# Iniciación al análisis de datos procedentes de técnicas de secuenciación masiva (NGS)

Unidad de Bioinformática (BU-ISCIII)
Unidades Comunes Científico Técnicas – SGAFI-ISCIII

17-21 Junio 2019, 7ª Edición
Programa Formación Continua, ISCIII

# OBJETIVOS DEL CURSO

- Aproximación a las técnicas de secuenciación masiva (NGS) y a sus aplicaciones
- Adquirir conocimientos básicos del entorno linux
- Familiarizarse con los formatos de ficheros generados en el análisis de datos procedentes de la SM
- Conocer el flujo de análisis de los datos procedentes de la SM

# Sesión 1 - Secuenciación Masiva Plataformas de Secuenciación

Isabel Cuesta

Unidad de Bioinformática
Unidades Comunes Científico Técnicas – SGAFI-ISCIII

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Programa Formación Continua, ISCIII

# INDICE

- Unidad de Bioinformática
  Servicios ofertados

- Evolución de la secuenciación

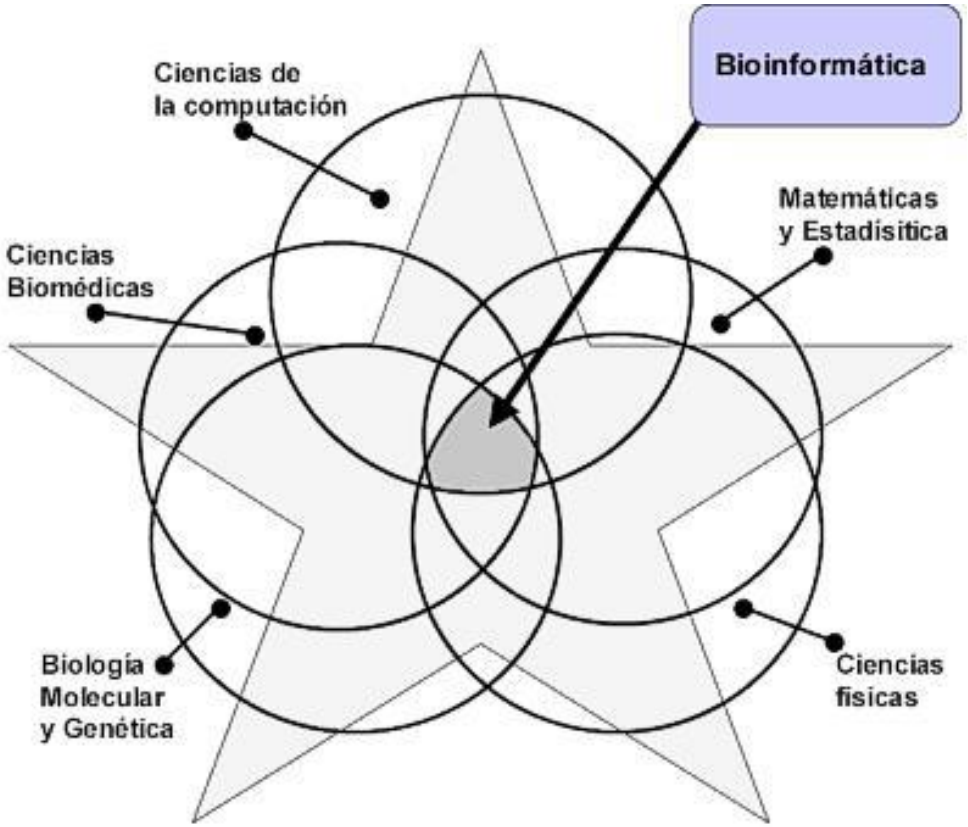
- Plataformas de secuenciación masiva (NGS)

**Bioinformatics** (i /ˌbaɪoʊˌɪnfərˈmætɪks/) is the application of statistics and computer science to the field of molecular biology.


(Quantitative+Computable) Molecular Biology

Bioinformatics now entails the creation and advancement of databases, algorithms, computational and statistical techniques and theory to solve formal and practical problems arising from the management and analysis of biological data.

# Bioinformática es multidisciplinar

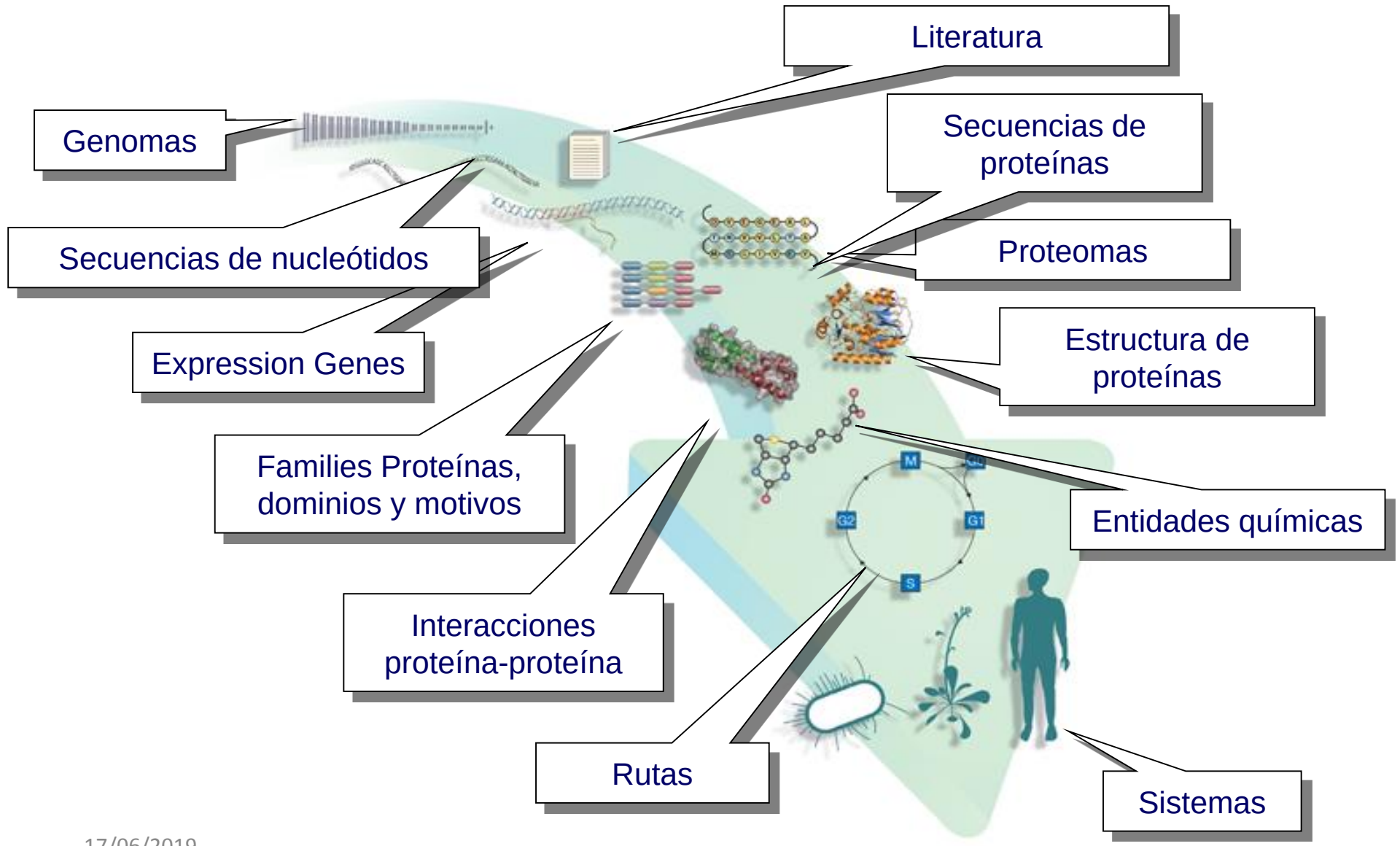


# PERSONAL DE LA UNIDAD DE BIOINFORMATICA

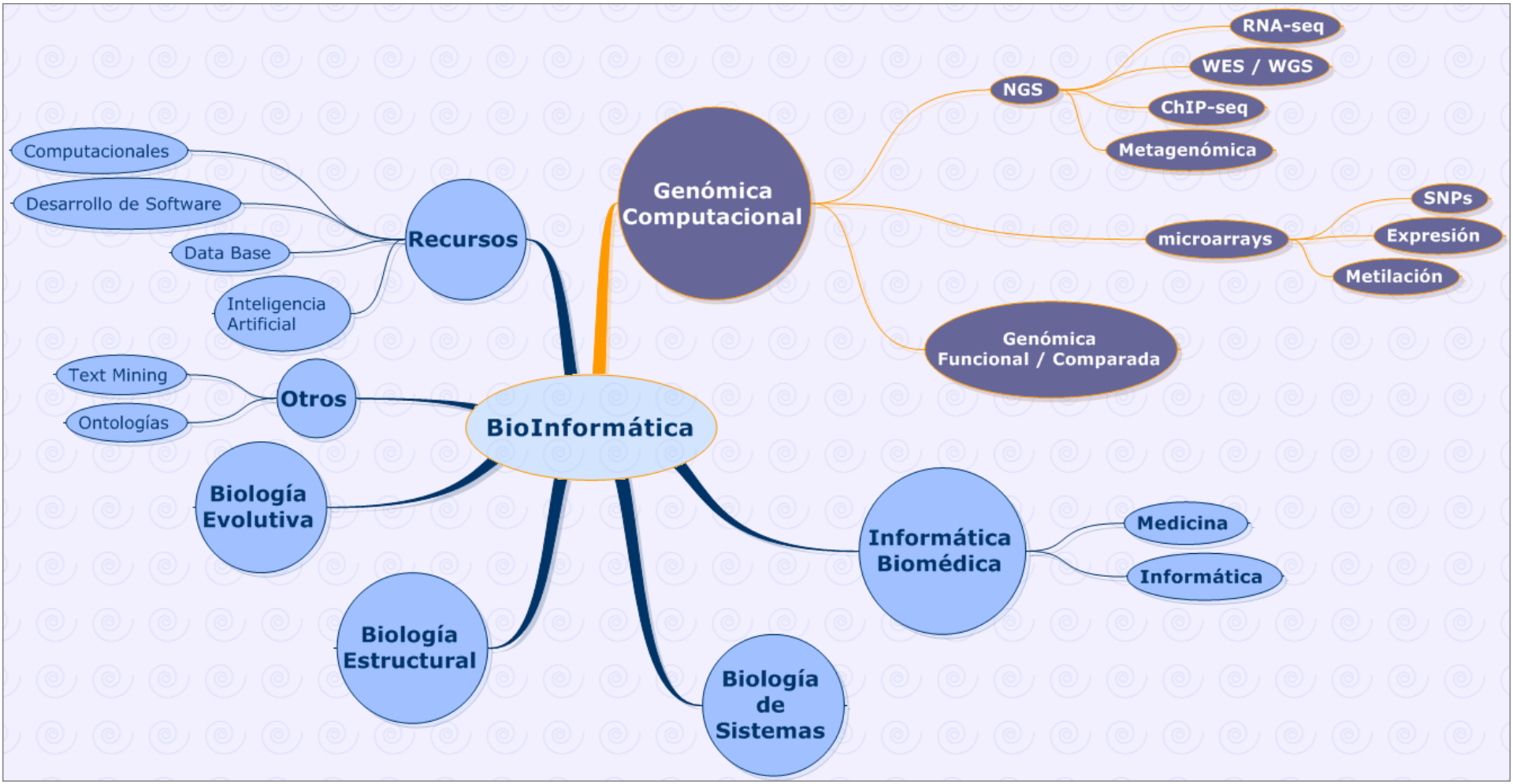
| | Disciplina | 2012 | 2013 | 2014 | 2015 | 2016 | 2017 | 2018 | 2019 | 2020 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Isabel Cuesta** | Dr. Biología Molecular | CIENTIFICO TITULAR OPIS | | | | | | | | |
| **Sara Monzón** | Biotecnología | **CIBERER** | | | | **PTA MINECO** | | | T.SUPERIOR OPIS | |
| **Bruno Lobo** | Administrador Sistemas | | **PROYECTO** | | **PTA MINECO** | | | **SISTEMAS** | | |
| **Jorge de la Barrera** | Informática | | **PTA FIS** | | | | | | | |
| **Miguel Juliá** | Matemáticas | | | | | | | **PTA MINECO** | | |
| **José Luis García** | Telecomunicaciones | | | | | | | **PROYECTO** | | |
| **Pedro Sola** | Biología | | | | | | **U. ANTIBIOTICOS** | | | |
| **Sarai Varona** | Bioquímica | | | | | | | | **PROYECTO** | |

Master en Bioinformática | CNM | IIER | BU-ISCIII | FUNCIONARIO

# Tipos de datos dan idea de la dimensión de la Bioinformática



# ESPECIALIZACIÓN



# RECURSOS INFORMÁTICOS

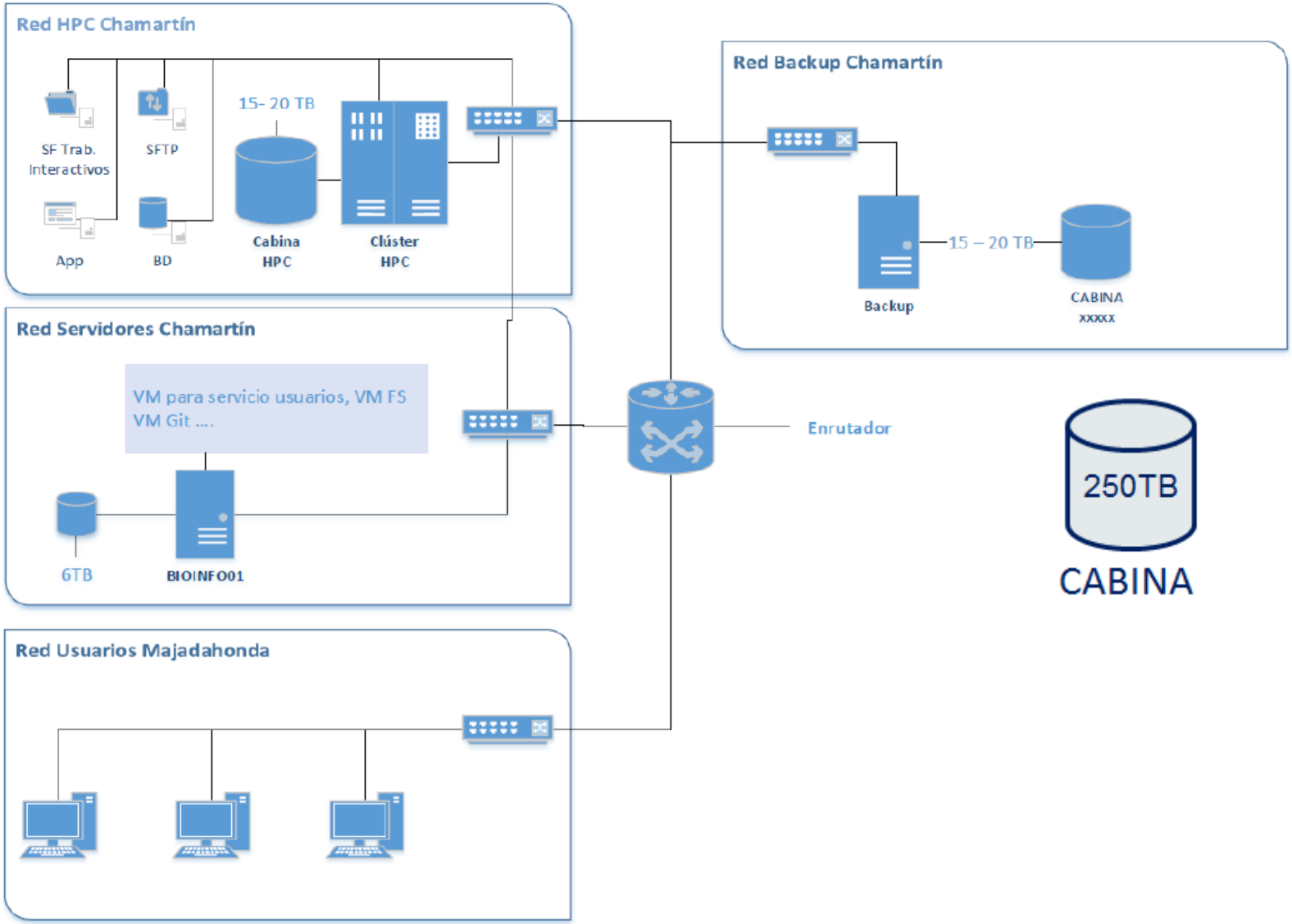
- Marco de relación con UTIC, establecido en 2013, administración compartida.
- Workstation (5), 4nucleos, 32Gb Ram, 4TB almacenamiento
- Servidor de la Unidad, 4-quad, 120Gb Ram,
- HPC 320 cores, 8TB RAM (16 nodos: Por nodo: 2 procesadores de 10 cores (20 threads) a 2,5 GHZ. 256 GB de RAM. Almacenamiento interno de 500 GB. 2 Interfaces de red a 10 Gbps.)
- 2 cabinas de almacenamiento, NetApp, 70 TB y 250TB escalable, ubicadas en el nuevo CPD del ISCIII.

Nuevo CPD

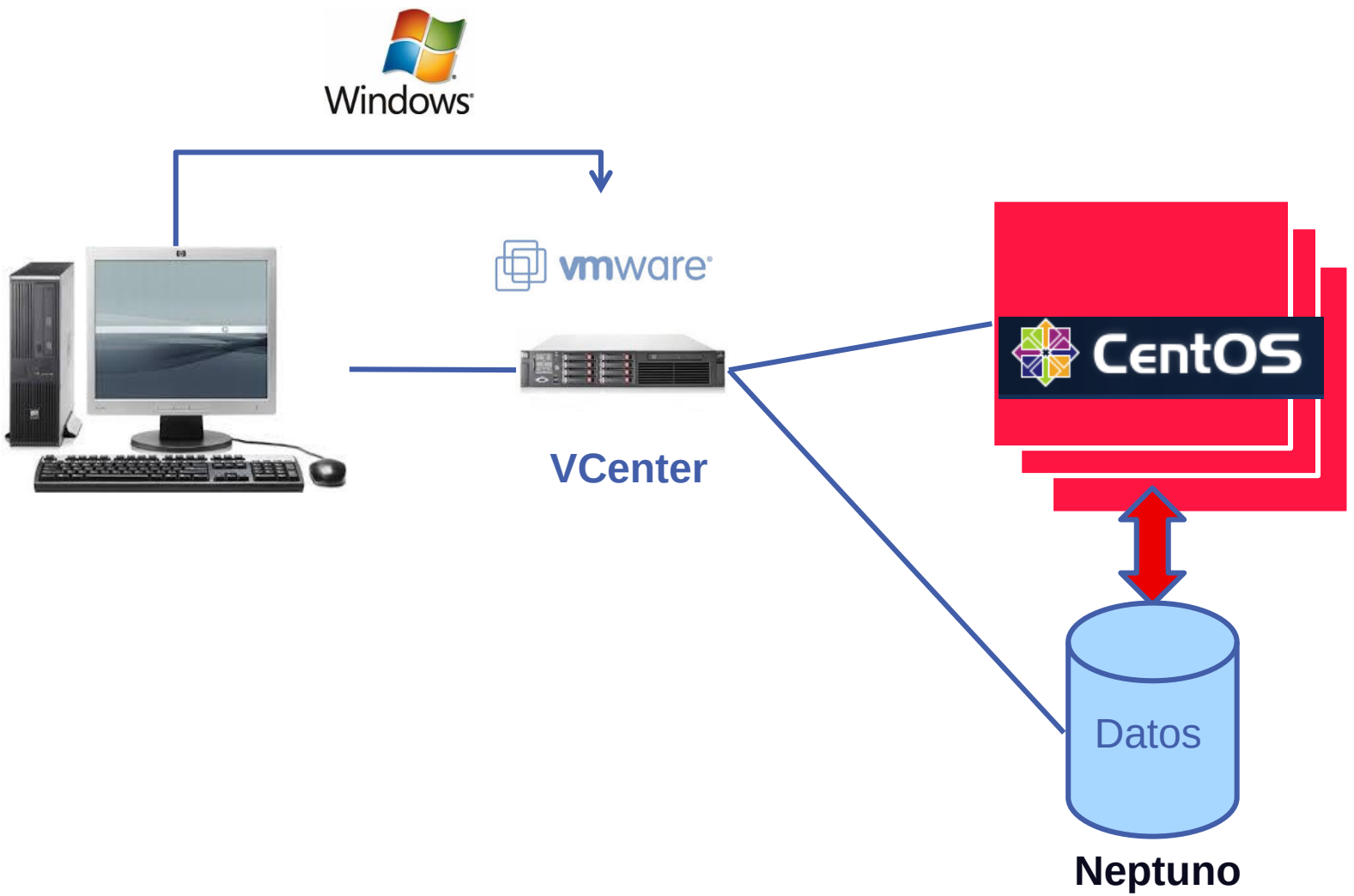
Pabellón 4 – planta semisótano

CPD respaldo Majadahonda

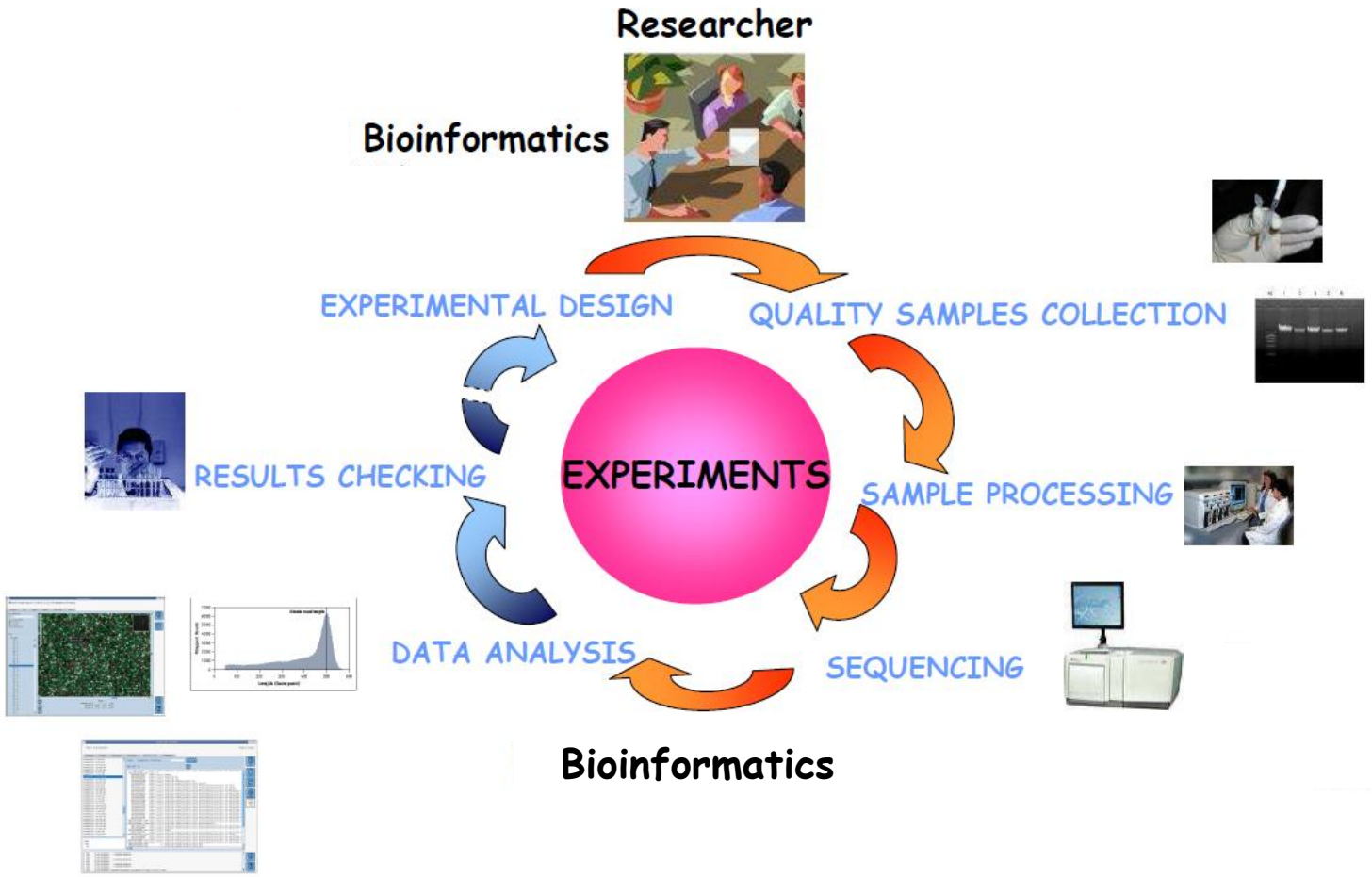
# INFRAESTRUCTURA



# Recursos Informáticos para el curso



# Workflow en NGS



# Cartera de Servicios

- **GENÓMICA COMPUTACIONAL: ANÁLISIS DE DATOS MASIVOS**
  Técnicas de secuenciación masiva (NGS)

