run duration = **30 hours.**

PACIFIC BIOSCIENCES™

Nanopore technology

- **Single molecule detection** system by passing single strand DNA through a **nanometric pore**.
- Base to base analysis using **electric properties** of the nanopore.

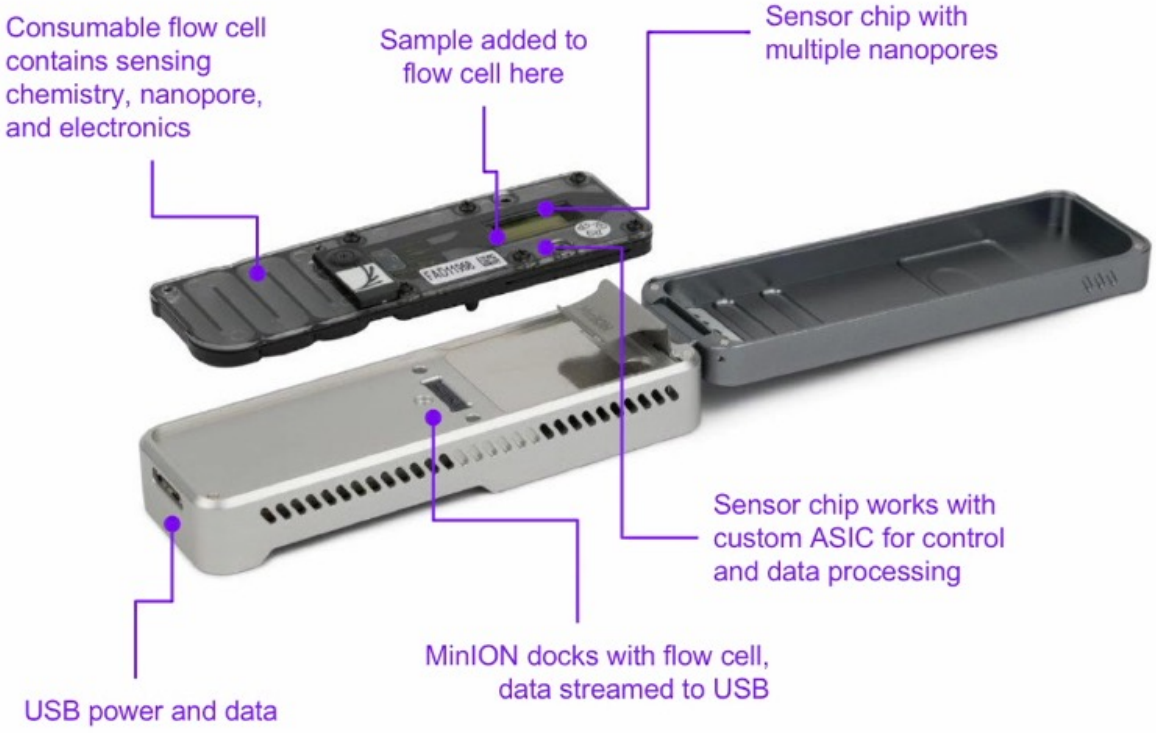
- DNA size sequencing of **kilobases**
- No limitation on the acid nucleic type to be detected (**DNA or RNA**).
- No amplification.



Greenwood (2013) *Popular Science*

The MiniON flow cell

- 1 flow cell = 1 membrane with 512 nanopores.
- Single molecule sequencing – up to 130 kb during up to 48 hours.