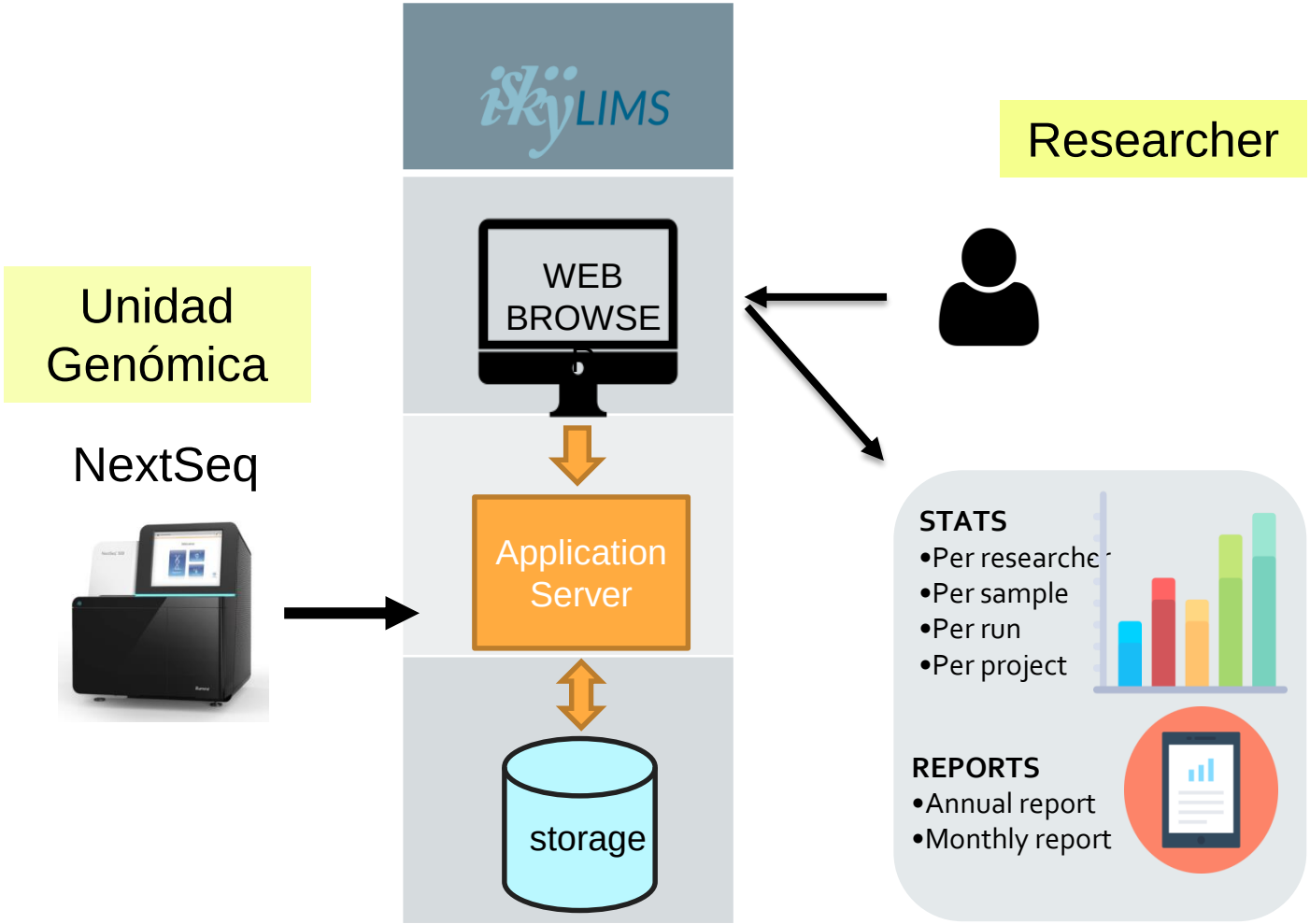
- **ASESORIA Y FORMACIÓN EN BIOINFORMÁTICA**
  Orientación en el análisis bioinformático
  Organización de cursos internos y externos

- **SOPORTE A USUARIOS**
  Generación y acceso a máquinas virtuales que contienen software bioinformático, ubicadas en los servidores de la Unidad

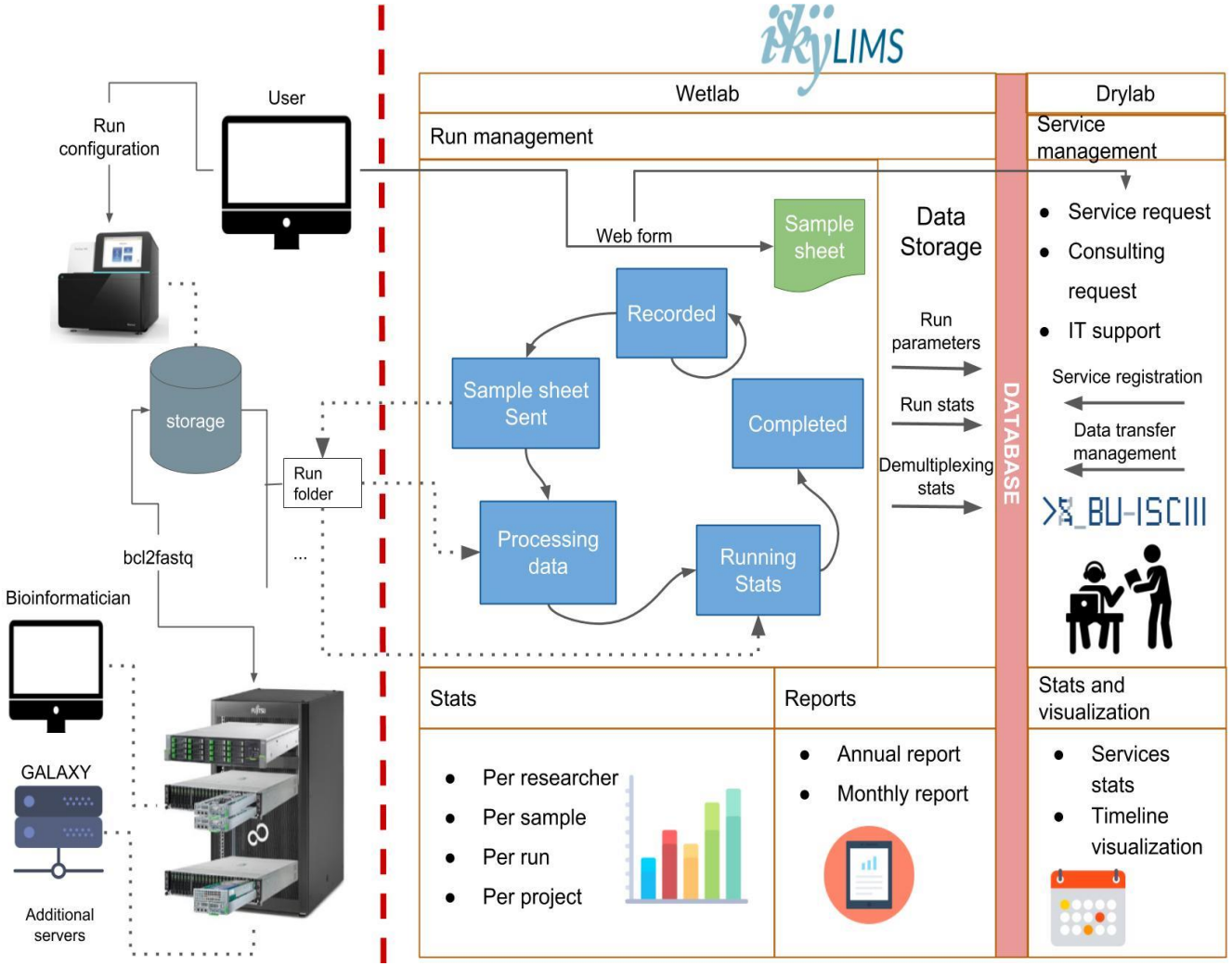
# Cartera de Servicios de la Unidad – Análisis de datos Secuenciación Masiva

| | | QC | Assembly | Reference based Mapping | Variant calling | Annotation | Pipelines |
|---|---|---|---|---|---|---|---|
| DNAseq | **HUMAN** | | | | | | |
| | WES Target –Panels | Report html | | (Bam file) | (Vcf file) | Desease model (Vcf file annotated) | .Trio / family .Tumor .Pampu caller |
| | **MICROBIAL** | | | | | | |
| | WGS Amplicon | Report html | *De novo* / Reference (fasta file) | MLST, Resistance g, Virulence g | SNPs Phylogenetic analysis | Structural Functional | WGSOutbraker Plasmid ID |
| RNAseq | **mRNA** | RSeQC Report html | *De novo* (fasta file) | Transcripts coverage / expression | Variants (Vcf file) | Transcripts annotation | mRNA seq |
| | **miRNA** | | | | | | miRNA seq |
| Metagenomics | **16S taxonomic profile** | Report html | *De novo* | Green genes DB | | species diversity | Qiime MeTRS Kaiju |
| | **Shotgun** | | | Genome Ref Seq | | Pathogen / Genome coverage | PikaVirus |

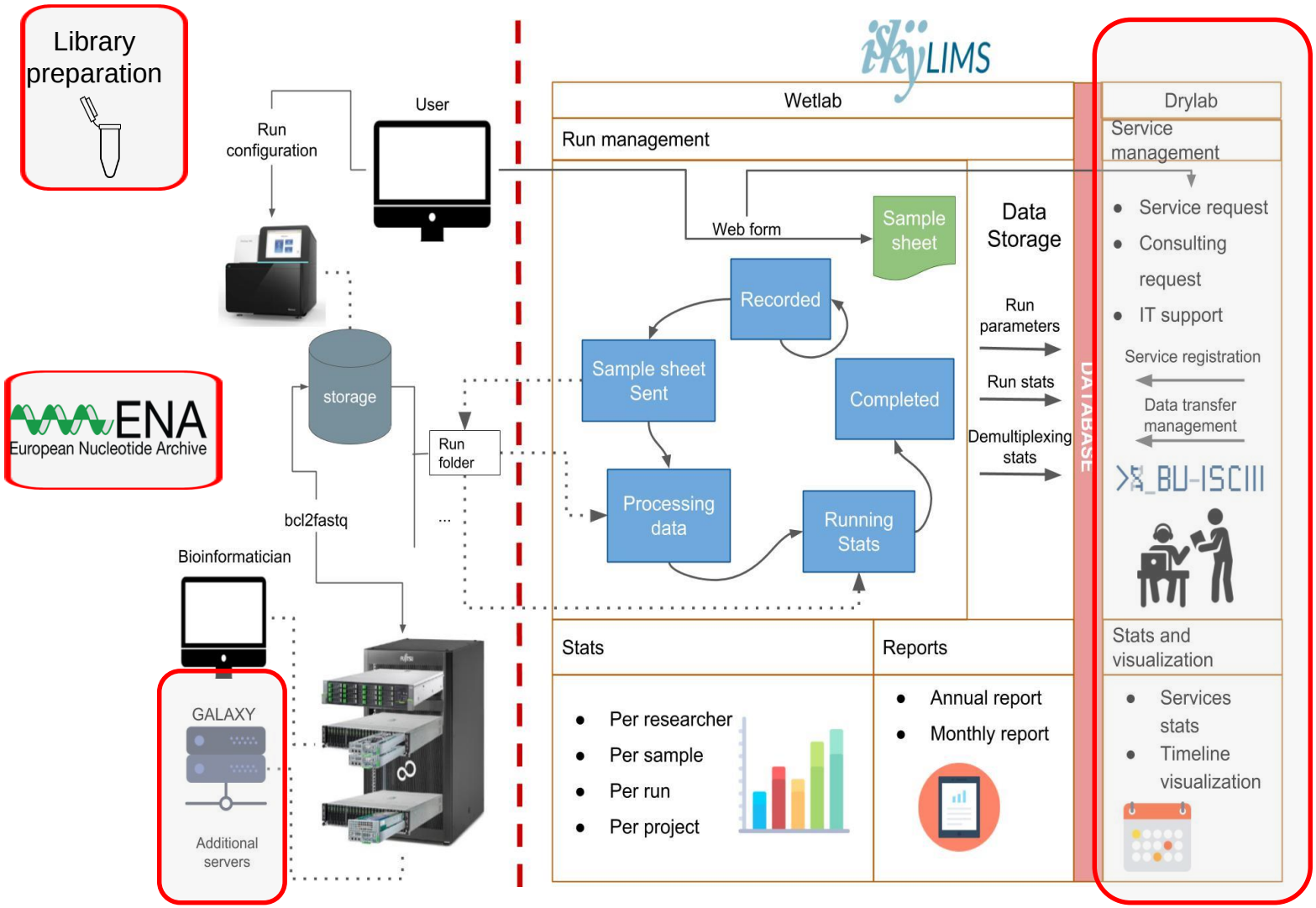
# iSkyLIMS


iSkyLIMS
Researcher
Unidad Genómica
WEB BROWSE
NextSeq
Application Server
storage
STATS
•Per researche
•Per sample
•Per run
•Per project
REPORTS
•Annual report
•Monthly report

# iSkyLIMS


iSkyLIMS
Wetlab
Drylab
Run management
Service management
User
Run configuration
Web form
Sample sheet
Data Storage
Service request
Consulting request
IT support
Recorded
Sample sheet Sent
Completed
Processing data
Running Stats
Run parameters
Run stats
Demultiplexing stats
DATABASE
Service registration
Data transfer management
storage
Run folder
bcl2fastq
...
Bioinformatician
GALAXY
Additional servers
Stats
Reports
Stats and visualization
Per researcher
Per sample
Per run
Per project
Annual report
Monthly report
Services stats
Timeline visualization

# iSkyLIMS


Library preparation
User
Run configuration
storage
ENA
European Nucleotide Archive
Run folder
bcl2fastq
Bioinformatician
GALAXY
Additional servers
iSkyLIMS
Wetlab
Run management
Web form
Sample sheet
Recorded
Sample sheet Sent
Completed
Processing data
Running Stats
Data Storage
Run parameters
Run stats
Demultiplexing stats
DATABASE
Stats
Per researcher
Per sample
Per run
Per project
Reports
Annual report
Monthly report
Drylab
Service management
Service request
Consulting request
IT support
Service registration
Data transfer management
BU-ISCIII
Stats and visualization
Services stats
Timeline visualization

# SERVICIOS DE LA UNIDAD

smonzon | Logout | My account

## BioInformatics

### iSkyLIMS: DryLab

**Welcome**

This section will allow you to check BU-ISCIII service activity. Available processes are request new services, colaborations, counseling and infrastructure. You will be able to check the status of your ongoing services.

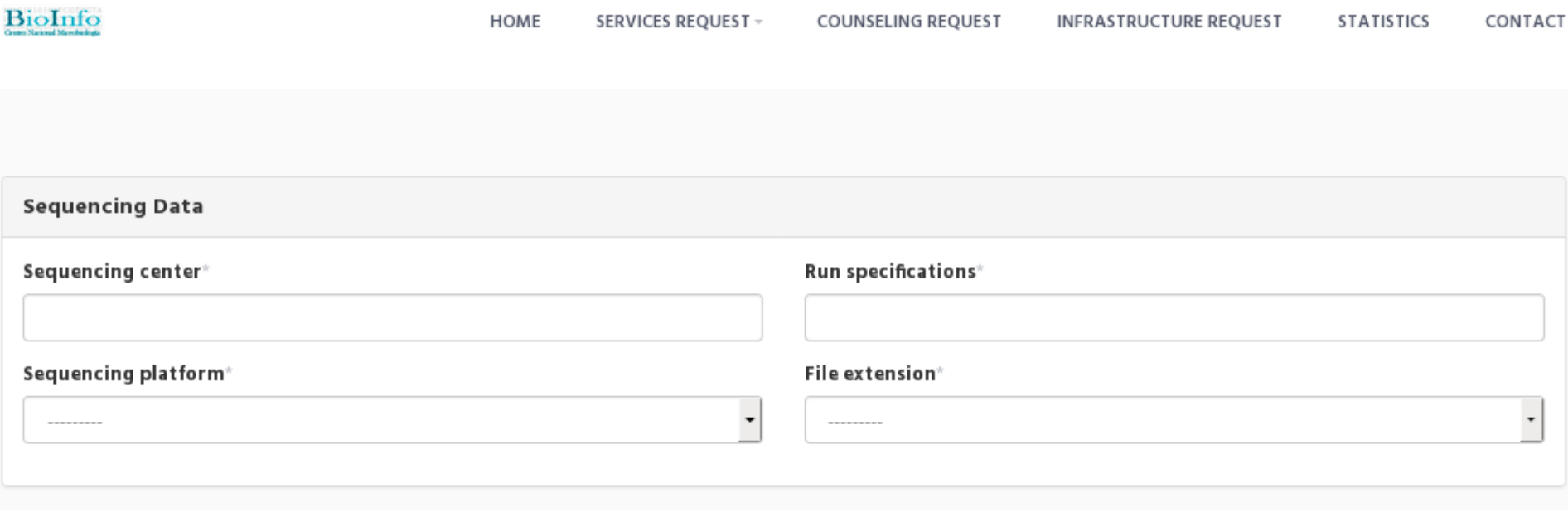
**Services ongoing and queued**

Under construction. This will be a table with services ongoing or queued

**Timeline of services**

Under construction. Kind of diagram with services dates.

# SERVICES REQUEST



# SERVICES REQUEST

**Service selection**

**AvailableServices** *

- ☐ Genomic Data Analysis
  - ☐ Download and quality analysis
    - ☐ Data download
    - ☐ Sequence quality analysis
    - ☐ Sequence pre-processing (quality filtering)
  - ☐ Next Generation Sequencing data analysis
    - ☐ DNAseq: Exome sequencing (WES) / Genome sequencing (WGS) / Target sequencing
      - ☐ Trio/family variant calling pipeline
      - ☐ Variant calling and annotation pipeline
      - ☐ Microbial: Whole genome outbreak analysis pipeline
      - ☐ Microbial: wgMLST
      - ☐ Microbial: MLST + virulence + AMR + plasmid analysis
      - ☐ Microbial: Assembly + automatic annotation
      - ☐ Microbial: plasmidID pipeline - strain plasmid characterization
    - ☐ RNAseq: Transcriptome sequencing
      - ☐ miRNA-Seq pipeline
      - ☐ mRNA-Seq pipeline
    - ☐ Amplicon sequencing (Deep sequencing)
      - ☐ Low frequency variant detection
      - ☐ Viral: assembly and minor variants detection
    - ☐ Metagenomics
      - ☐ 16S taxonomic profiling
      - ☐ Shotgun metagenomics

# SERVICES REQUEST


**Service Description**

**Service description file***

Browse... No file selected.

**Service Notes***

Save

# COUNSELING REQUEST

**Service selection**

**AvailableServices** *

- [ ] **Bioinformatics consulting and training**
  - [ ] **Bioinformatics analysis consulting**
  - [ ] **In-house and outer course organization**
  - [ ] **Student training in colaboration: Master thesis, research visit,...**

**Service Description**

**Service description file***

Browse... No file selected.

**Service Notes***

# INFRASTRUCTURE REQUEST

**Service selection**

**AvailableServices** *

- [ ] **User support**
  - [ ] **Installation and support of bioinformatic software on Linux OS**
  - [ ] **Installation and access to Virtual machines in the Unit server containing bioinformatic software**
  - [ ] **Code snippets development**

**Service Description**

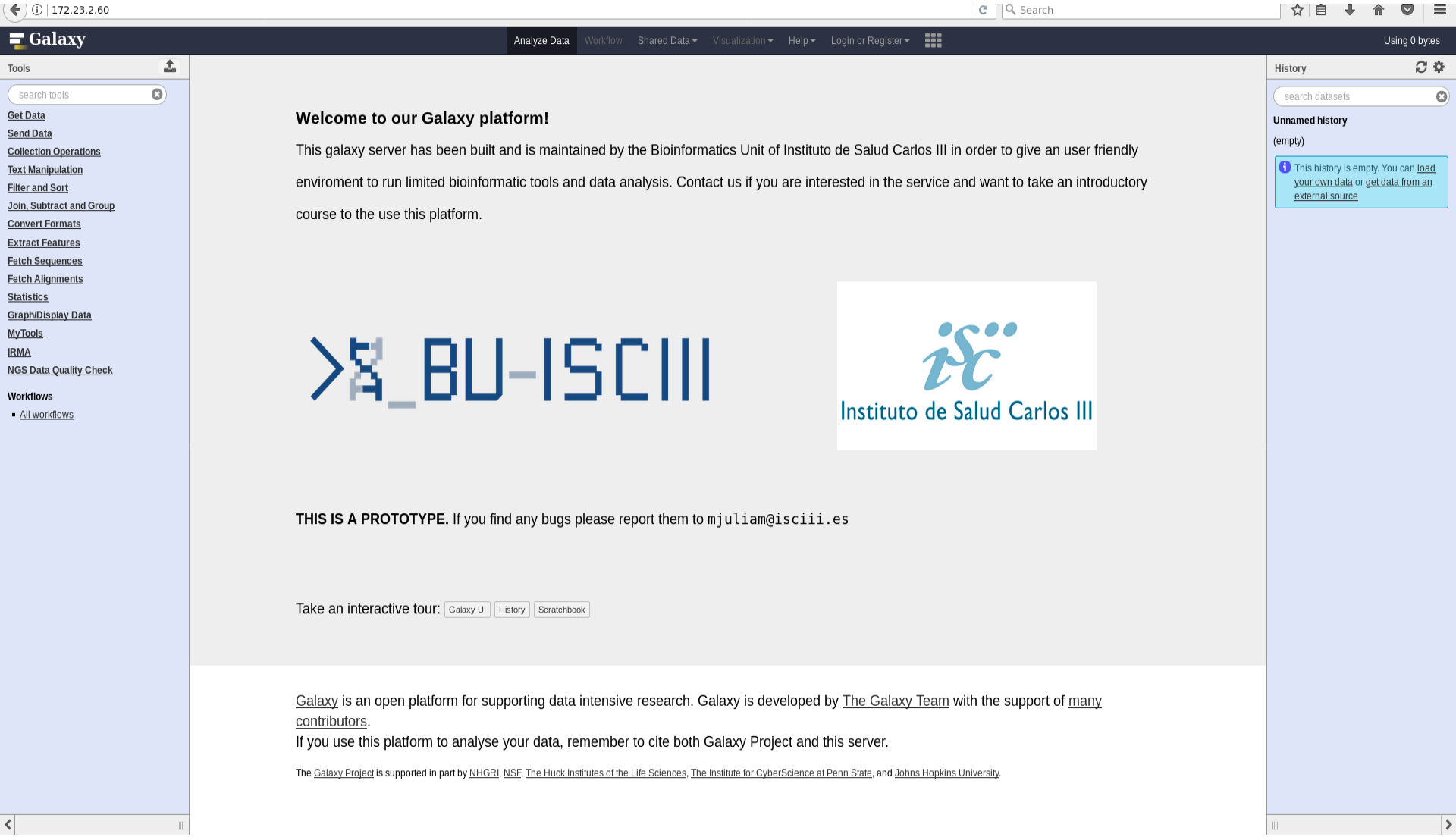
**Service description file***

Browse... No file selected.