@NanoporeConf

Applications

They cover a lot of already existing techniques

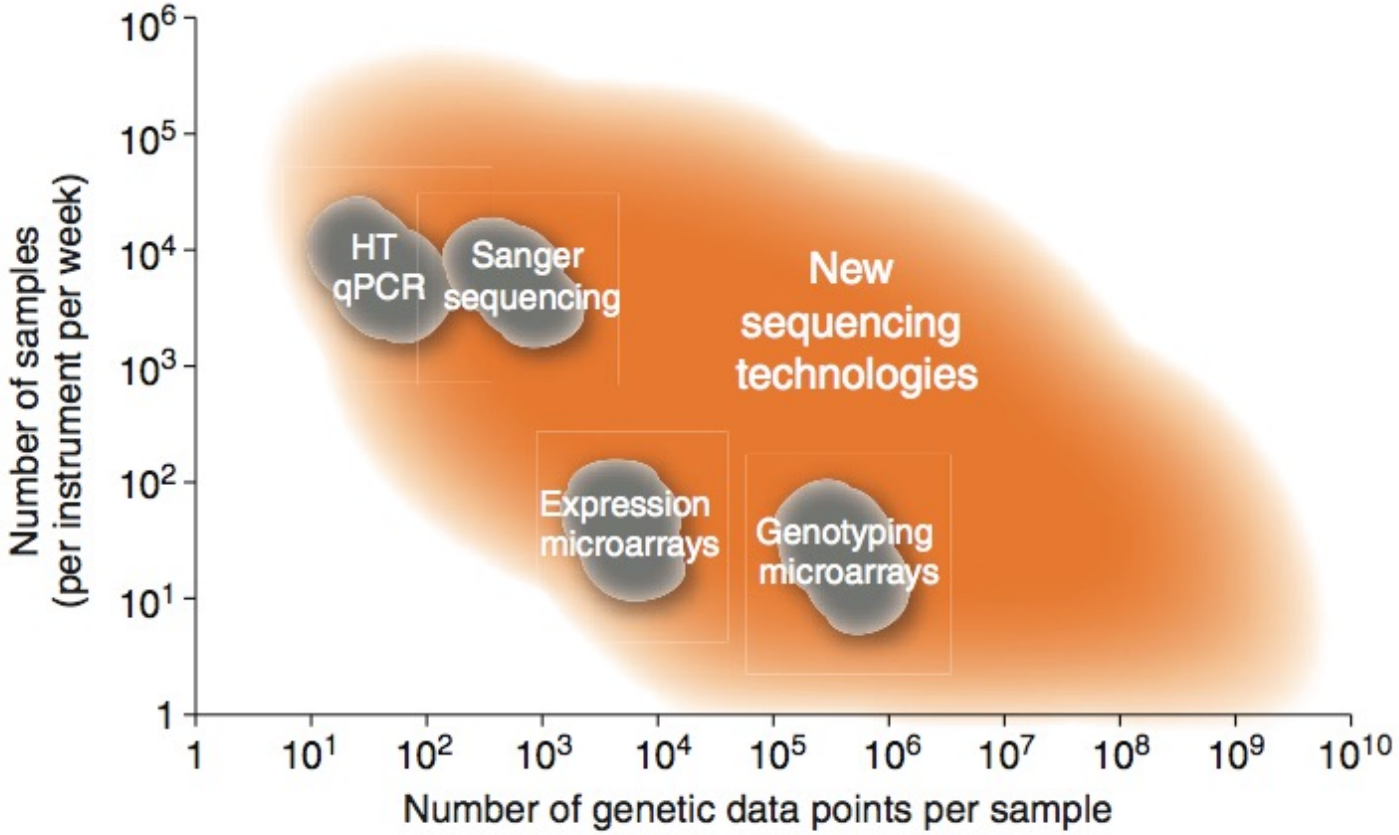


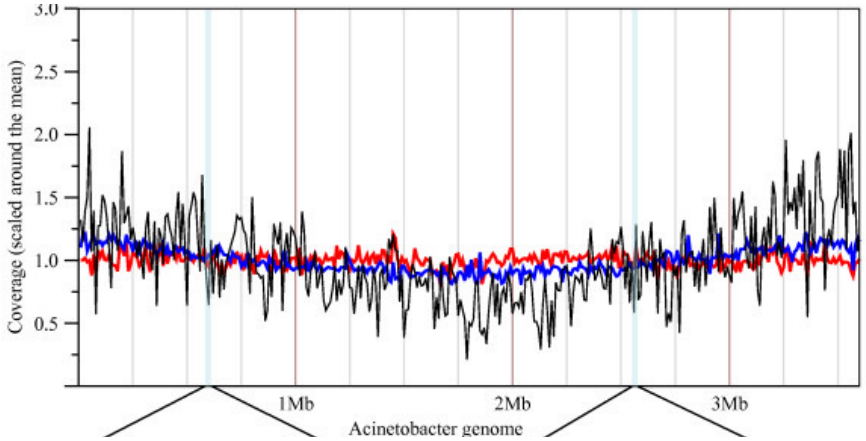
Figure 2 Relative sample and data throughputs for different nucleic acid detection and sequencing technologies. A rough estimate of the number of samples that can be run on a single instrument in one week with the resultant data points is shown on a logarithmic scale for different technologies. This is intended for comparative scale and is not exact.