**Service Notes***

Save

# Galaxy


172.23.2.60
Search
Galaxy
Analyze Data
Workflow
Shared Data
Visualization
Help
Login or Register
Using 0 bytes
Tools
search tools
Get Data
Send Data
Collection Operations
Text Manipulation
Filter and Sort
Join, Subtract and Group
Convert Formats
Extract Features
Fetch Sequences
Fetch Alignments
Statistics
Graph/Display Data
MyTools
IRMA
NGS Data Quality Check
Workflows
All workflows
Welcome to our Galaxy platform!
This galaxy server has been built and is maintained by the Bioinformatics Unit of Instituto de Salud Carlos III in order to give an user friendly enviroment to run limited bioinformatic tools and data analysis. Contact us if you are interested in the service and want to take an introductory course to the use this platform.
BU-ISCIII
Instituto de Salud Carlos III
THIS IS A PROTOTYPE. If you find any bugs please report them to mjuliam@isciii.es
Take an interactive tour:
Galaxy UI
History
Scratchbook
Galaxy is an open platform for supporting data intensive research. Galaxy is developed by The Galaxy Team with the support of many contributors.
If you use this platform to analyse your data, remember to cite both Galaxy Project and this server.
The Galaxy Project is supported in part by NHGRI, NSF, The Huck Institutes of the Life Sciences, The Institute for CyberScience at Penn State, and Johns Hopkins University.
History
search datasets
Unnamed history
(empty)
This history is empty. You can load your own data or get data from an external source

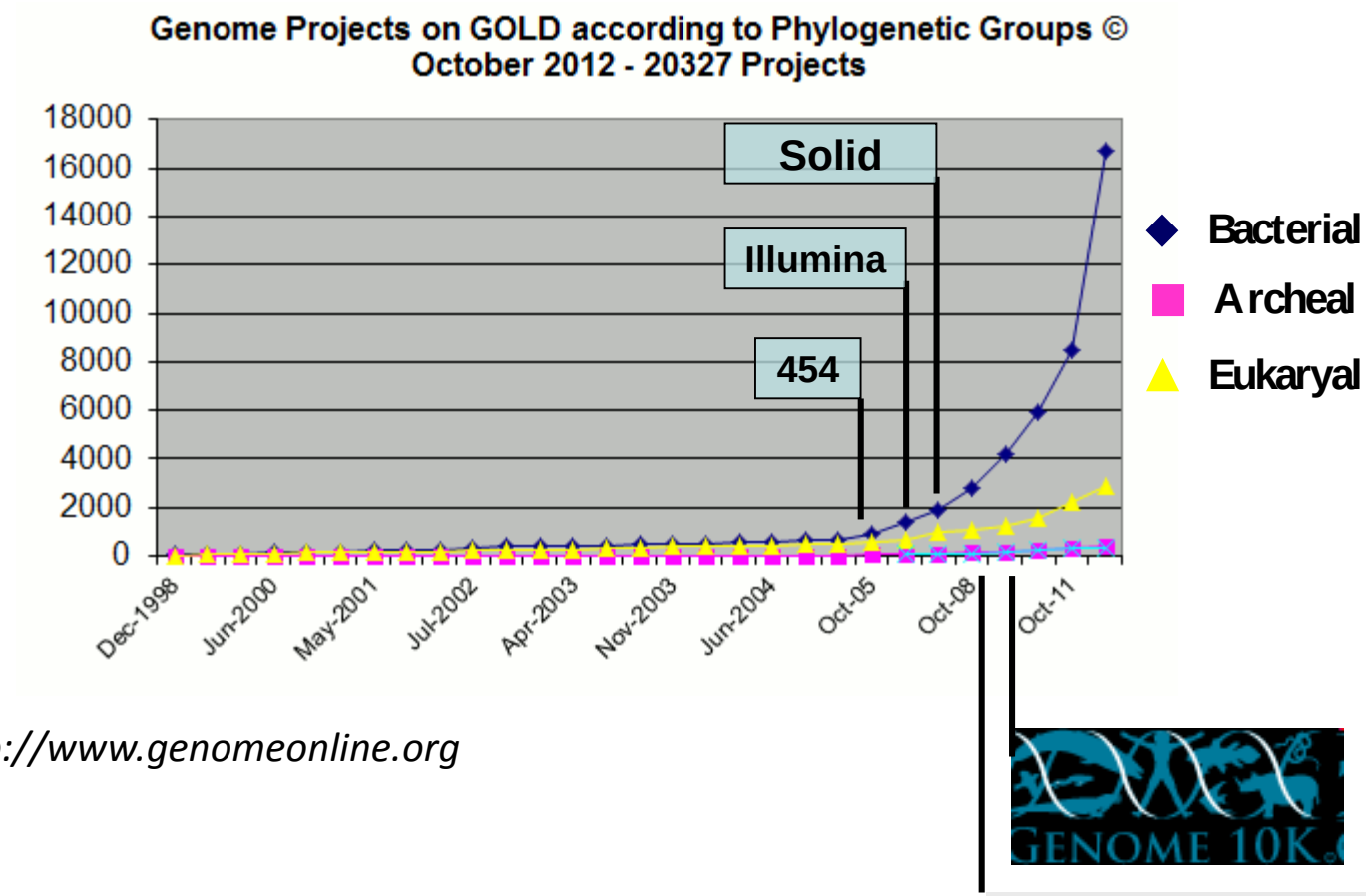
# INDICE

- Unidad de Bioinformática
  Servicios ofertados

- Evolución de la secuenciación

- Plataformas de secuenciación masiva (NGS)

# Genomics Revolution Era

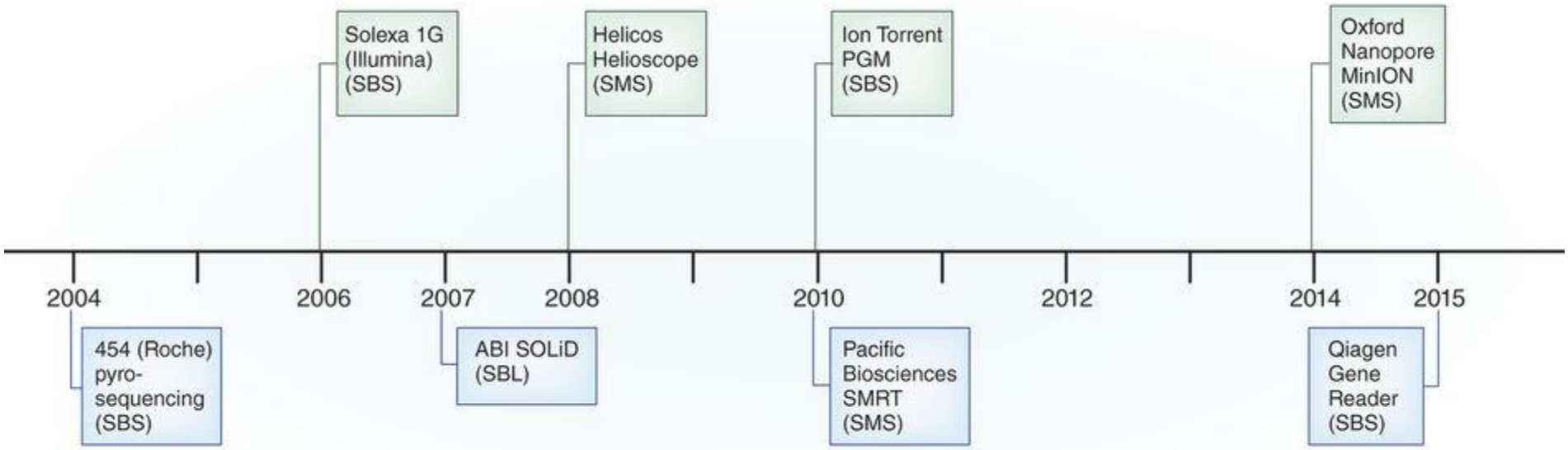



*Source: http://www.genomeonline.org*

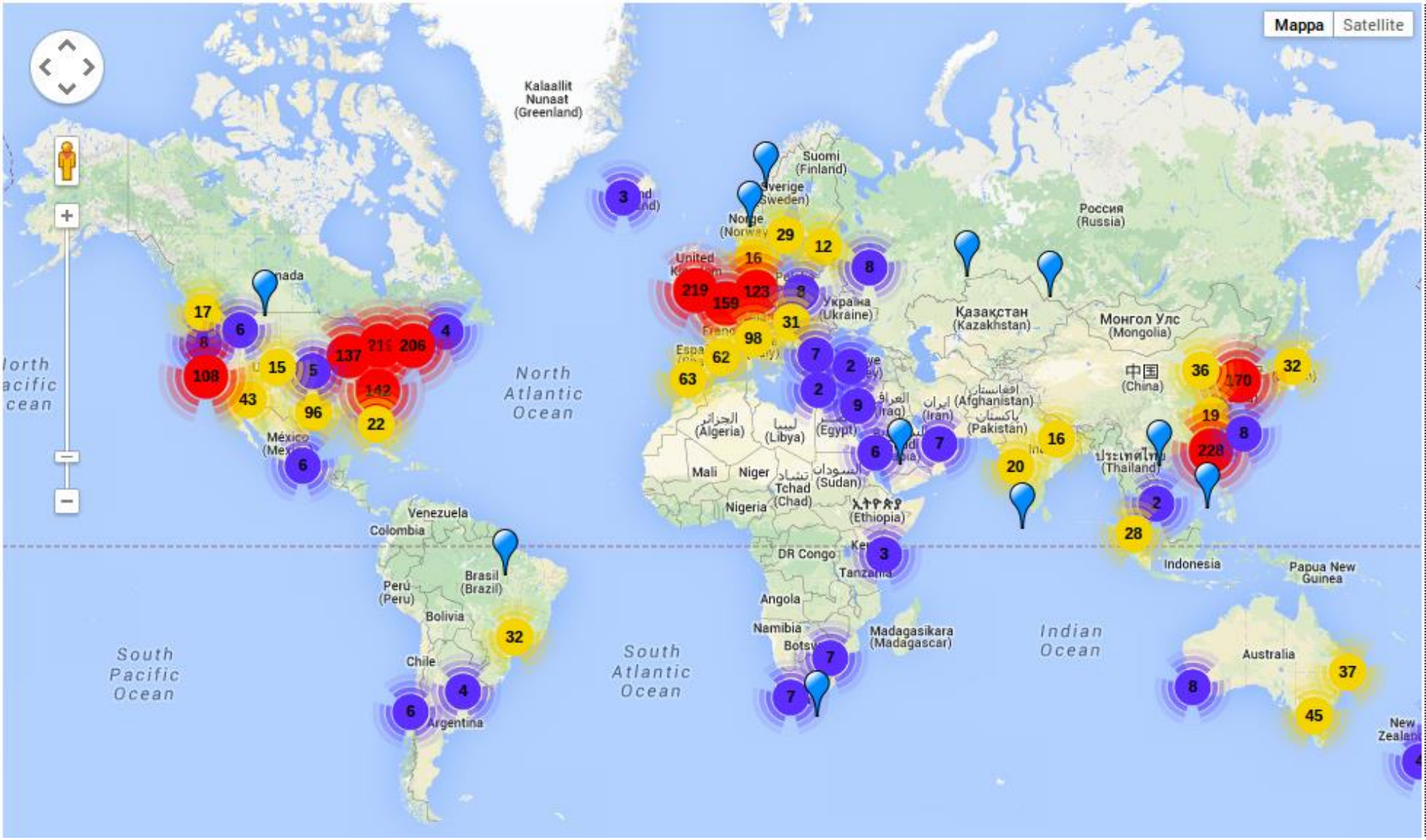
GENOME 10K

**1000 Genomes Project**

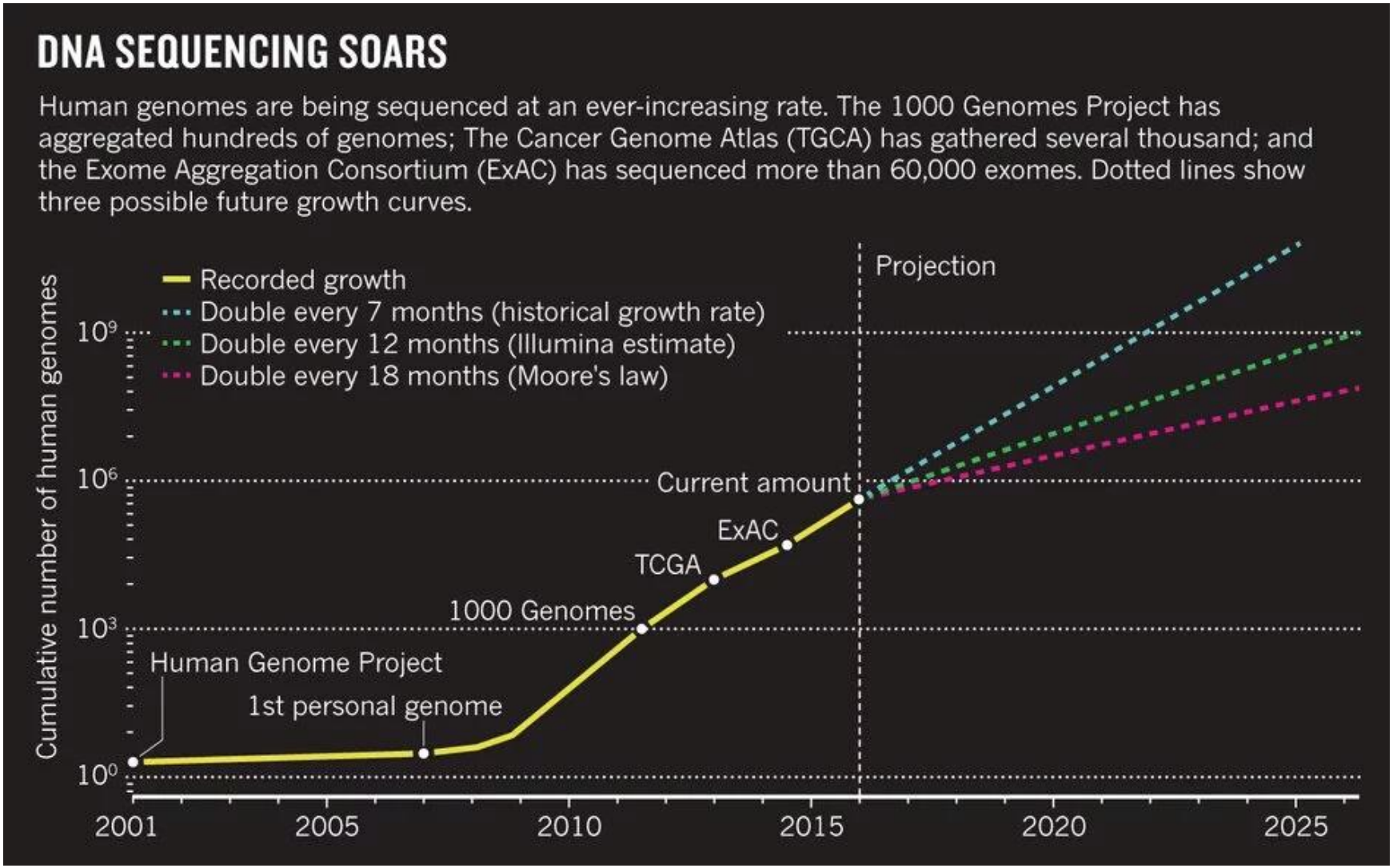
# NGS PLATFORMS: TIMELINE



# Democratización de la secuenciación. Mapa de los secuenciadores de alto rendimiento



# SEQUENCING PROJECTS



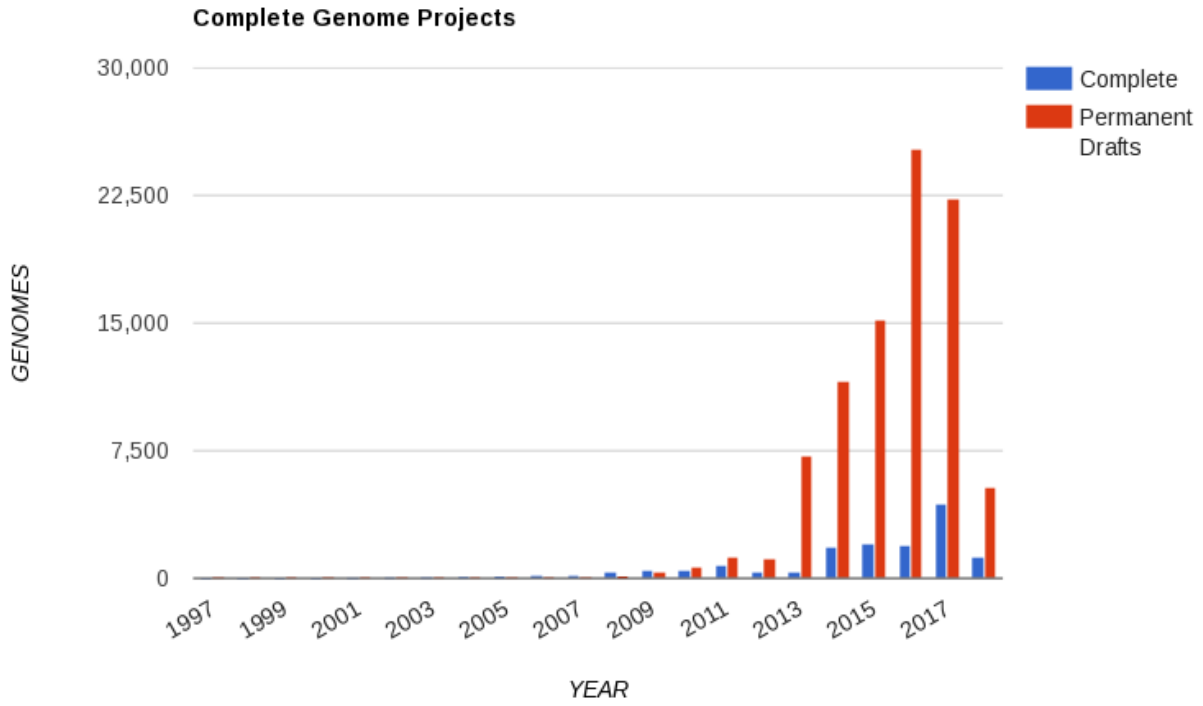
# Genomics Revolution Era

JGI GOLD GENOMES ONLINE DATABASE

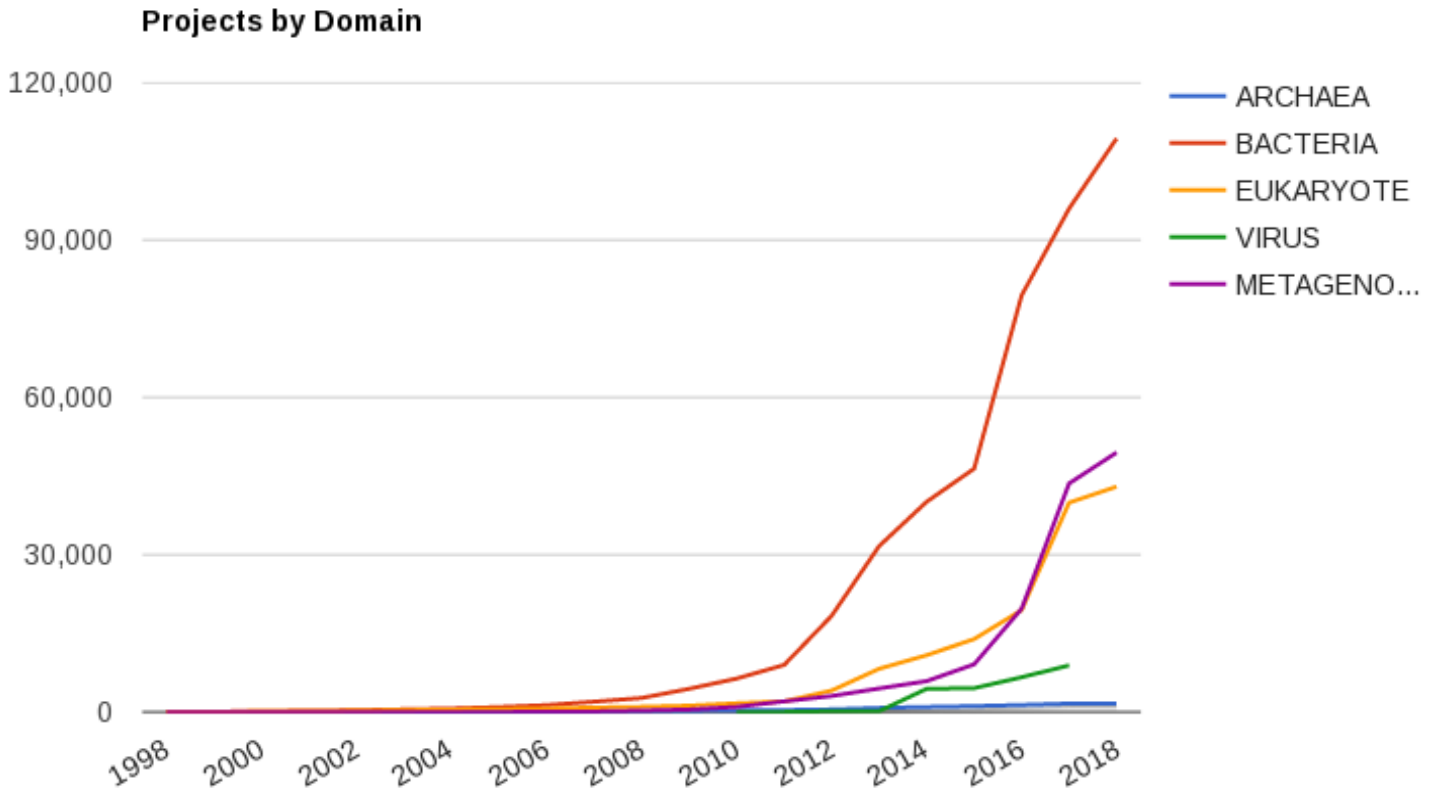
JGI HOME LOG IN

Home Search Distribution Graphs Biogeographical Metadata Statistics References Team Help News