Kahvejian et al. (2008) *Nat. Biotech.*

De novo sequencing

- **Quicker and cheaper** sequencing than Sanger.
- But **reads are smaller.**
- Combination of different methods allow to obtain **better quality sequencing drafts.**

  => Combining 454 and Illumina.
- Low error rate and **homogenous coverage** due to no cloning biases compared to the Sanger method.
- Errors are not same with the two high throughput sequencing methods.



Genomic DNA

↓ Roche-454 sequenced paired-end library to a ~7x fragment size coverage (for 3 kb fragments)

↓ Add 454 unpaired data to a final 25x coverage

Newbler Assembly

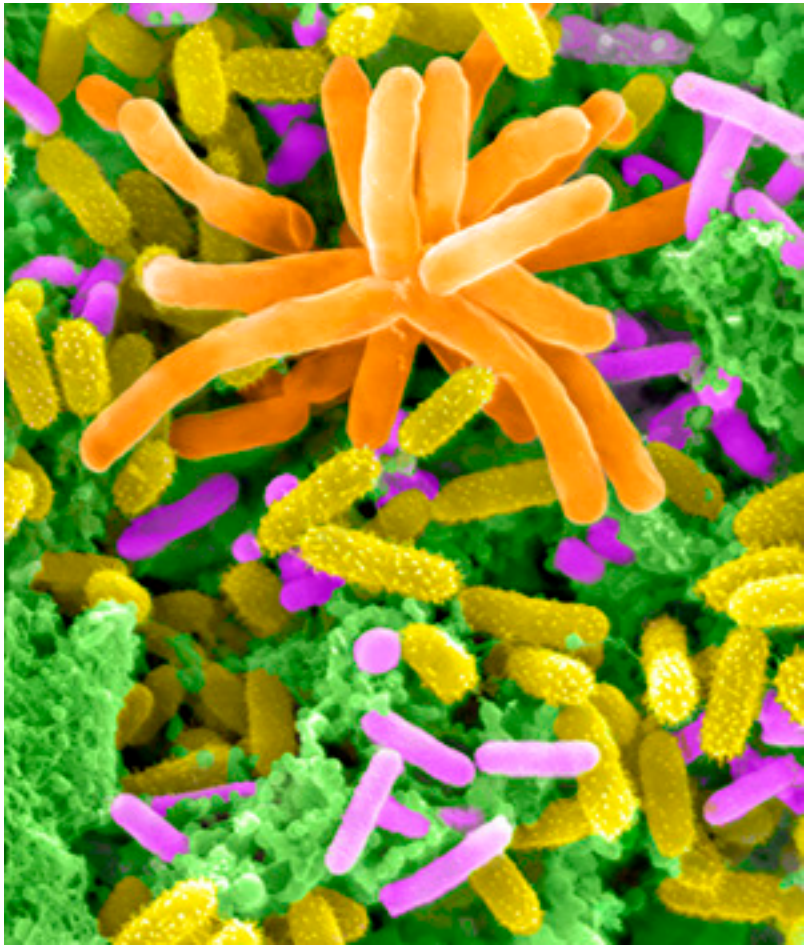
↓ Correct errors with ~50x Solexa-Illumina short reads data

High quality draft (<10^{-4} error rate)