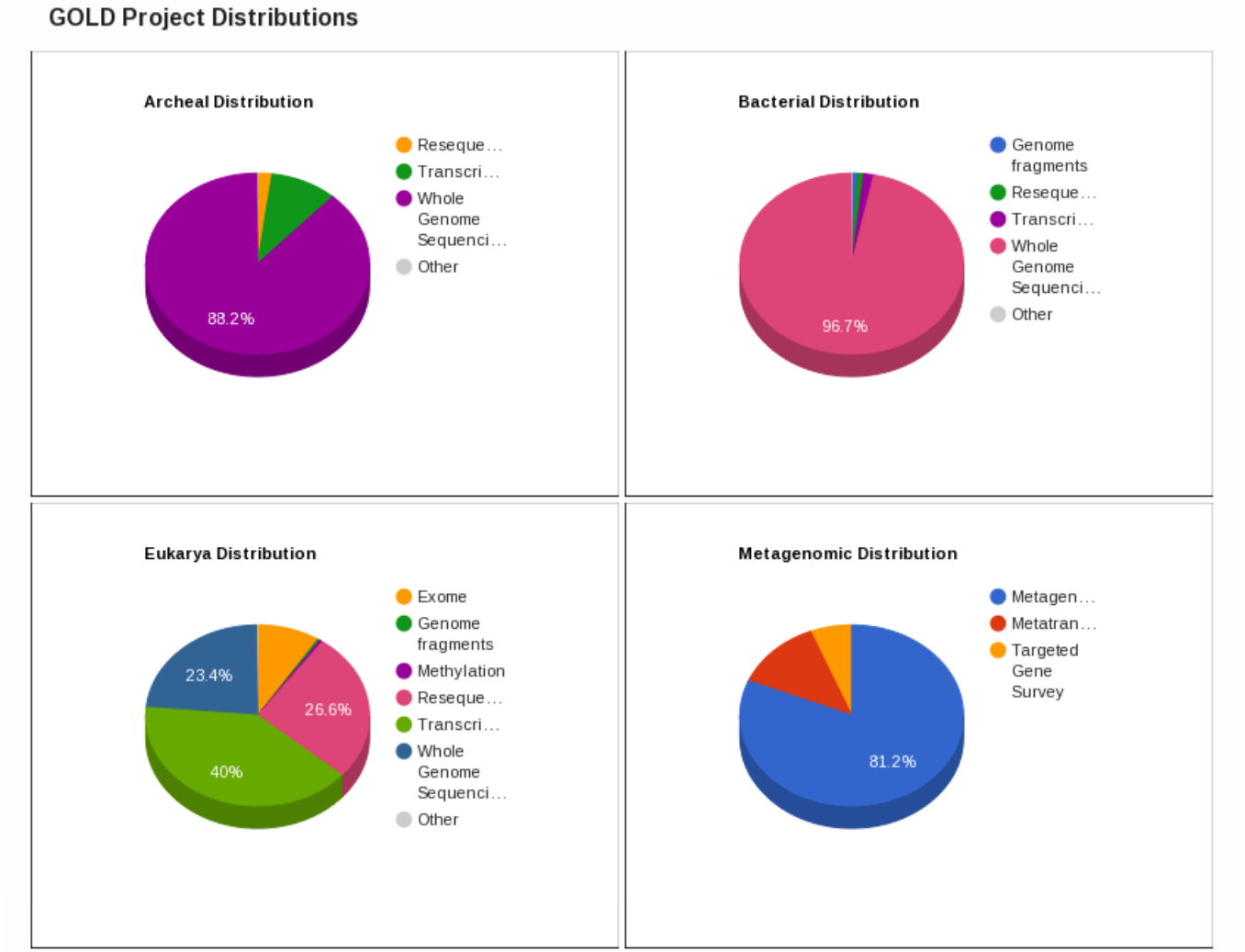
*Source: https://gold.jgi.doe.gov/statistics*



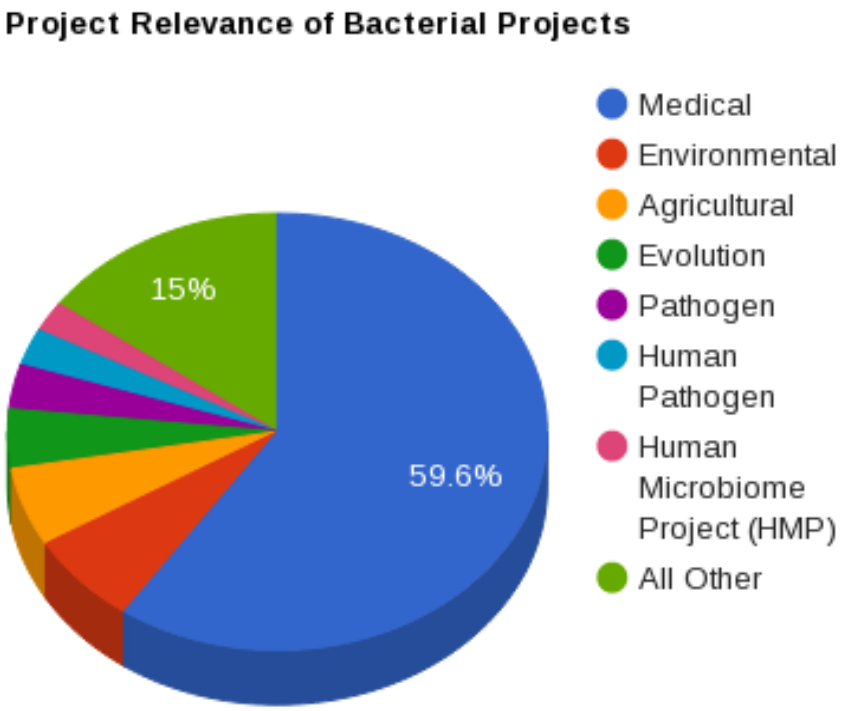
# Genomics Revolution Era



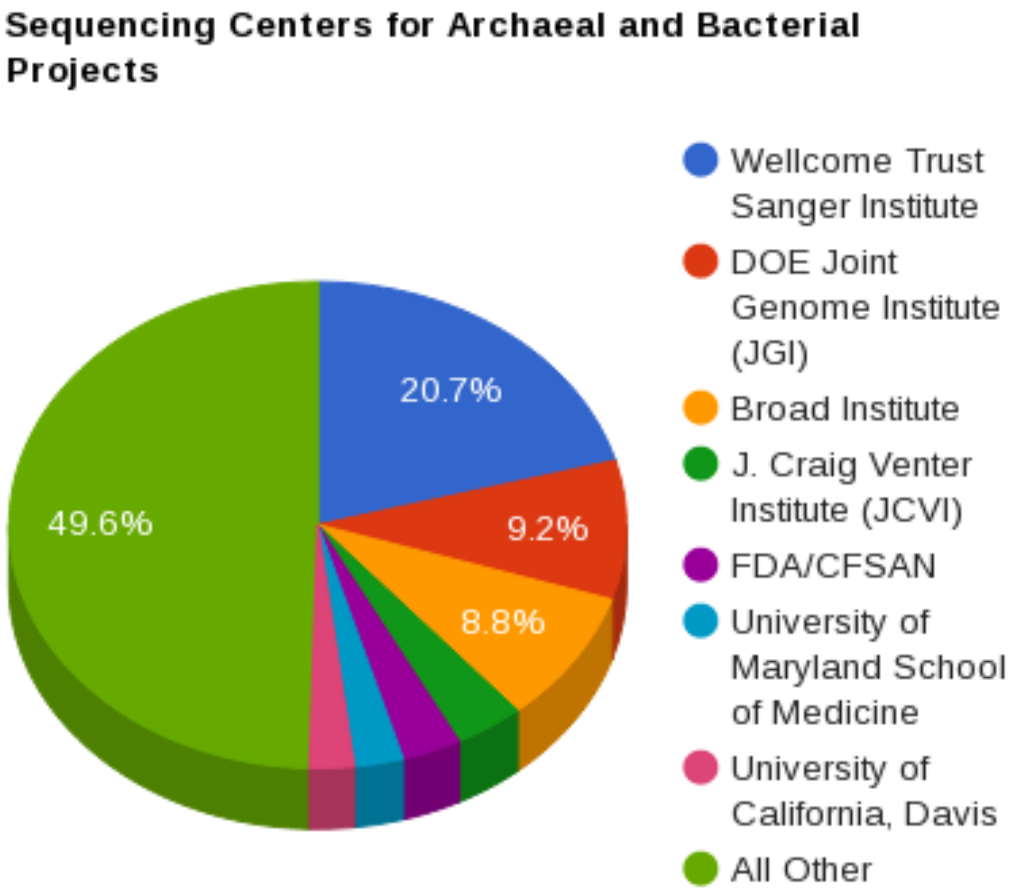
# Genomics Revolution Era



# Genomics Revolution Era



# Genomics Revolution Era



# Secuenciadores

## Primera generación

- Sanger

## Segunda Generación

- 454/Roche
- Solexa/Illumina
- Solid
- Ion Torrent

## Tercera Generación

- Pacific Biosciences
- Nanopore

# High-Throughput Sequencing Platforms

GS-FLX System

Genome Analyzer IIx

SOLID 3 Plus/4

| | | | |
|---|---|---|---|
| **Sequencing Chemistry** | Sequencing by synthesis, pyrosequencing | Sequencing by synthesis with reversible terminators | Sequencing by ligation |
| **Amplification approach** | Emulsion PCR | Cluster amplification | Emulsion PCR |
| **DNA support** | 25-35 µm bead | Flow cell surface | Bead (Solid 3 Plus/4)<br>Flow cell surface (GA5500w) |

*Mardis et al., Trends in Genetics 2008, 24:3*

# High-Throughput Sequencing Platforms

Roche 454 SEQUENCING

GS-FLX System

illumina

Genome Analyzer IIx

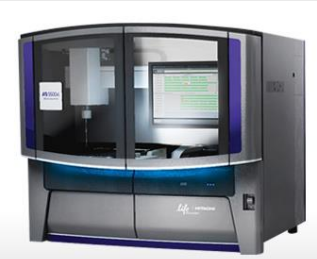
Hiseq 2000/1000

Hiseq 2500/1500

NextSeq

life technologies™

SOLID 3 Plus/4



Genetic Analyzers 5500W Series

# Benchtop High-Throughput Sequencing Platforms

Roche 454 SEQUENCING

**Roche 454 GS Junior**

illumina

**MiSeq**

**iSeq 100**

**MiniSeq**

life technologies™

**Ion Proton™ System**

**Ion PGM™ System**

# Illumina Benchtop Sequencers

https://emea.illumina.com/systems/sequencing-platforms.html




| | 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 | | ● | ● | ● |
| **Run Time** | 9–17.5 hours | 4–24 hours | 4–55 hours | 12–30 hours |
| **Maximum Output** | 1.2 Gb | 7.5 Gb | 15 Gb | 120 Gb |
| **Maximum Reads Per Run** | 4 million | 25 million | 25 million † | 400 million |
| **Maximum Read Length** | 2 × 150 bp | 2 × 150 bp | 2 × 300 bp | 2 × 150 bp |

# Illumina Production-Scale Sequencers

| | Benchtop Sequencers | Production-Scale Sequencers | | |
|---|---|---|---|---|
| |  NextSeq Series ✪ |  HiSeq Series ✪ |  HiSeq X Series† |  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 | ● | ● | | ● |
| **Run Time** | 12–30 hours | < 1–3.5 days (HiSeq 3000/HiSeq 4000)<br>7 hours–6 days (HiSeq 2500) | < 3 days | 16–36 hours (Dual S2 flow cells)<br>44 hours (Dual S2 flow cells) |
| **Maximum Output** | 120 Gb | 1500 Gb | 1800 Gb | 6000 Gb§ |
| **Maximum Reads Per Run** | 400 million | 5 billion | 6 billion | 20 billion‖ |
| **Maximum Read Length** | 2 × 150 bp | 2 × 150 bp | 2 × 150 bp | 2 × 150 bp |

# High-Throughput Single Molecule Sequencing Platforms

## 3ª GENERACIÓN

Helicos BioSciences Corporation

**Helicos Genetic Analysis System**

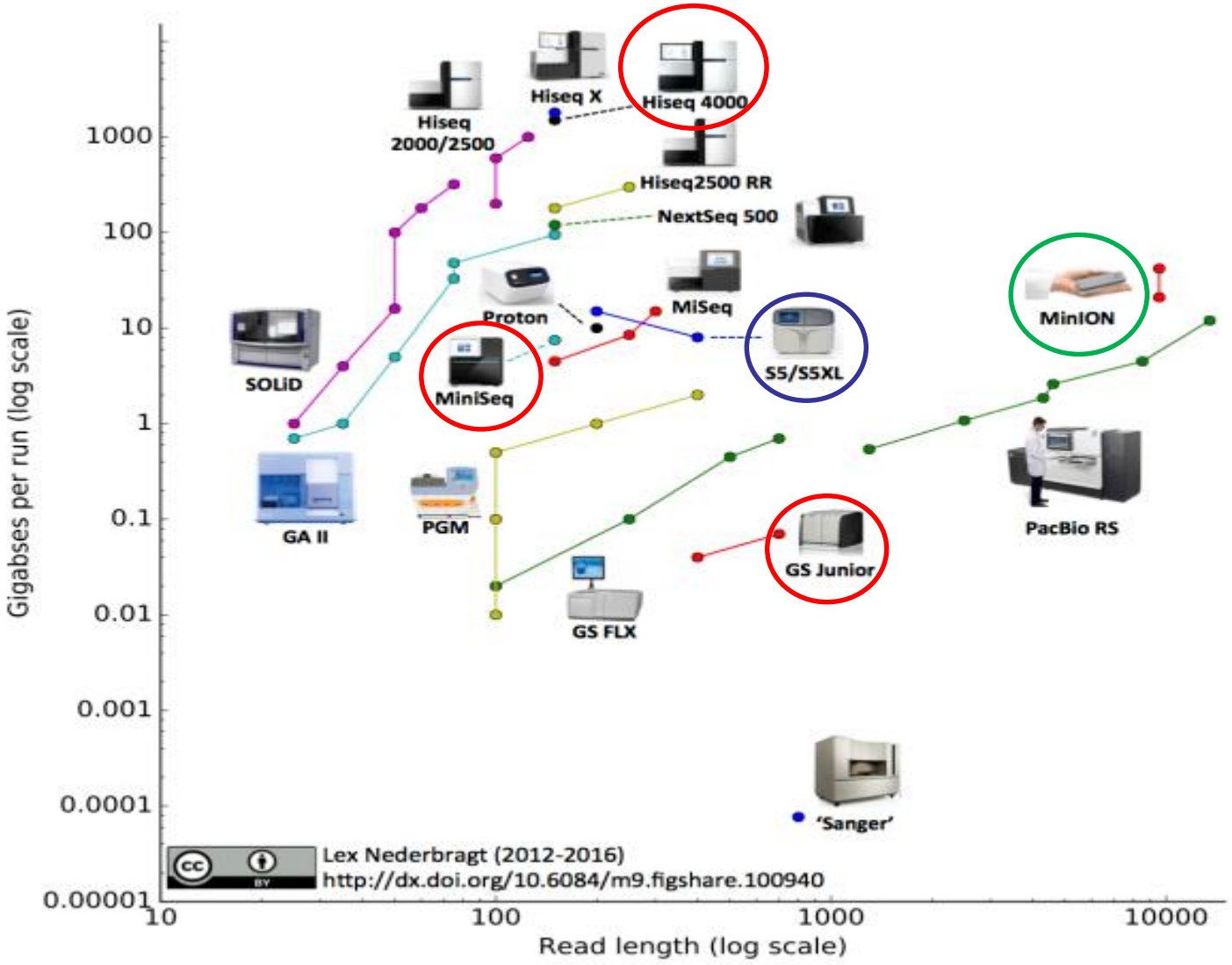
PACIFIC BIOSCIENCES

**PacBio RS II is a Single Molecule, Real-Time (SMRT®) DNA Sequencing System**

Oxford Nanopore

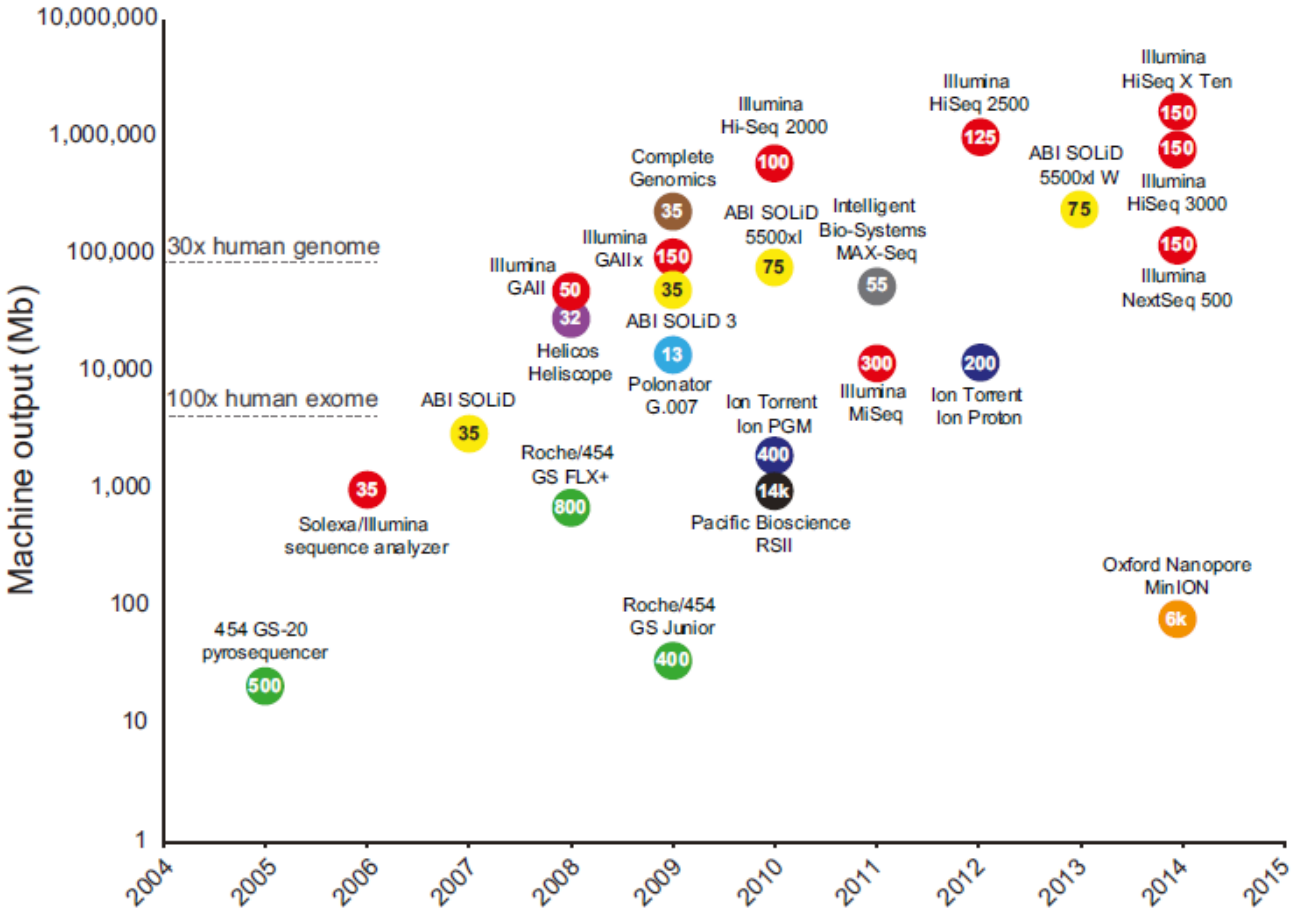
**MinIon™ Device**

# PLATAFORMAS DE SECUENCIACIÓN. 2016 Edition




https://flxlexblog.wordpress.com/

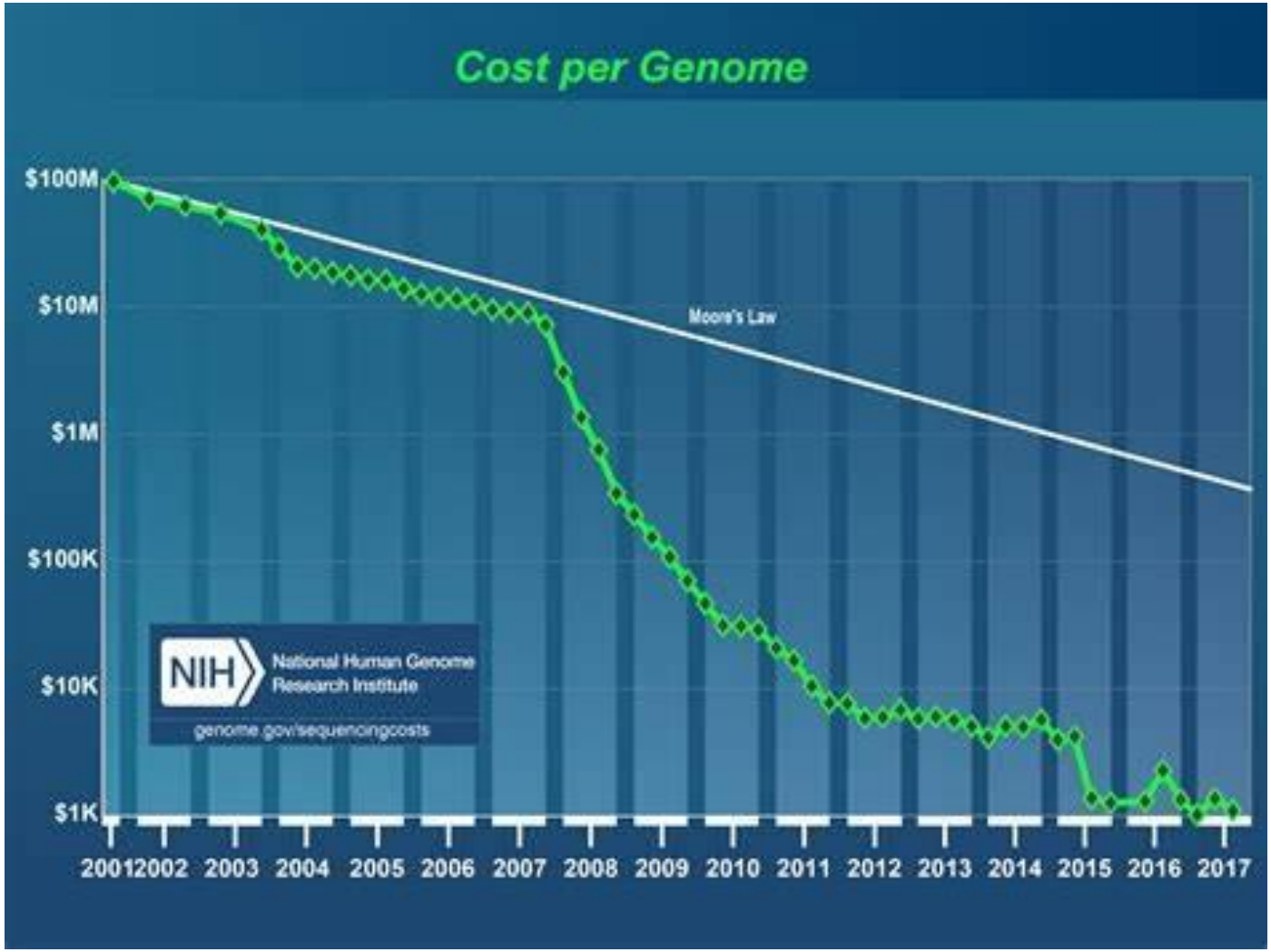
# High-Throughput Sequencing Technologies



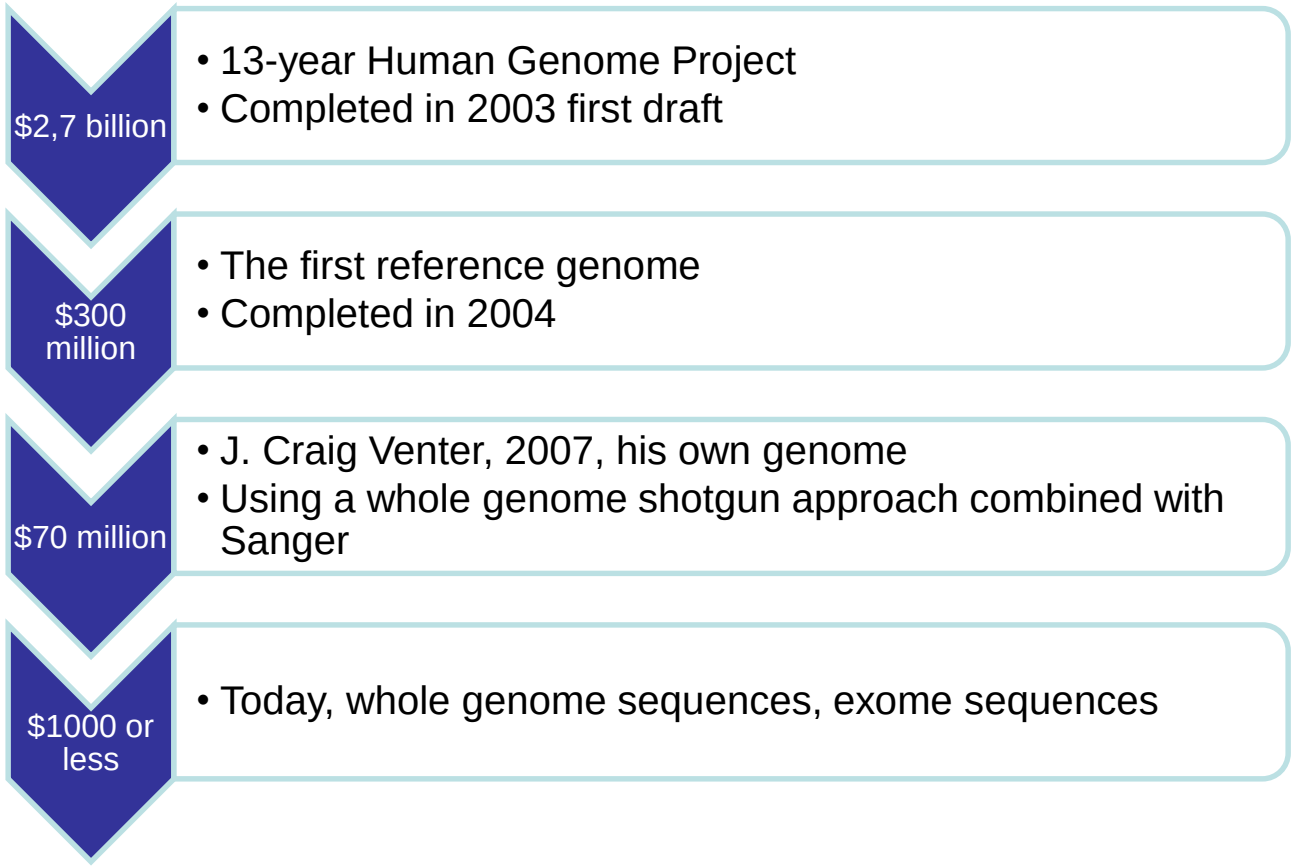

Numbers inside data points denote current read lengths. Sequencing platforms are color coded.

Reuter et al., Mol Cell 2015

# Coste actual de la secuenciación



# Evolución del coste de la secuenciación de un genoma humano




https://www.aacc.org/publications/cln/articles/2012/april/sequencing

## Differences Between Sanger and Next Gen
### samples, tracking, and data relationships

| | Sanger | Next Gen |
|---|---|---|
| **Sequencing Samples** | Clones, PCR | DNA libraries |
| **Sample Tracking** | Many samples in 96, 384 well plates | Few |
| **Preparation Steps** | Few, sequencing reactions clean up | Many, complex procedures |
| **Data collection** | Samples in plates 96, 384 | Samples on slides 1 - 16+ |
| **Data** | One read / sample | Thousands and millions reads / sample |

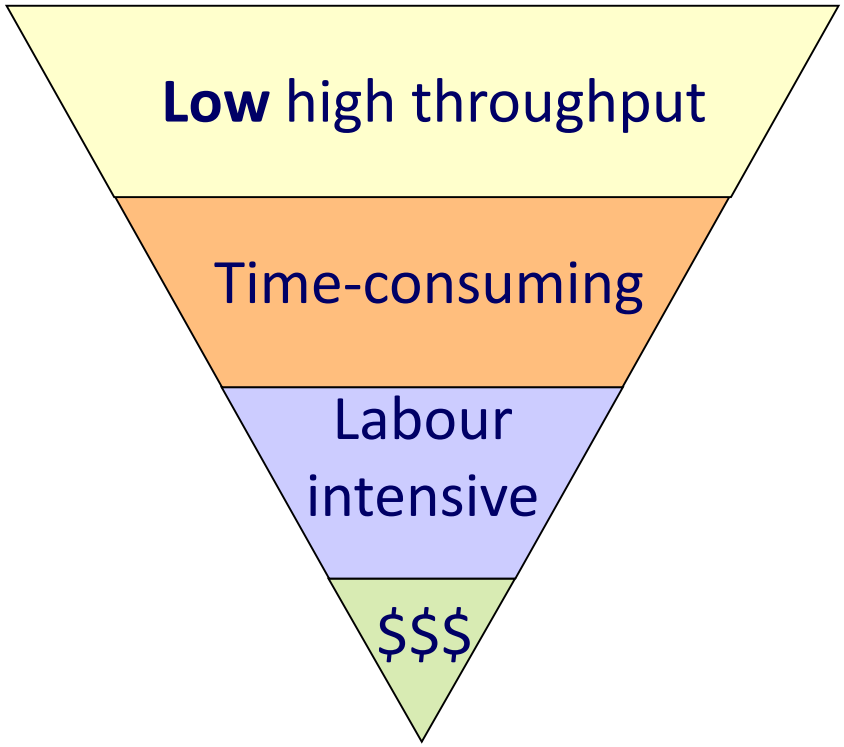
En 2008, hoy 96+

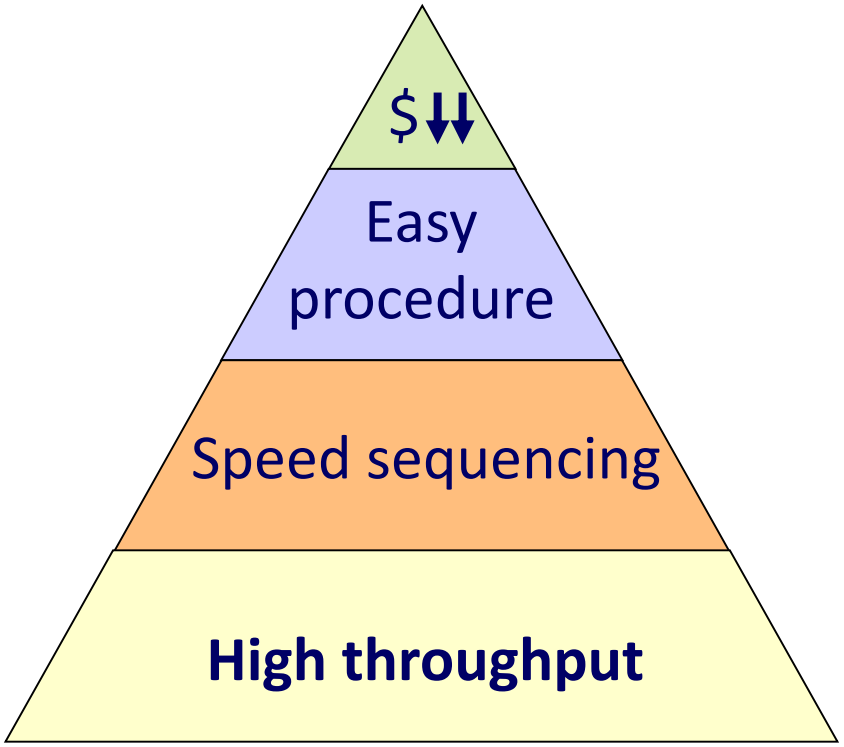
Kits comerciales

geospiza™ FROM SAMPLE TO RESULTS™

# Sanger vs NGS

advantages of new technologies

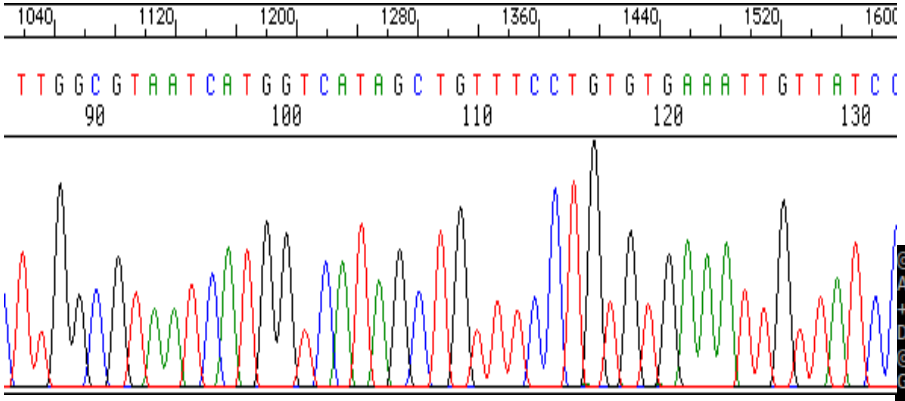



Semiautomatic **Sanger** capillary-based sequencing technology

NGS
**Next** Generation Sequencing =
**Now** Generation Sequencing

# Nuevo escenario en el análisis de datos, BIG DATA

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+
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+
B:B:?BB/:=55177#55877<775EDD>E=B?BBBBGGGDDAG@G>GGGGGG@)EEEEBEG>GGGGGGGAAA?<D
@HWUSI-EAS1752R:21:FC64JUKAAXX:3:1:3185:1033 1:N:0:ACAGTG
TCTGGGACATTGCTCNTGGCTGGGAGTCACCTGTCTGGGACATTGCTCAGGGCTGGGAGACACGTGTTGGAGGGAC
+
BC??A66;)74781<#7??;452.27'64(8,851DDG8GB?##############################
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# Secuenciadores