Aury et al. (2008) *BMC Genomics*

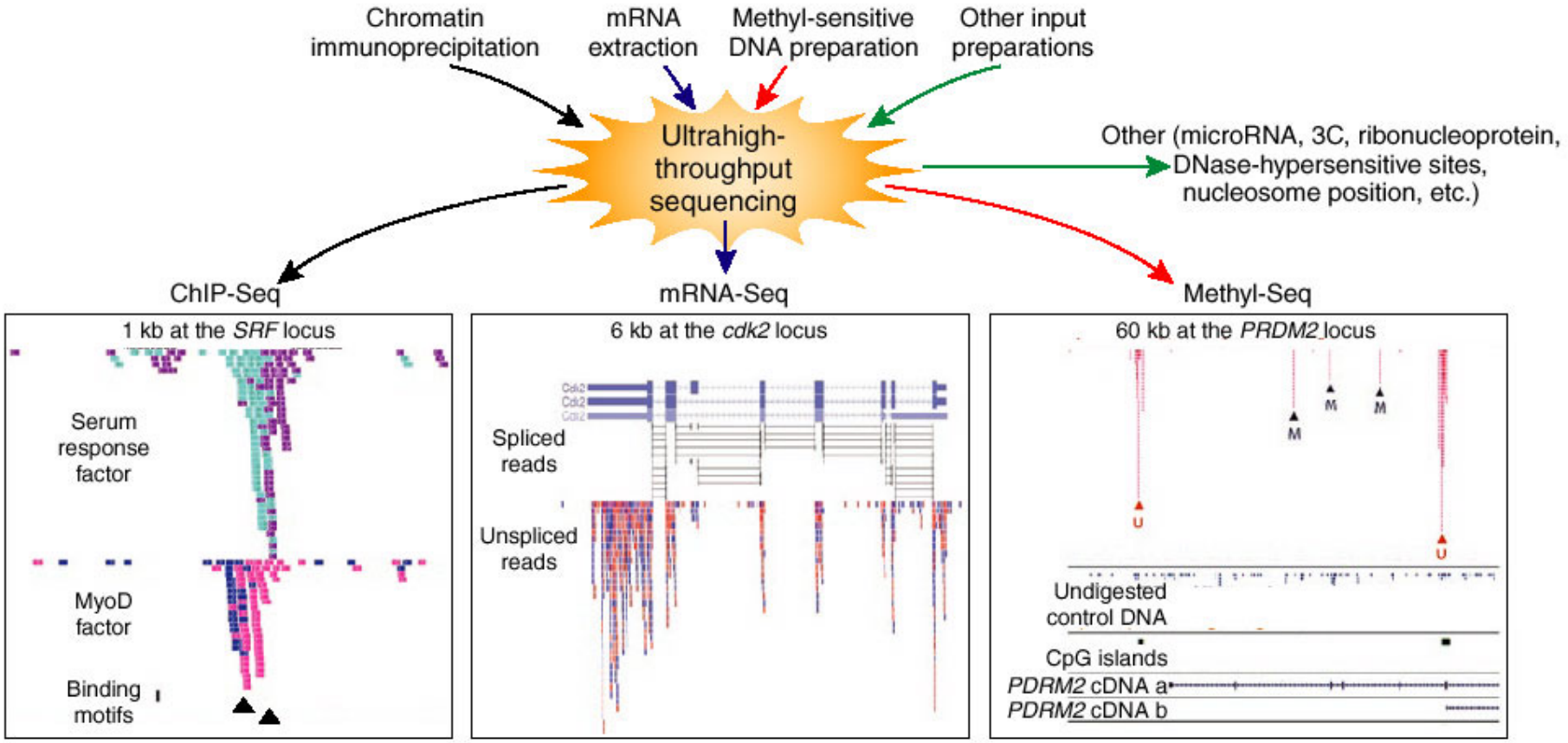
Resequencing applications

- Goal: analyze various genomes compared to a reference one.
- **Search for polymorphisms and structural variants** in populations, mutation identification in biotechnology, organism evolution analyses, cell differentiation along time, ancient DNA discovery…
- **Metagenomics**: genome characterisation in samples.
- A wide range of applications: characterise pathogen micro-organisms in patient tissues, definition of the species found in environment samples, understand species evolution…



From JGI DOE

Functional genomic applications



Wold et al. (2008) *Nat. Methods*

High throuput sequencing outcome

Avantages

- **No cloning steps** in bacteria
  - no more bias;
  - library construction easier.
- Each sequence come from a unique molecule:
  - quantification;
  - larger dynamic range.
- **High resolution** for a wide range of different applications.
- Huge **improvements** in term of **speed** and **cost** compared to the former Sanger method.

Drawbacks

- **Sequences are shorter**
  - compare to Sanger;
  - new base calling parameters;
  - new bioinformatics analyses.
- The amount of results generated is a problem for data management:
  - several To by run;
  - CPU usage;
  - what can we store?
- A **costly** still **evolving technology**.
- **Library preparation** is a **limiting step**.