## Primera generación

- Sanger

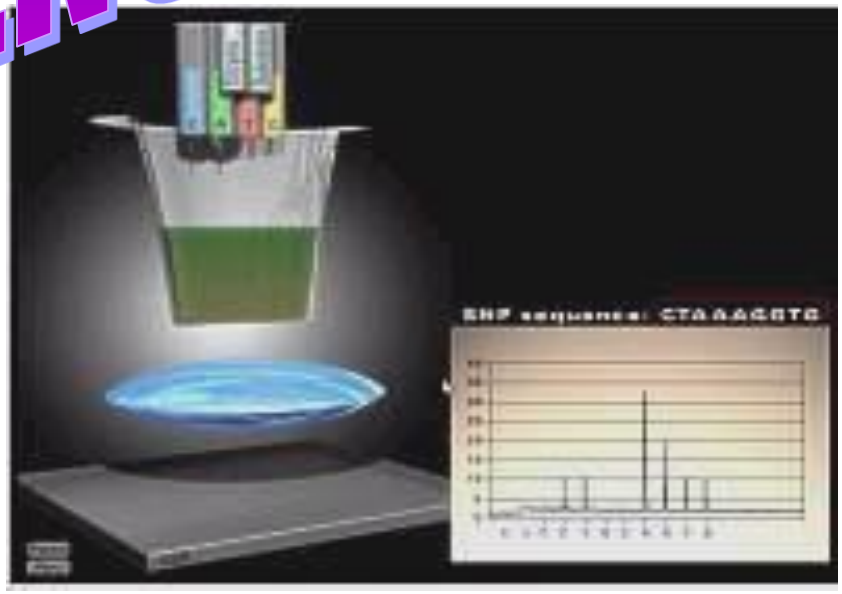
## Segunda Generación

- 454/Roche
- Solexa/Illumina
- Solid
- Ion Torrent

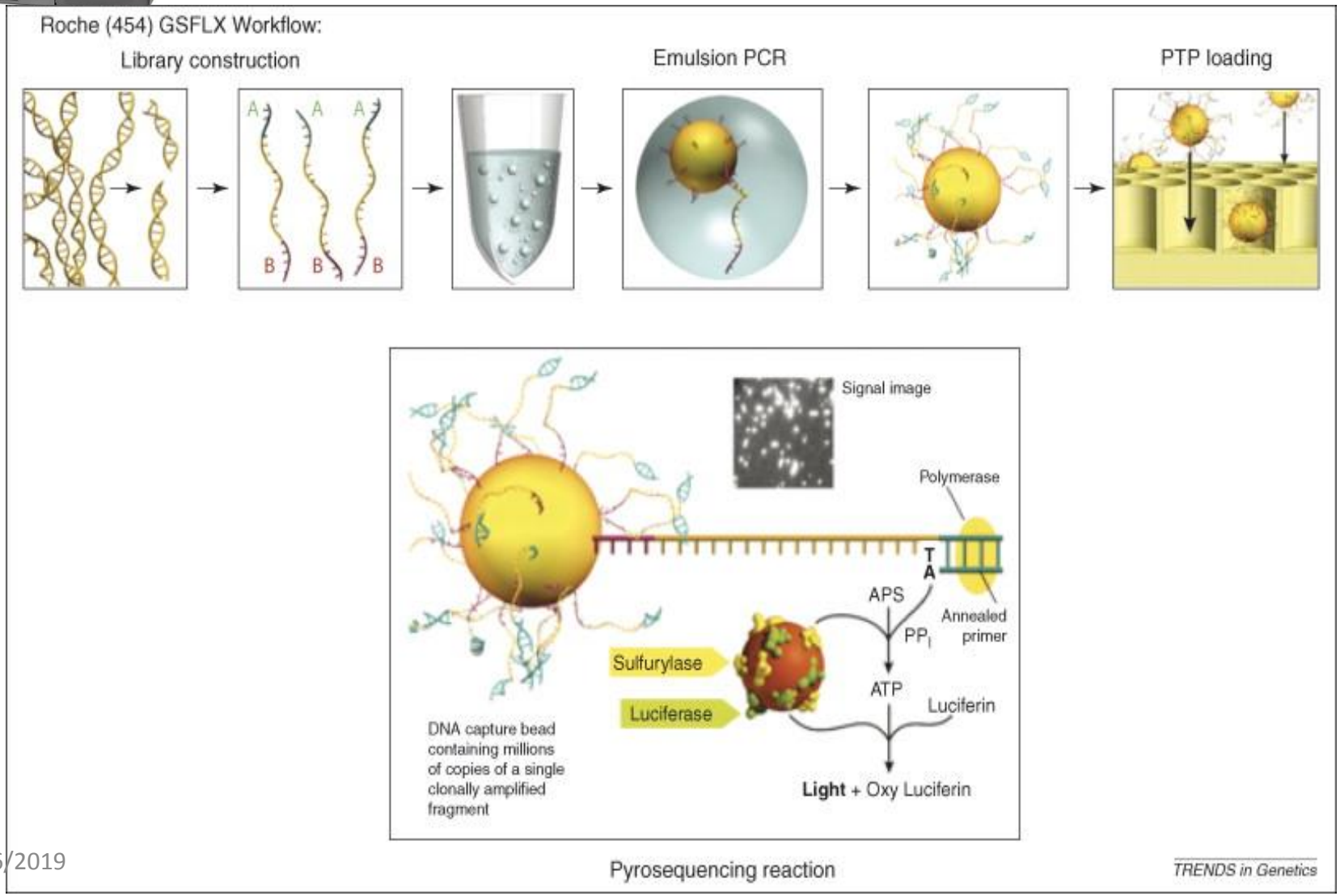
## Tercera Generación

- Pacific Biosciences
- Nanopore

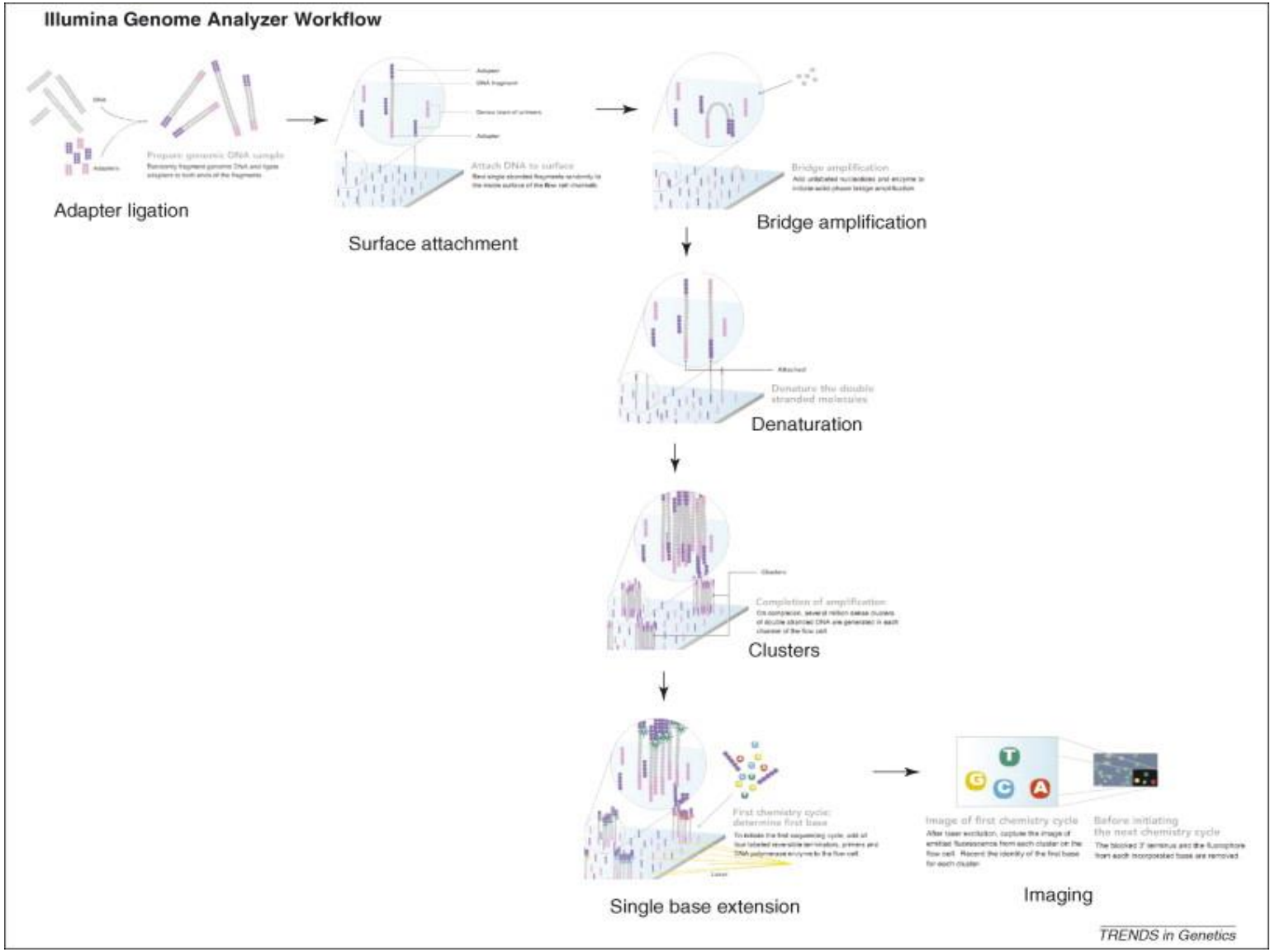
# PIROSECUENCIACIÓN

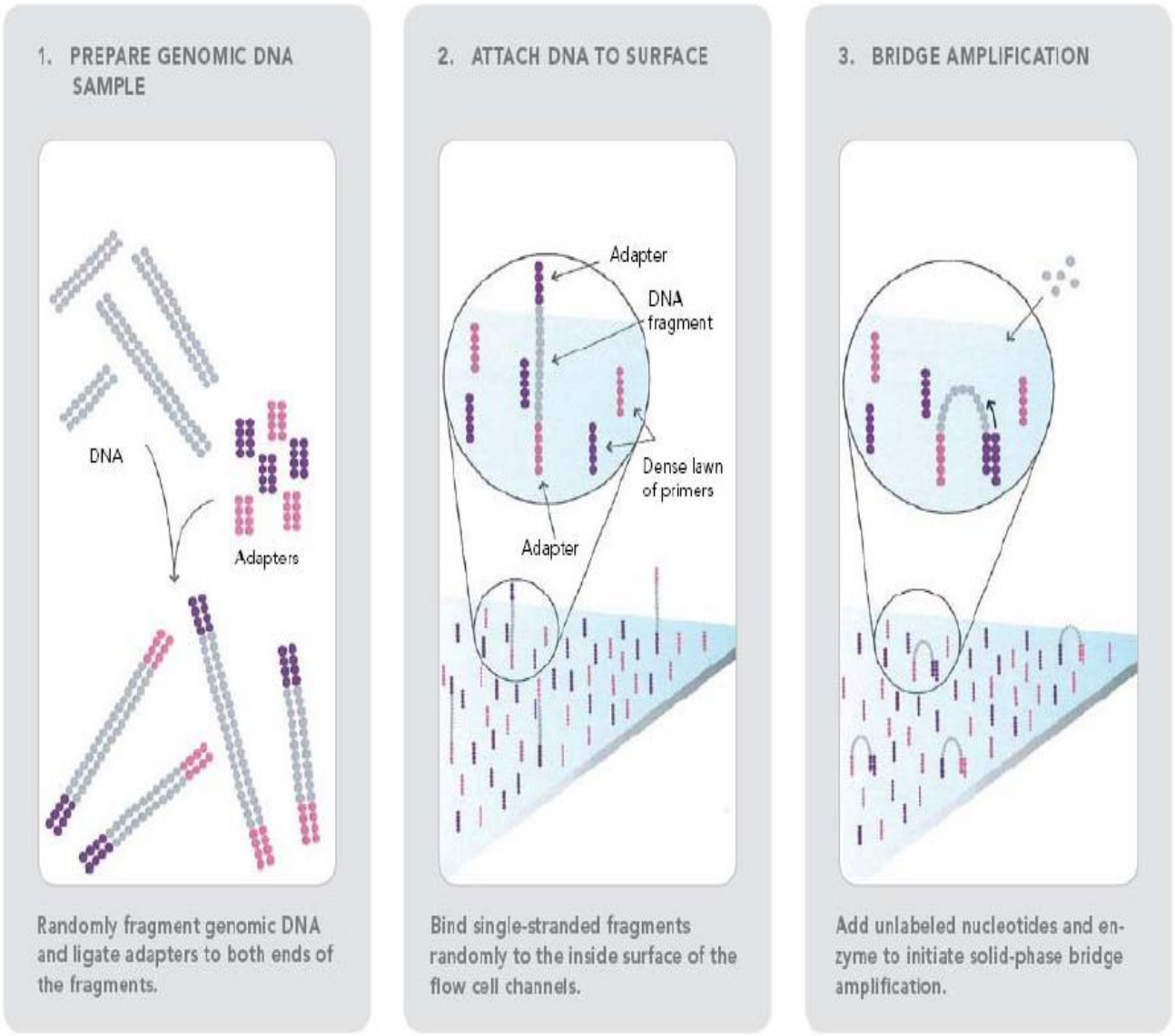


# Roche (454) Workflow

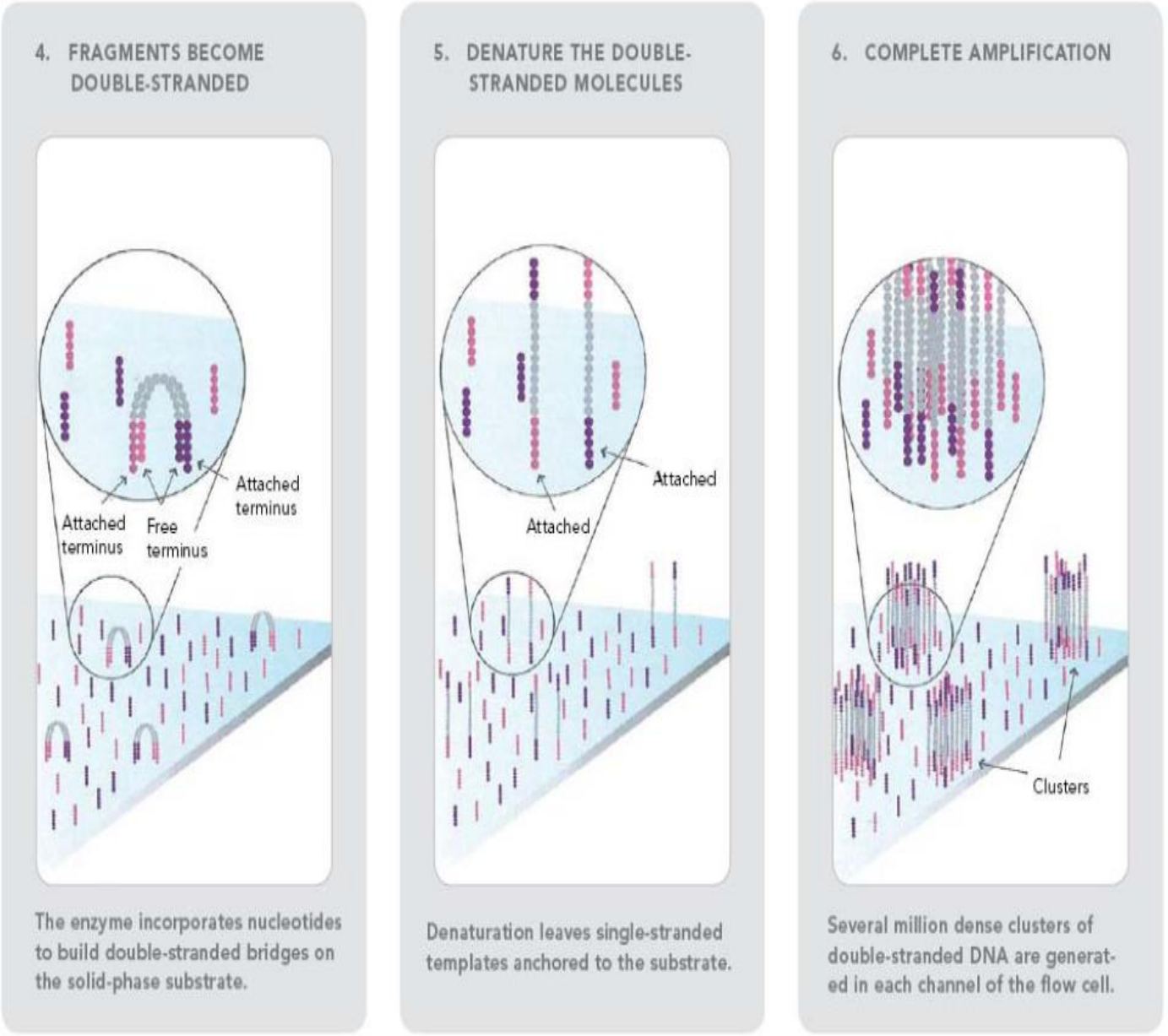


# Illumina (Solexa) Workflow

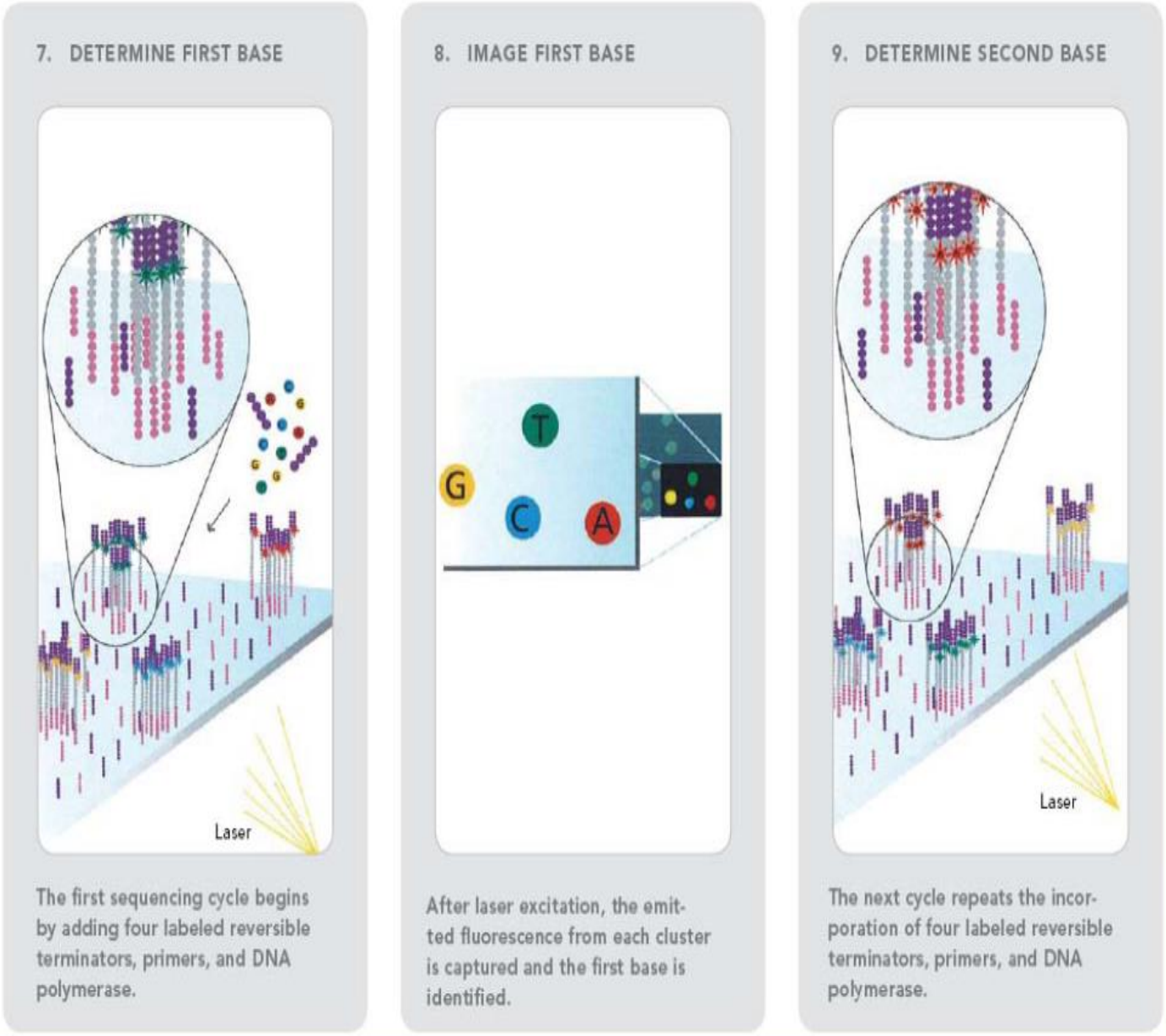





*amplificación en fase sólida*

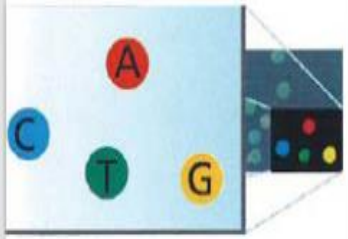



*> 1000 copies in ≤ 1 µm;  $10^7$ clusters per $cm^2$*



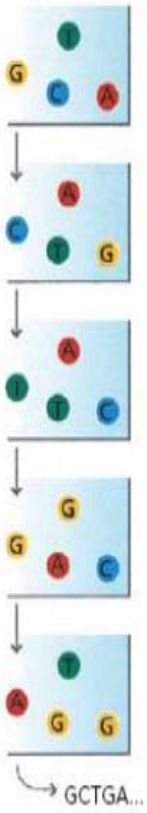

*Secuenciación con nucleótidos terminadores reversibles*

10. IMAGE SECOND CHEMISTRY CYCLE



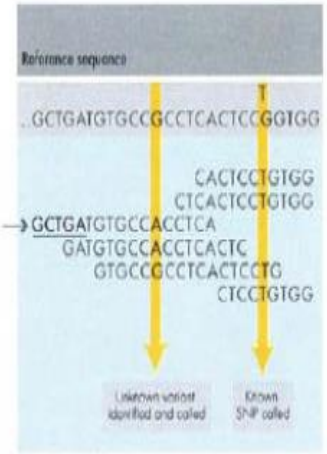

After laser excitation, the image is captured as before, and the identity of the second base is recorded.

11. SEQUENCING OVER MULTIPLE CHEMISTRY CYCLES



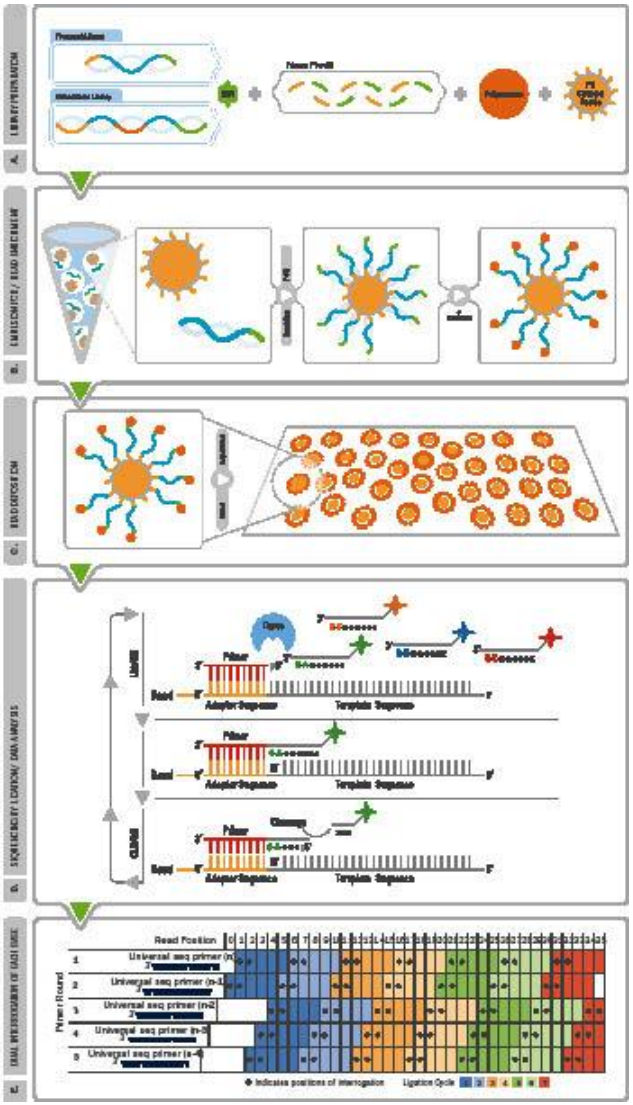

The sequencing cycles are repeated to determine the sequence of bases in a fragment, one base at a time.

12. ALIGN DATA




The data are aligned and compared to a reference, and sequencing differences are identified.

# Life Technologies / Solid



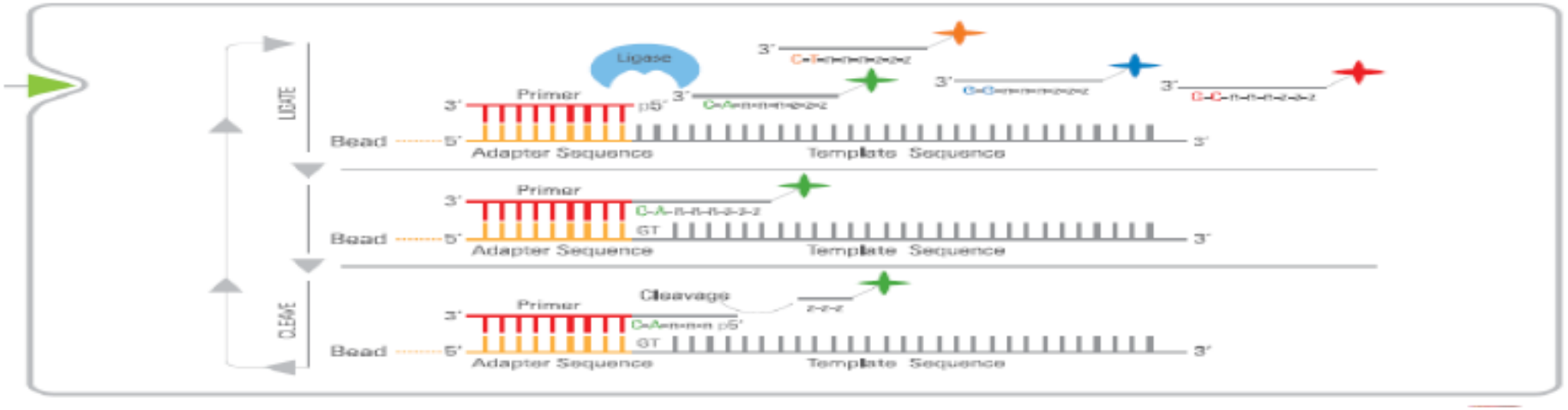
Library Construction

Emulsion PCR

The beads are deposited onto a slide

# Life Technologies / Solid

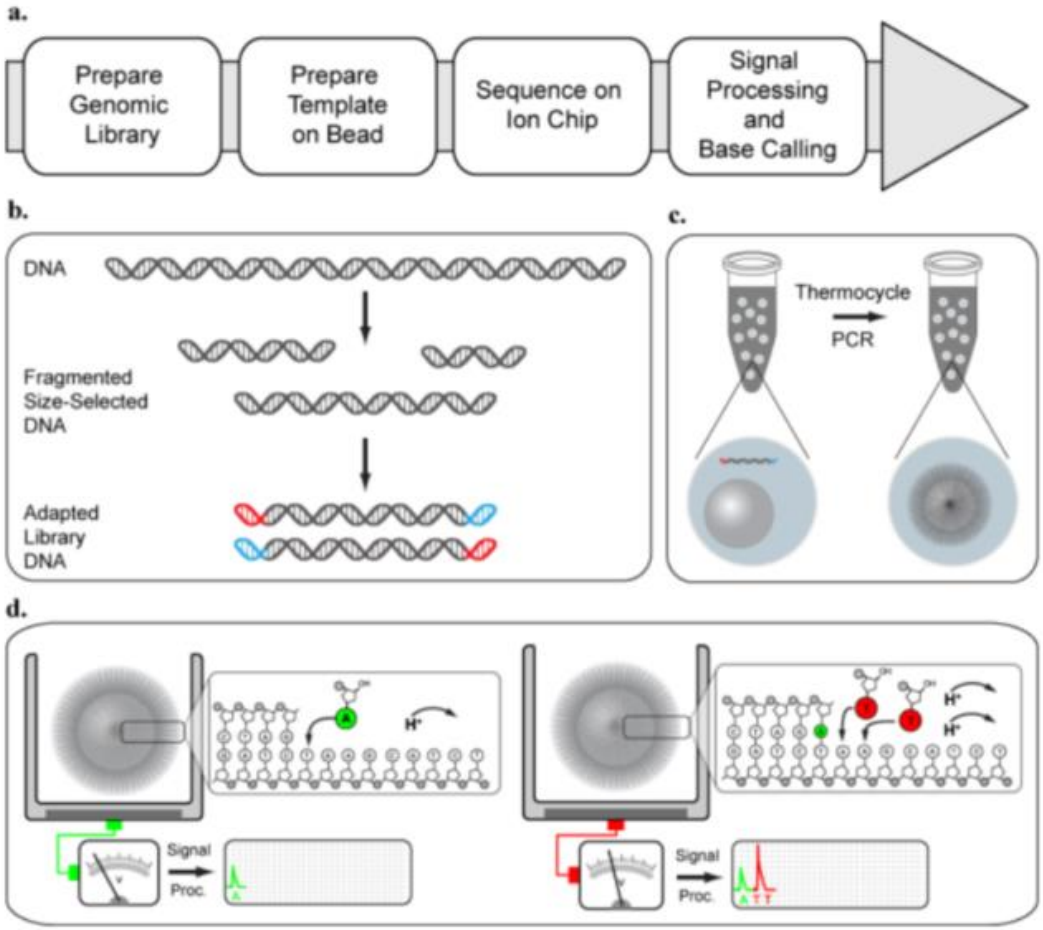
## Sequencing by Ligation




Primers hybridize to the P1 adapter sequence within the library template.
A set of four fluorescently labeled di-base probes compete for ligation to the sequencing primer. Specificity of the di-base probe is achieved by interrogation every 1st and 2nd base in each ligation reaction.
Multiple cycles of ligation, detection and cleavage are performed.

# Ion Torrent PGM

Personal Genome Machine




# Secuenciadores

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- Solid
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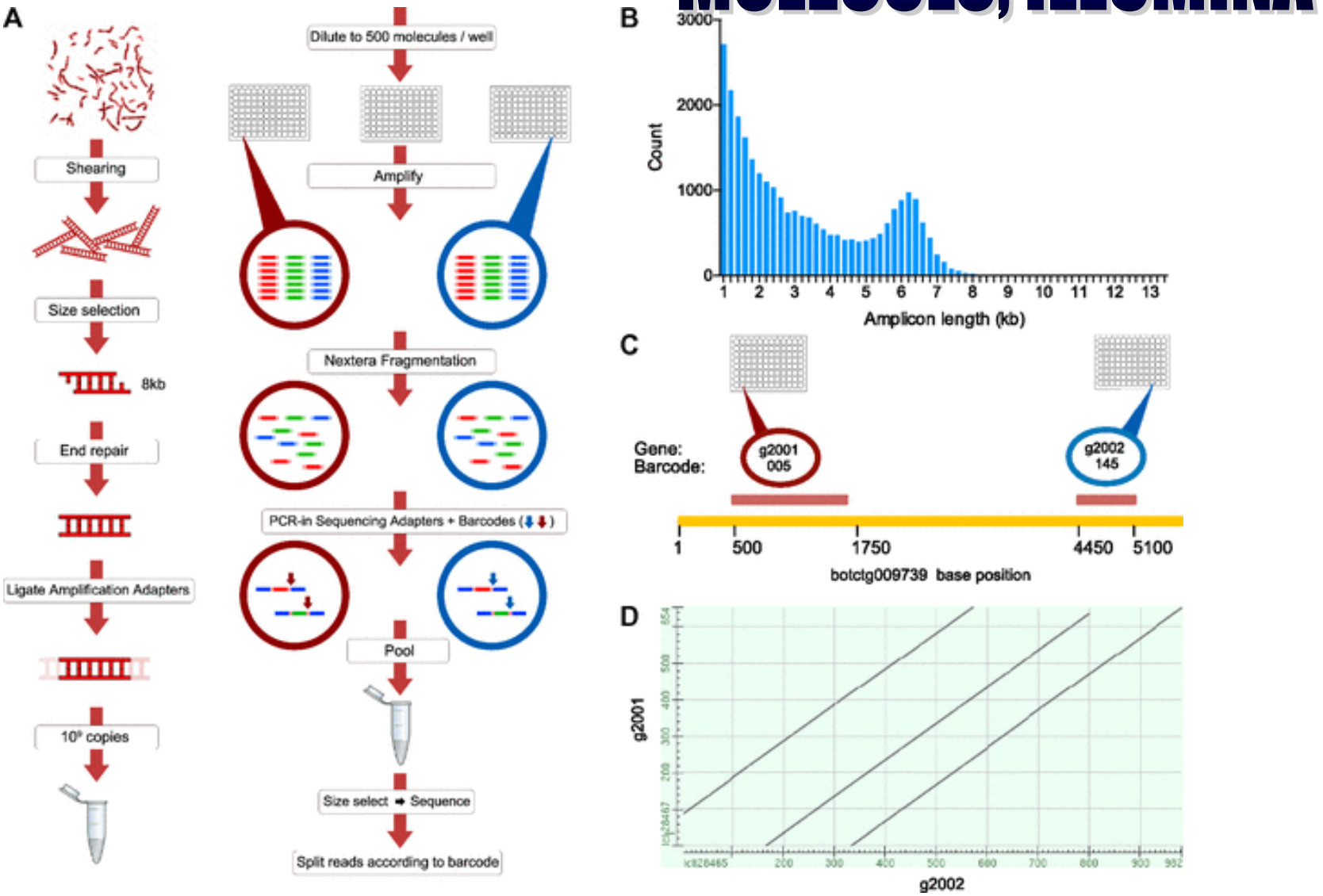
## Tercera Generación

- Pacific Biosciences
- Nanopore

# 3ª GENERACIÓN: LECTURAS MAS LARGAS Y MOLECULA ÚNICA

- PacBio, PACIFIC BIOSCIENCE
- Moleculo, ILLUMINA
- MinION, GridION, OXFORD NANOPORE

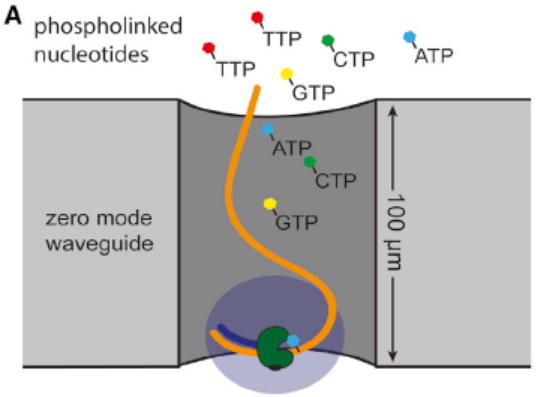
# MOLECULO, ILLUMINA

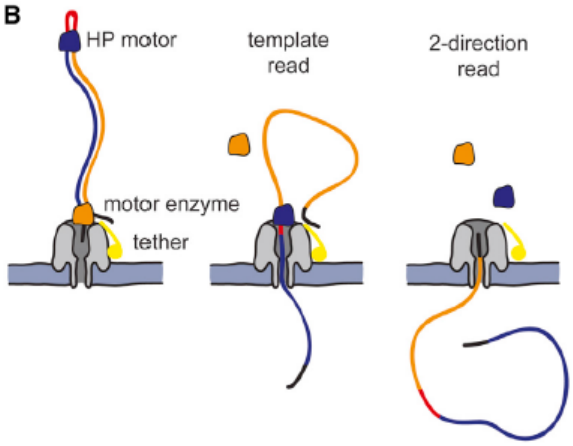


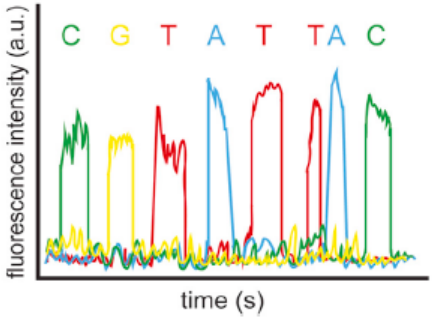

Moleculo, acquired by Illumina in late 2012, developed an innovative technology for generating long reads that combines a new library prep method and genome analysis tools

Voskoboynik et al., elife 2013,2:e00569

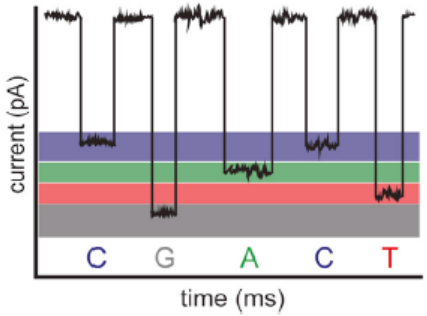
# The Third-generation Sequencing Technologies




Pacific Bioscience's SMRT sequencing




Oxford Nanopore's sequencing strategy

Reuter et al., Mol Cell 2015

**Single Molecule Sequencing Platforms**

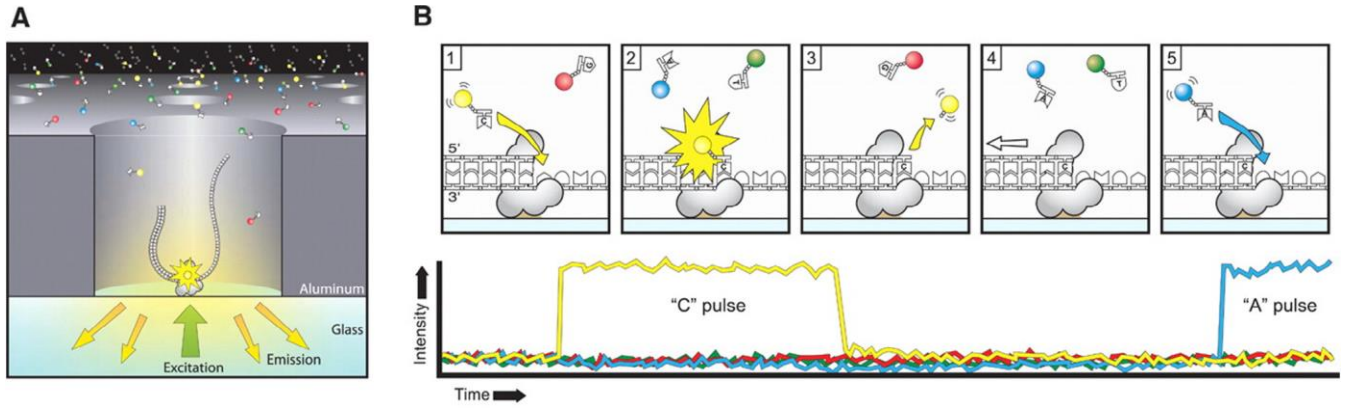
# PacBio sequencing and its applications

Rhoads & Au, Gen Prot Bioinf 2015

**SMRTbell template**: is a closed, single-stranded circular DNA that is created by ligating hairpin adaptors to both ends of a target dsDNA

**Sequencing by light pulses:** The replication processes in all ZMWs of a SMRTcell are recorder by a movie of light pulses, and the pulses corresponding to each ZMW can be interpreted to be a sequence of bases (**continuous long read, CLR**).
Both strands can be sequenced multiple times (passes) in a single CLR. CLR can be split to multiple reads (subreads) and CCS is the consensus sequence of multiple subreads

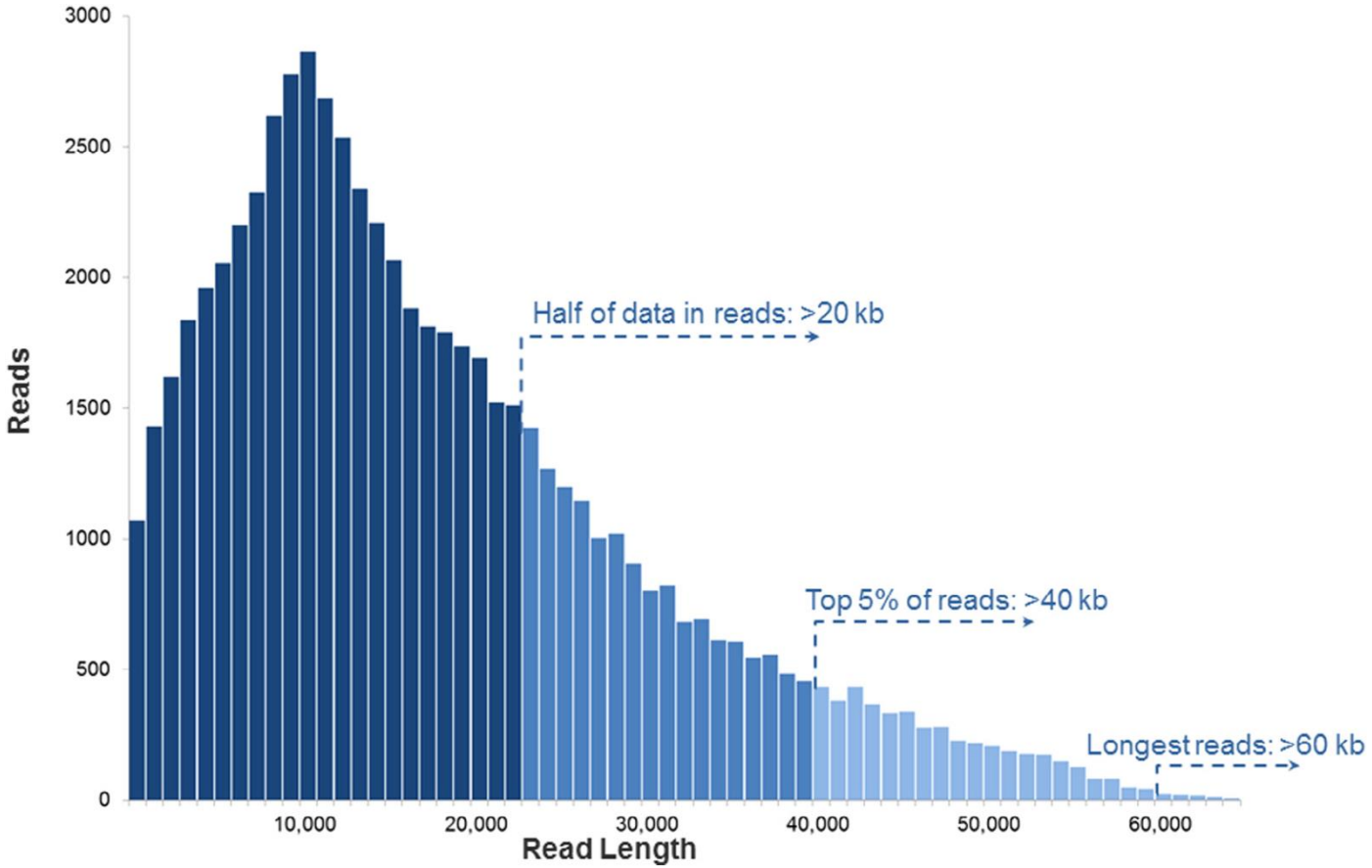
**A single SMRT cell:** this contains 150000 ZMWs (zero-mode waveguide). A SMRTbell diffuses into a ZMW.
Approx 35000 -75000 ZMWs produce a read in a run lasting 0,5-4h resulting in 0,5-1Gb.



# PacBio sequencing and its applications

Rhoads & Au, Gen Prot Bioinf 2015

**PacBio RS II read length distribution** using P6-C4 chemistry. Data are based on a 20kb size-selected E. coli library using a 4-h movie. A SMRTcell produces 0,5-1 billion bases.



# PacBio sequencing and its applications

Rhoads & Au, Gen Prot Bioinf 2015

Table 2 *De novo* genome assemblies using hybrid sequencing or PacBio sequencing alone

| Species | Method | Tools | SMRT cells | Coverage | Contigs | Achievements | Ref. |
|---|---|---|---|---|---|---|---|
| *Clostridium autoethanogenum* | PacBio | HGAP | 2 | 179× | 1 | 21 fewer contigs than using SGS; no collapsed repeat regions (⩾4 using SGS) | [7] |
| *Potentilla micrantha* (choloroplast) | PacBio | HGAP, Celera, minimus2, SeqMan | 26 | 320× | 1 | 6 fewer contigs than with Illumina; 100% coverage (Illumina: 90.59%); resolved 187 ambiguous nucleotides in Illumina assembly; unambiguously assigned small differences in two >25 kb inverted repeats | [33] |
| *Escherichia coli* | PacBio | PBcR, MHAP, Celera, Quiver | 1 | 85× | 1 | 4.6 CPU hours for genome assembly (10× improvement over BLASR) | [31] |
| *Saccharomyces cerevisiae* | PacBio | PBcR, MHAP, Celera | 1[2] | 117× | 21 | 27 CPU hours for genome assembly (8× improvement over BLASR); improved current reference of telomeres | [31] |
| *Arabidopsis thaliana* | PacBio | PBcR, MHAP, Celera | 46 | 144× | 38 | 1896 CPU hours for genome assembly | [31] |
| *Drosophila melanogaster* | PacBio | PBcR, MHAP, Celera, Quiver | 42 | 121× | 132 | 1060 CPU hours for genome assembly (593× improvement over BLASR); improved current reference of telomeres | [31] |
| *Homo sapiens* (CHM1htert) | PacBio | PBcR, MHAP, Celera | 275 | 54× | 3434 | 262,240 CPU hours for genome assembly; potentially closed 51 gaps in GRCh38; assembled MHC in 2 contigs (60 contigs with Illumina); reconstructed repetitive heterochromatic sequences in telomeres | [31] |
| *Homo sapiens* (CHM1tert) | PacBio | BLASR, Celera, Quiver | 243 | 41× | N/A (local assembly) | Closed 50 gaps and extended into 40 additional gaps in GRCh37; added over 1 Mb of novel sequence to the genome; identified 26,079 indels at least 50 bp in length; cataloged 47,238 SV breakpoints | [32] |
| *Melopsittacus undulatus* | Hybrid | PBcR, Celera | 3 | 5.5× PacBio + 15.4× 454 = 3.83× corrected | 15,328 | 1st assembly of >1 Gb parrot genome; N50 = 93,069 | [34] |
| *Vibrio cholerae* | Hybrid | BLASR, Bambus, AHA | 195 | 200× PacBio + 28× Illumina + 22× 454 | 2 | No N's in contigs; 99.99% consensus accuracy; N50 = 3.01 Mb | [30] |
| *Helicobacter pylori* | PacBio | HGAP, Quiver, PGAP | 8 per strain | 446.5× average among strains | 1 per strain | 1 complete contig for each of 8 strains; methylation analysis associated motifs with genotypes of virulence factors | [35] |

*Note:* N50, the contig length for which half of all bases are in contigs of this length or greater; MHC, major histocompatibility complex; SV, structural variation.

# PacBio sequencing and its applications

Rhoads & Au, Gen Prot Bioinf 2015

**Advantage**

Closes gaps and completes genomes due to longer reads

Identifies non-SNP SVs

**Achievements**

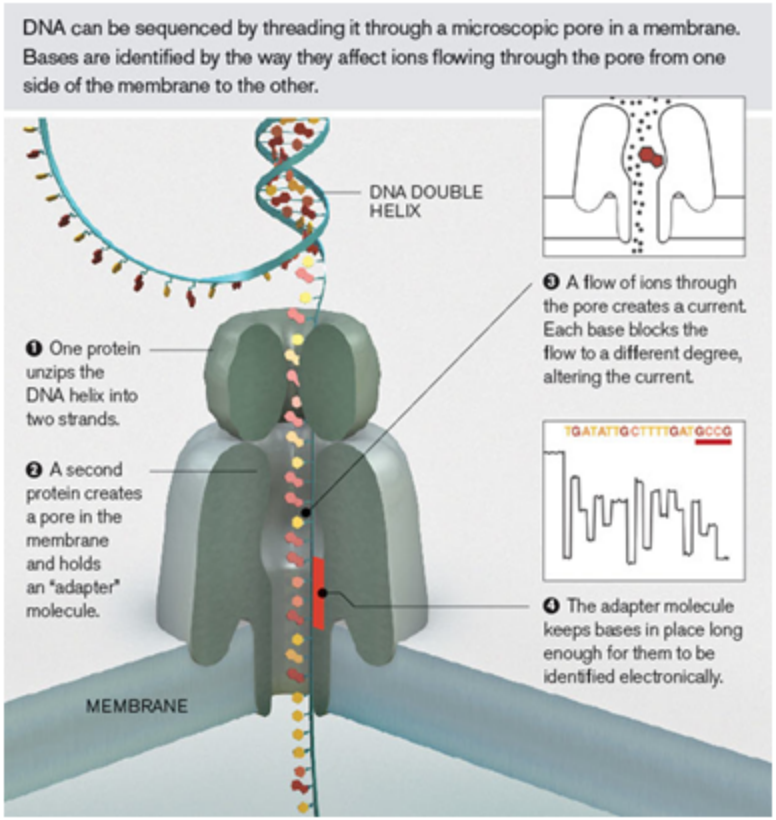
Produced highly-contiguous assemblies of bacterial and eukaryotic genomes

Discovered STRs (short tandem repeats)

**Limitations**

Both strands can be sequenced several times if the lifetime of the polymerase is long enough.

# Nanopore-based fourth-generation DNA sequencing technology. ONT, Oxford Nanopore Technologies



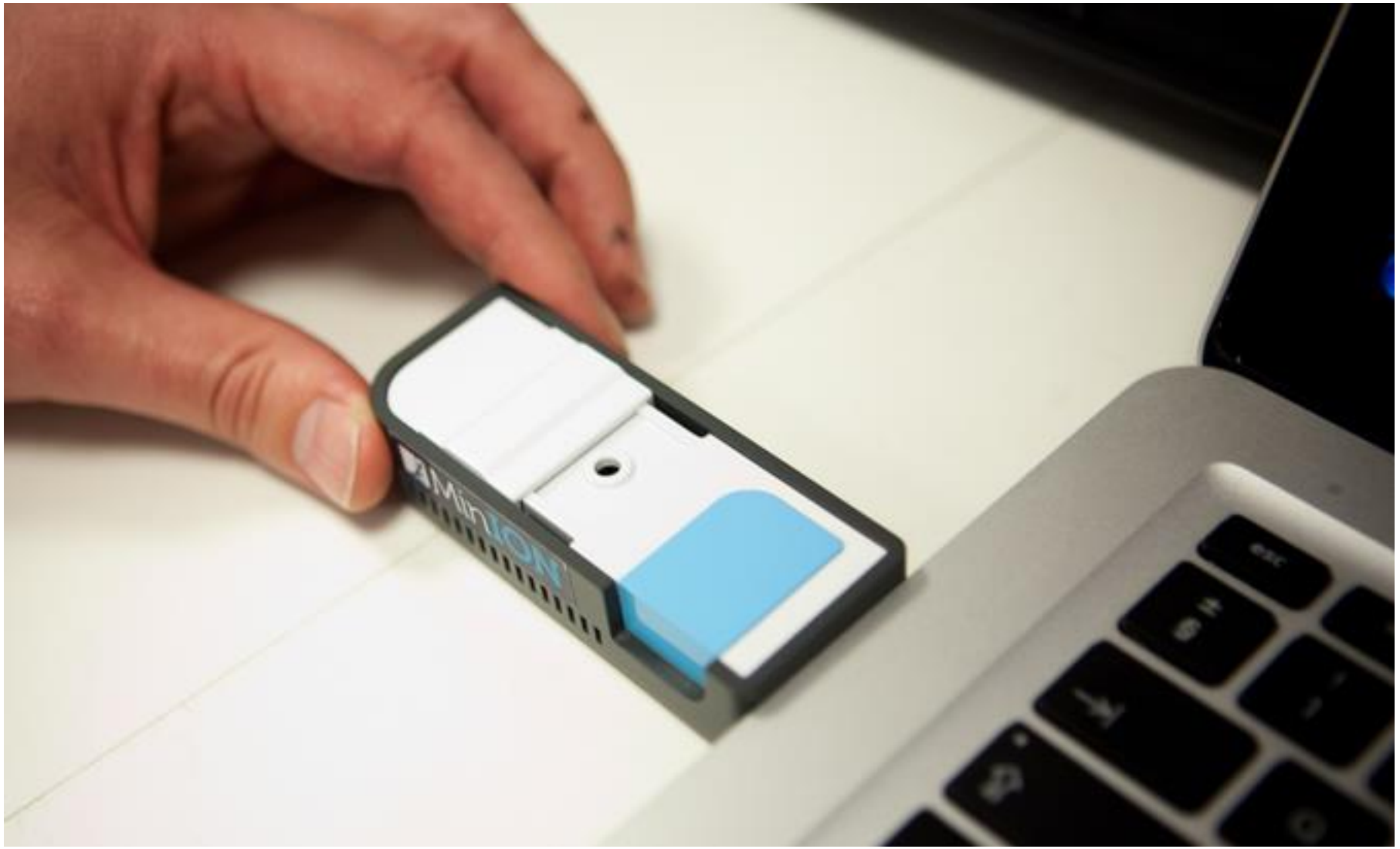

Strand sequencing' is a technique that passes intact DNA polymers through a protein nanopore, sequencing in real time as the DNA translocates the pore.

Nanopore sequencing also offers, for the first time, direct RNA sequencing, as well as PCR or PCR-free cDNA sequencing.

https://nanoporetech.com/applications/dna-nanopore-sequencing

Feng et al , Gen Prot Bioinf 2015

# MinIon, OXFORD NANOPORE




https://nanoporetech.com/news/movies#movie-24-nanopore-dna-sequencing

# Oxford Nanopore Technologies, MinION

The MinION is a portable sequencer; flow cells contain up to 512 nanopore sensors.

The Oxford Nanopore system processes the reads that are presented to it rather than generating read lengths. Sample-prep dependent, the longest read reported by a MinION user to date is >1 Mb.

Long reads confer many advantages, including simpler assembly and in the analysis of repetitive regions, phasing or CNVs.

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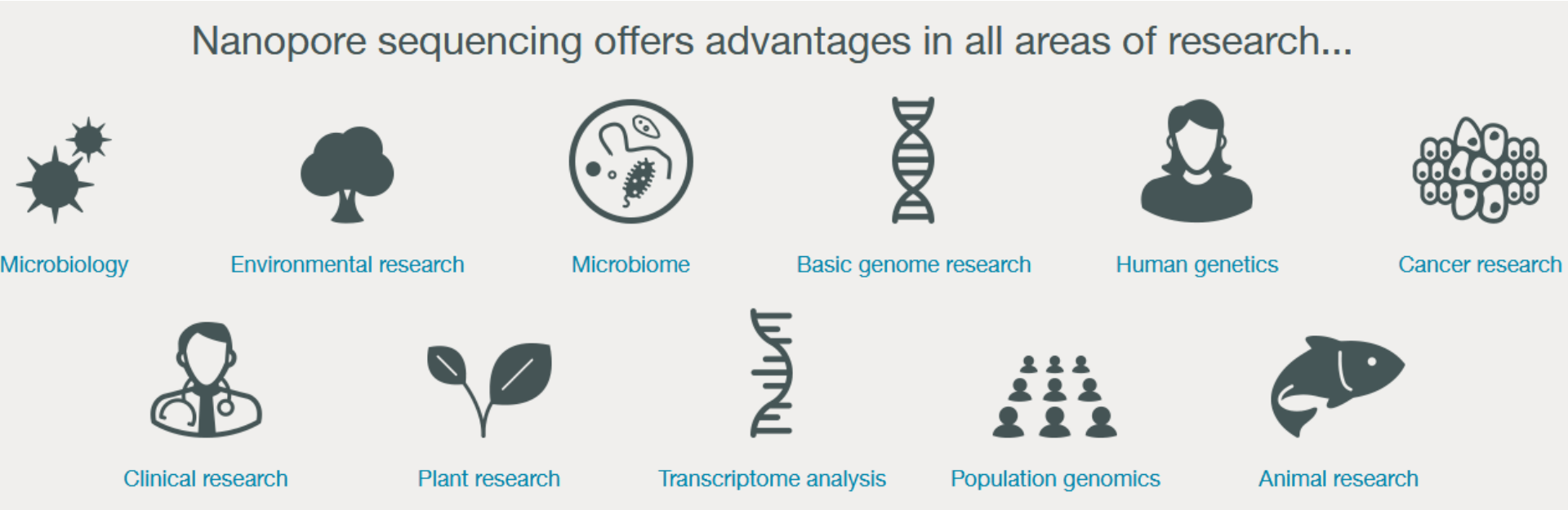
Long reads confer many advantages, including simpler assembly and in the analysis of repetitive regions, phasing or CNVs.

# Oxford Nanopore Technologies


| Flongle | MinION | GridION | PromethION |
|---|---|---|---|
| Long read, direct DNA/RNA/epigenetic sequencing, scalable, real time/rapid, on-demand sequencing that is easy to use and install. | | | |
| ✔ Your portable device for smaller, individual, rapid tests.<br>✔ When you don't want to multiplex samples or start a larger run.<br>✔ Amplicons, panels/targeted sequencing, quality testing and more.<br>✔ For use with MinIT or a laptop. | ✔ Your personal sequencer, putting you in control.<br>✔ Whether in your lab or out in the field.<br>✔ Whole genomes/exomes, metagenomics, targeted sequencing, whole transcriptome (cDNA), smaller transcriptomes (direct RNA), multiplexing for smaller samples and more.<br>✔ For use with MinIT or a laptop. | ✔ High throughput sequencing, in modular form (up to 5 flow cells) to be on-demand.<br>✔ For your lab or to offer as a service.<br>✔ Larger genomes or projects, whole transcriptomes (direct RNA or cDNA) or where you have larger numbers of samples and more.<br>✔ Compute included for real time data analysis and easy installation. | ✔ Very high throughput sequencing, in modular form (up to 48 flow cells) to be on-demand.<br>✔ For your lab or as a service.<br>✔ Larger genomes or projects, whole transcriptomes (direct RNA or cDNA), very large numbers of samples and more.<br>✔ Compute included for real time data analysis and easy installation. |

# Nanopore sequencing applications



# Library preparation


Oxford Nanopore has developed VolTRAX – a small device designed to perform library preparation automatically, so that a user can get a biological sample ready for analysis, hands-free. VolTRAX is designed as an alternative to a range of lab equipment, to allow consistent and varied, automated library prep options.

VolTRAX V2 Starter Pack

$8,000.00

VolTRAX V2 is designed to automate all laboratory processes associated with Nanopore Sequencing from sample extraction to library preparation.

# MinIT, Analysis

Eliminating the need for a dedicated laptop for nanopore sequencing with MinION.
$2400

**MinIT Specifications:**

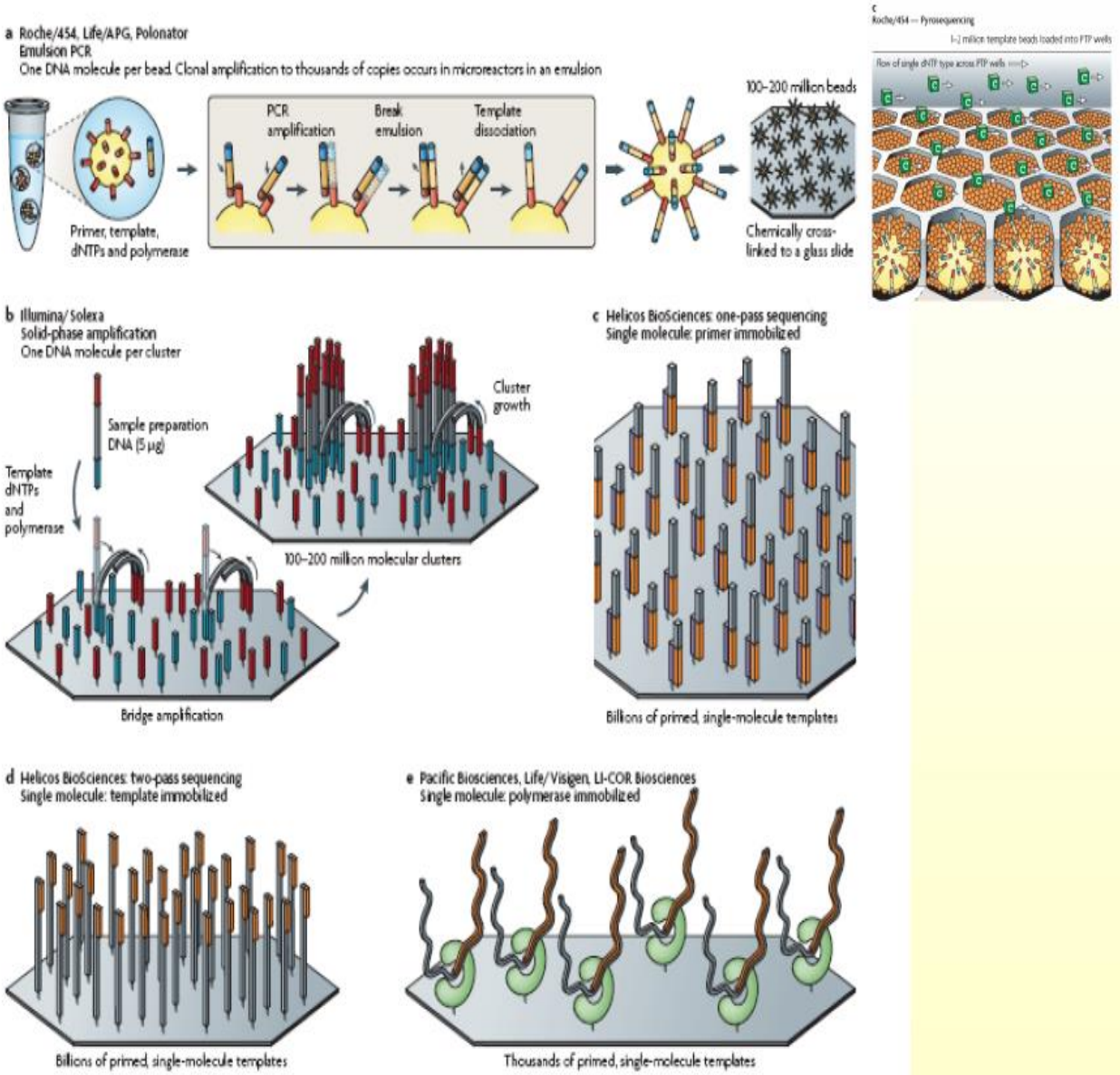
Pre-installed software: Linux OS, MinKNOW, Guppy, EPI2ME
Bluetooth and Wi-Fi enabled; you can control your experiments using a laptop, tablet or smartphone
fastq or fast5 files are written to Onboard storage: 512 GB SSD
Processing: GPU accelerators (ARM processor 6 cores, 256 Core GPU), 8 GB RAM.
Small footprint, 290g
1 x USB 2.0 port, 1 x USB 3.0 port and 1 x Ethernet port (1 Gbit capacity)

# SmidgION, Mobile analysis

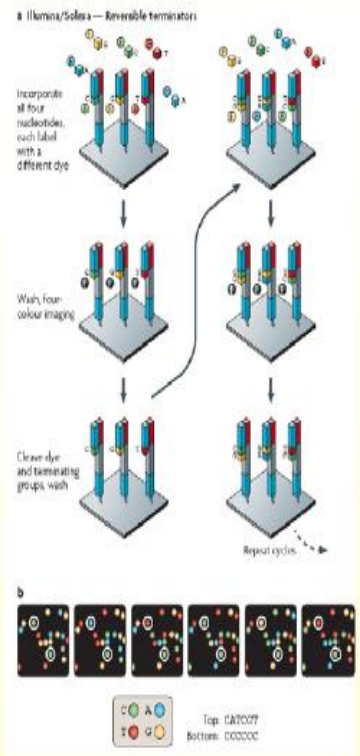
Oxford Nanopore has now started developing an even smaller device, SmidgION.

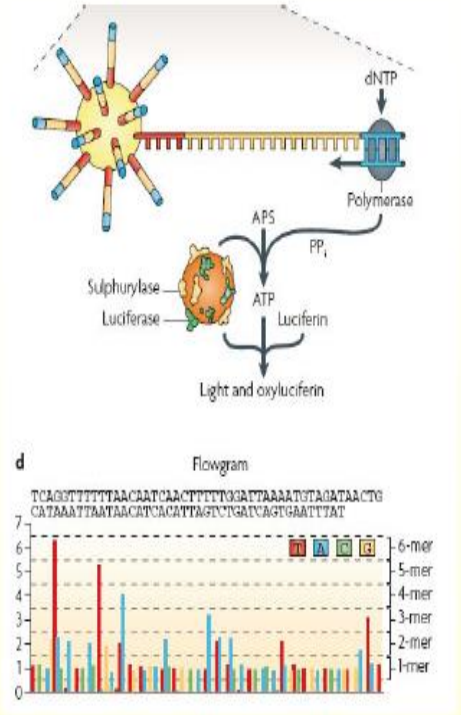
**potential applications** may include remote monitoring of pathogens in a breakout or infectious disease; the on-site analysis of environmental samples such as water/metagenomics samples, real time species ID for analysis of food, timber, wildlife or even unknown samples; field-based analysis of agricultural environments, and much more.

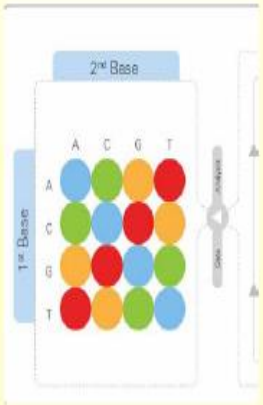
# ESTRATEGIAS DE INMOVILIZACIÓN

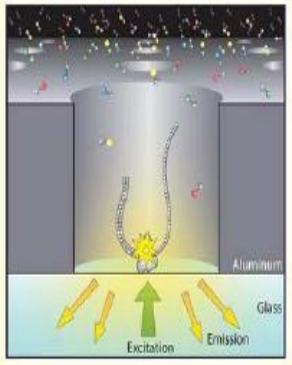


# ESTRATEGIAS DE LECTURA

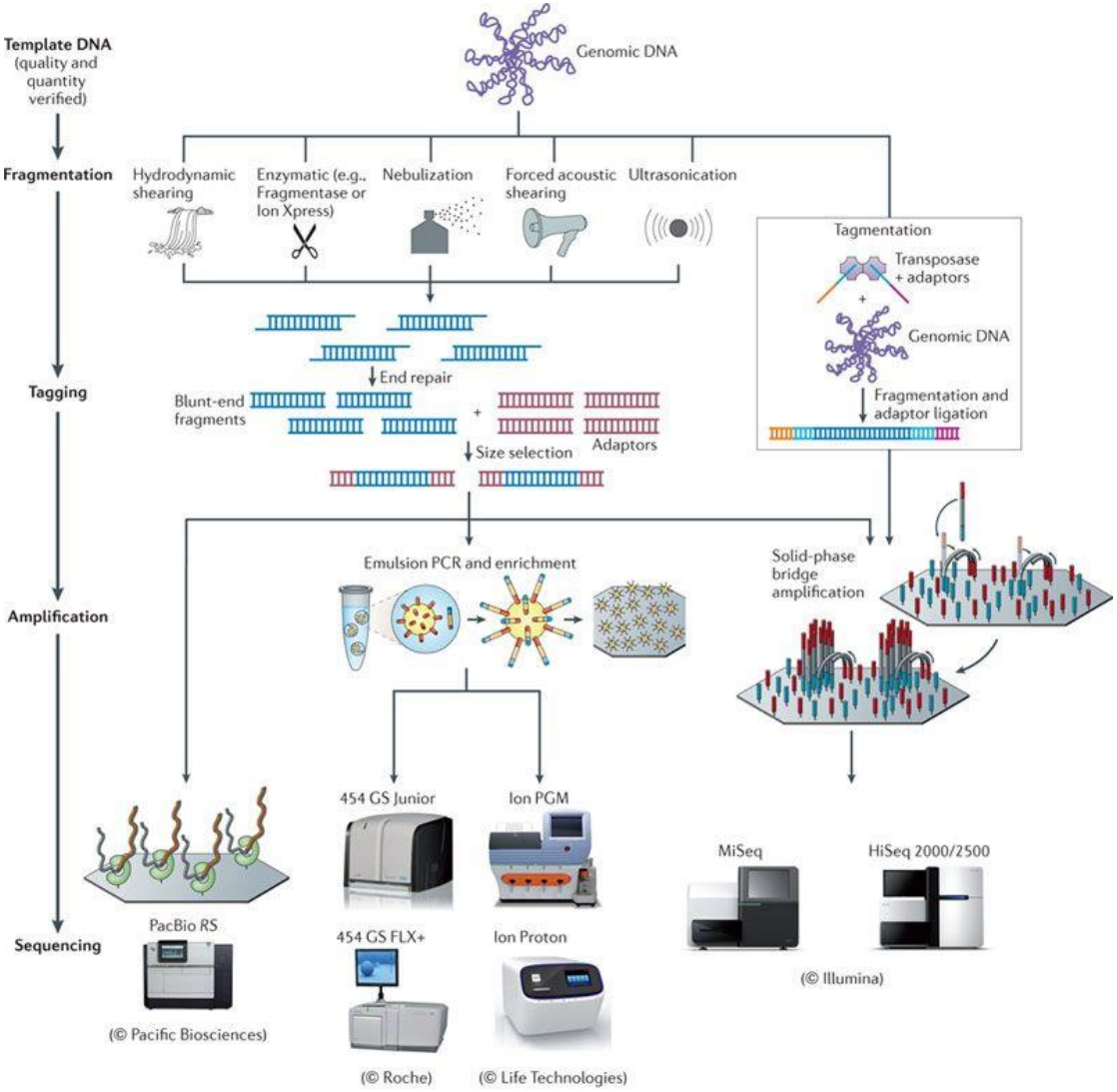


# High-throughput sequencing platforms




Nature Reviews | Microbiology Loman et al, 2012

# PacBio sequencing and its applications

Rhoads & Au, Gen Prot Bioinf 2015

**Performance comparison of sequencing platforms of various generations**

| Method | Generation | Read length (bp) | Single pass error rate (%) | No. of reads per run | Time per run | Cost per million bases (USD) | Refs. |
|---|---|---|---|---|---|---|---|
| Sanger ABI 3730×1 | 1st | 600–1000 | 0.001 | 96 | 0.5–3 h | 500 | [14,18–21] |
| Ion Torrent | 2nd | 200 | 1 | $8.2 \times 10^7$ | 2–4 h | 0.1 | [15,25] |
| 454 (Roche) GS FLX+ | 2nd | 700 | 1 | $1 \times 10^6$ | 23 h | 8.57 | [14,17,27] |
| Illumina HiSeq 2500 (High Output) | 2nd | $2 \times 125$ | 0.1 | $8 \times 10^9$ (paired) | 7–60 h | 0.03 | [9,16,26] |
| Illumina HiSeq 2500 (Rapid Run) | 2nd | $2 \times 250$ | 0.1 | $1.2 \times 10^9$ (paired) | 1–6 days | 0.04 | [9,16,26] |
| SOLiD 5500×1 | 2nd | $2 \times 60$ | 5 | $8 \times 10^8$ | 6 days | 0.11 | [14,24] |
| PacBio RS II: P6-C4 | 3rd | $1.0–1.5 \times 10^4$ on average | 13 | $3.5–7.5 \times 10^4$ | 0.5–4 h | 0.40–0.80 | [5,12,15] |
| Oxford Nanopore MinION | 3rd | $2–5 \times 10^3$ on average | 38 | $1.1–4.7 \times 10^4$ | 50 h | 6.44–17.90 | [22,23] |

# Characteristics, strengths and weaknesses of commonly used sequencing platforms

**Table 2**
Characteristics, strengths and weaknesses of commonly used sequencing platforms

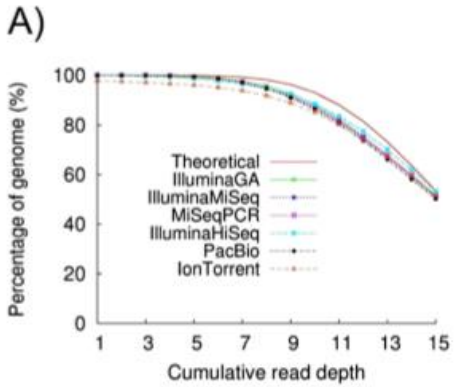
| *Platform* \ Instrument | Throughput range (Gb)[a] | Read length (bp) | Strength | Weakness |
|---|---|---|---|---|
| *Sanger sequencing* | | | | |
| ABI 3500/3730 | 0.0003 | Up to 1 kb | Read accuracy and length | Cost and throughput |
| *Illumina* | | | | |
| MiniSeq | 1.7–7.5 | 1×75 to ×150 | Low initial investment | Run and read length |
| MiSeq | 0.3–15 | 1×36 to 2×300 | Read length, scalability | Run length |
| NextSeq | 10–120 | 1×75 to 2×150 | Throughput | Run and read length |
| HiSeq (2500) | 10–1000 | ×50 to ×250 | Read accuracy, throughput, | High initial investment, run |
| NovaSeq 5000/6000 | 2000–6000 | 2×50 to ×150 | Read accuracy, throughput | High initial investment, run |
| *IonTorrent* | | | | |
| PGM | 0.08–2 | Up to 400 | Read length, speed | Throughput, homopolymers[c] |
| S5 | 0.6–15 | Up to 400 | Read length, speed, | Homopolymers[c] |
| Proton | 10–15 | Up to 200 | Speed, throughput | Homopolymers[c] |
| *Pacific BioSciences* | | | | |
| PacBio RSII | 0.5–1[b] | Up to 60 kb | Read length, speed (Average 10 kb, N50 20 kb) | High error rate and initial |
| Sequel | 5–10[b] | Up to 60 kb | Read length, speed (Average 10 kb, N50 20 kb) | High error rate |
| *Oxford Nanopore* | | | | |
| MInION | 0.1–1 | Up to 100 kb | Read length, portability | High error rate, run length, |

[a] The throughput ranges are determined by available kits and run modes on a per run basis. As an example of a 15-GB throughput, thirty-five 5-MB genomes can be sequenced to a minimum coverage of 40× on the Illumina MiSeq using the v3 600 cycle chemistry.
[b] Per one single-molecule real-time cell.
[c] Results in increased error rate (increased proportion of reads containing errors among all reads) which in turn results in false-positive variant calling.

*Besser et al., Clin Micr Infect, 2018*

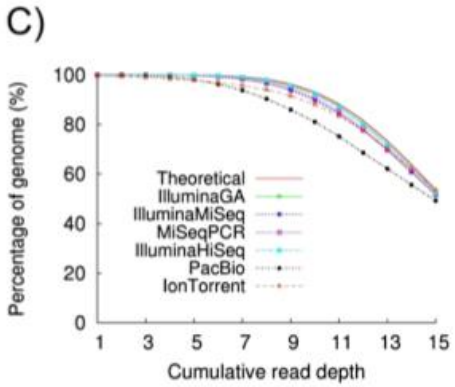
# Uniformidad de cobertura a lo largo del genoma

%GC



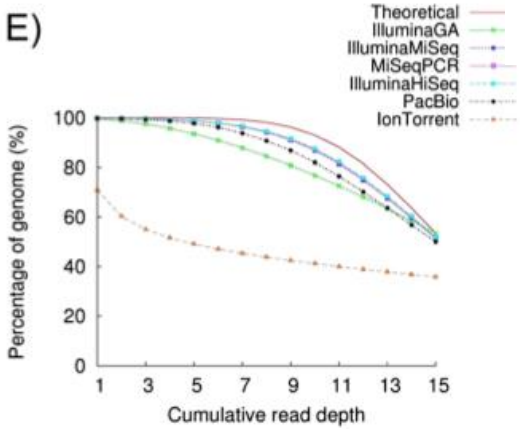

*Bordetella pertussis*

%GC




*Staphylococcus aureus*

%AT




*Plasmodium falciparum*



*Quail et al., BMC Genomics 2012, 13:341*

# Variación en la cobertura dependiendo del Sistema de Secuenciación

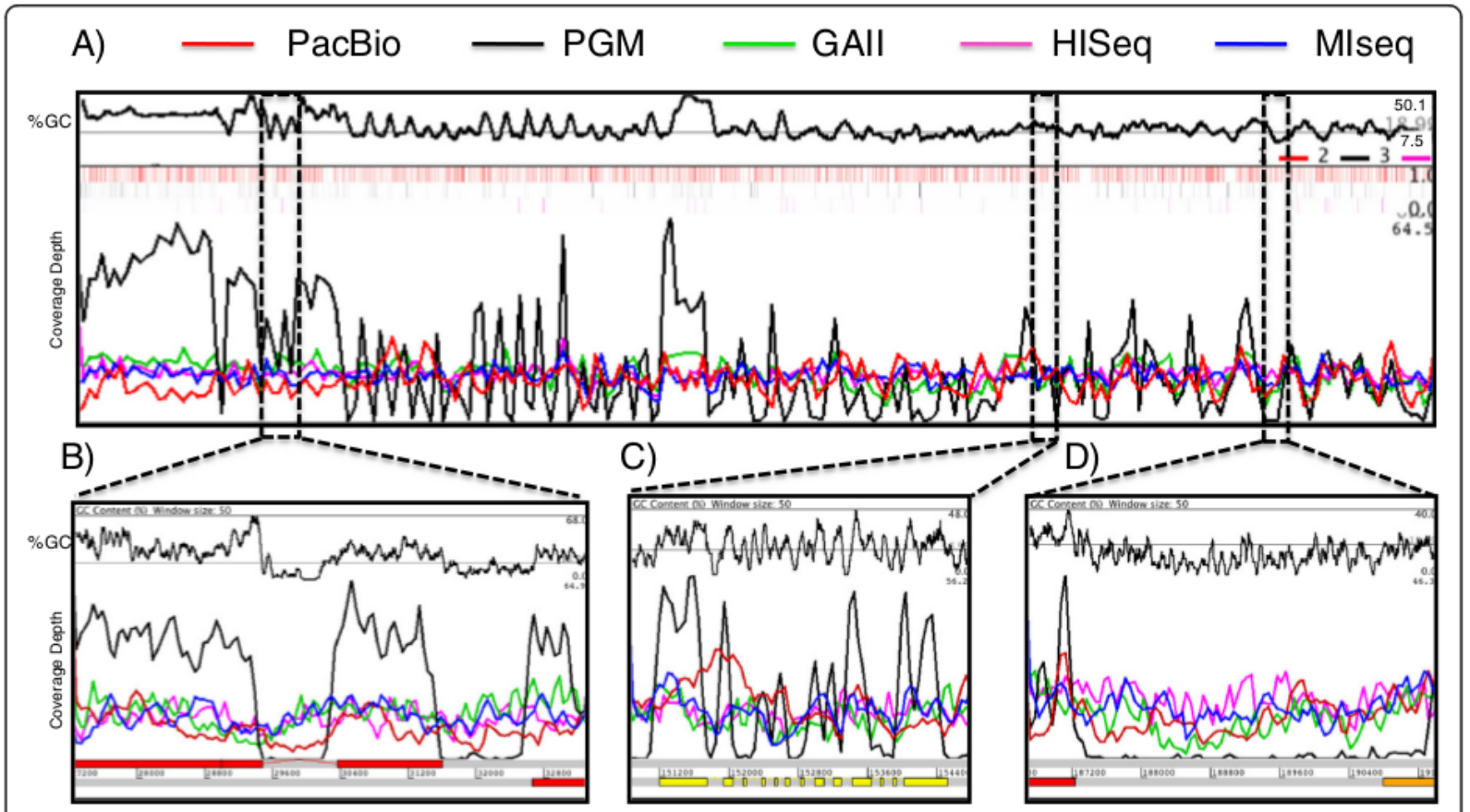



**Figure 2 Artemis genome browser** [8] **screenshots illustrating the variation in sequence coverage of a selected region of *P. falciparum* chromosome 11, with 15x depth of randomly normalized sequence from the platforms tested.** In each window, the top graph shows the percentage GC content at each position, with the numbers on the right denoting the minimum, average and maximum values. The middle graph in each window is a coverage plot for the dataset from each instrument; the colour code is shown above graph a). Each of the middle graphs shows the depth of reads mapped at each position, and below that in B-D are the coordinates of the selected region in the genome with gene models on the (+) strand above and (–) strand below. **A**) View of the first 200 kb of chromosome 11. Graphs are smoothed with window size of 1000. A heatmap of the errors, normalized by the amount of mapping reads is included just below the GC content graph (PacBio top line, PGM middle and MiSeq bottom). **B**) Coverage over region of extreme GC content, ranging from 70% to 0%. **C**) Coverage over the gene PF3D7_1103500. **D**) Example of intergenic region between genes PF3D7_1104200 and PF3D7_1104300. The window size of B, C and D is 50 bp.



*Quail et al., BMC Genomics 2012, 13:341*

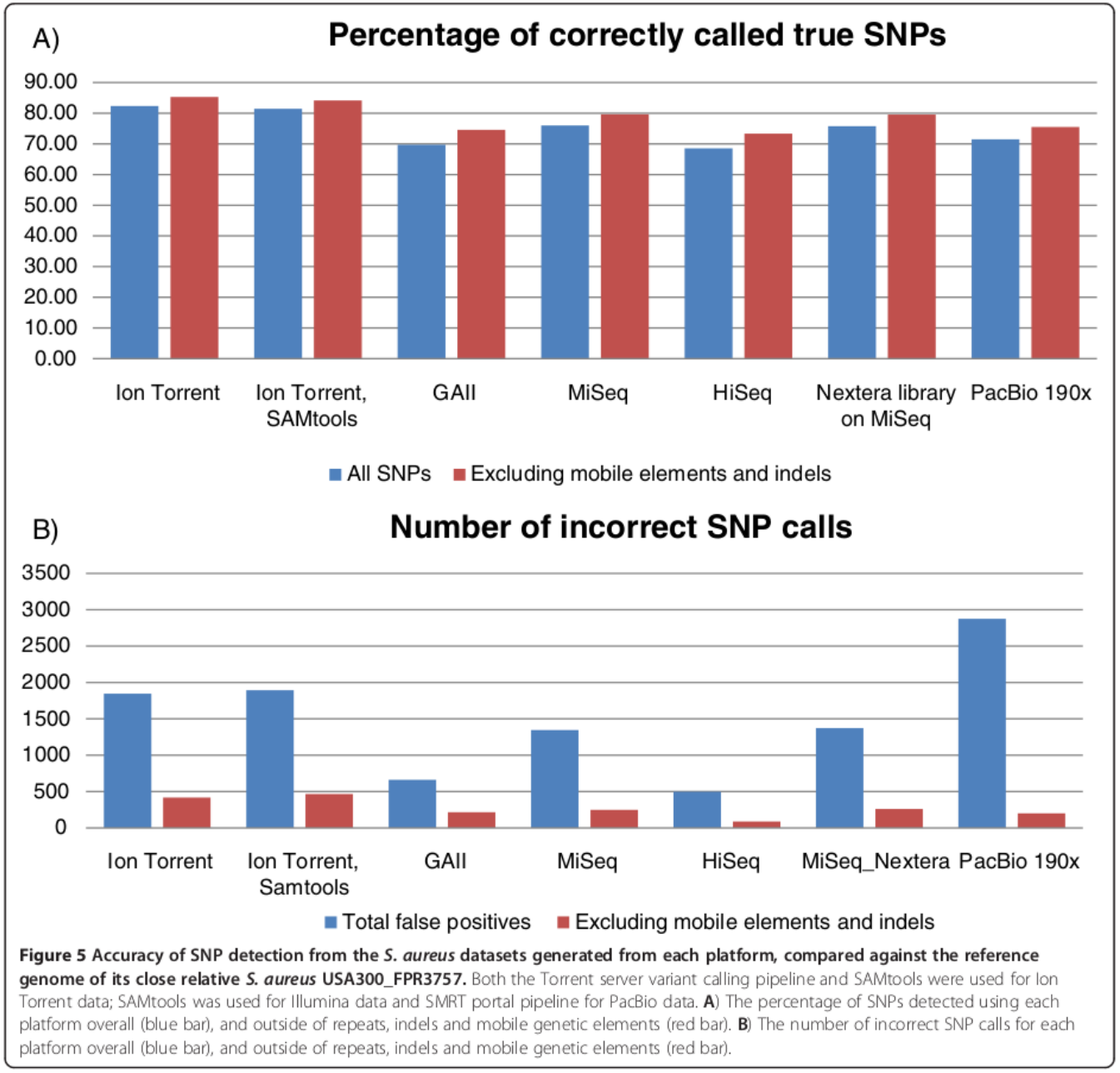



**Figure 5 Accuracy of SNP detection from the *S. aureus* datasets generated from each platform, compared against the reference genome of its close relative *S. aureus* USA300_FPR3757.** Both the Torrent server variant calling pipeline and SAMtools were used for Ion Torrent data; SAMtools was used for Illumina data and SMRT portal pipeline for PacBio data. **A**) The percentage of SNPs detected using each platform overall (blue bar), and outside of repeats, indels and mobile genetic elements (red bar). **B**) The number of incorrect SNP calls for each platform overall (blue bar), and outside of repeats, indels and mobile genetic elements (red bar).



*Quail et al., BMC Genomics 2012, 13:341*

# Errores específicos de plataforma

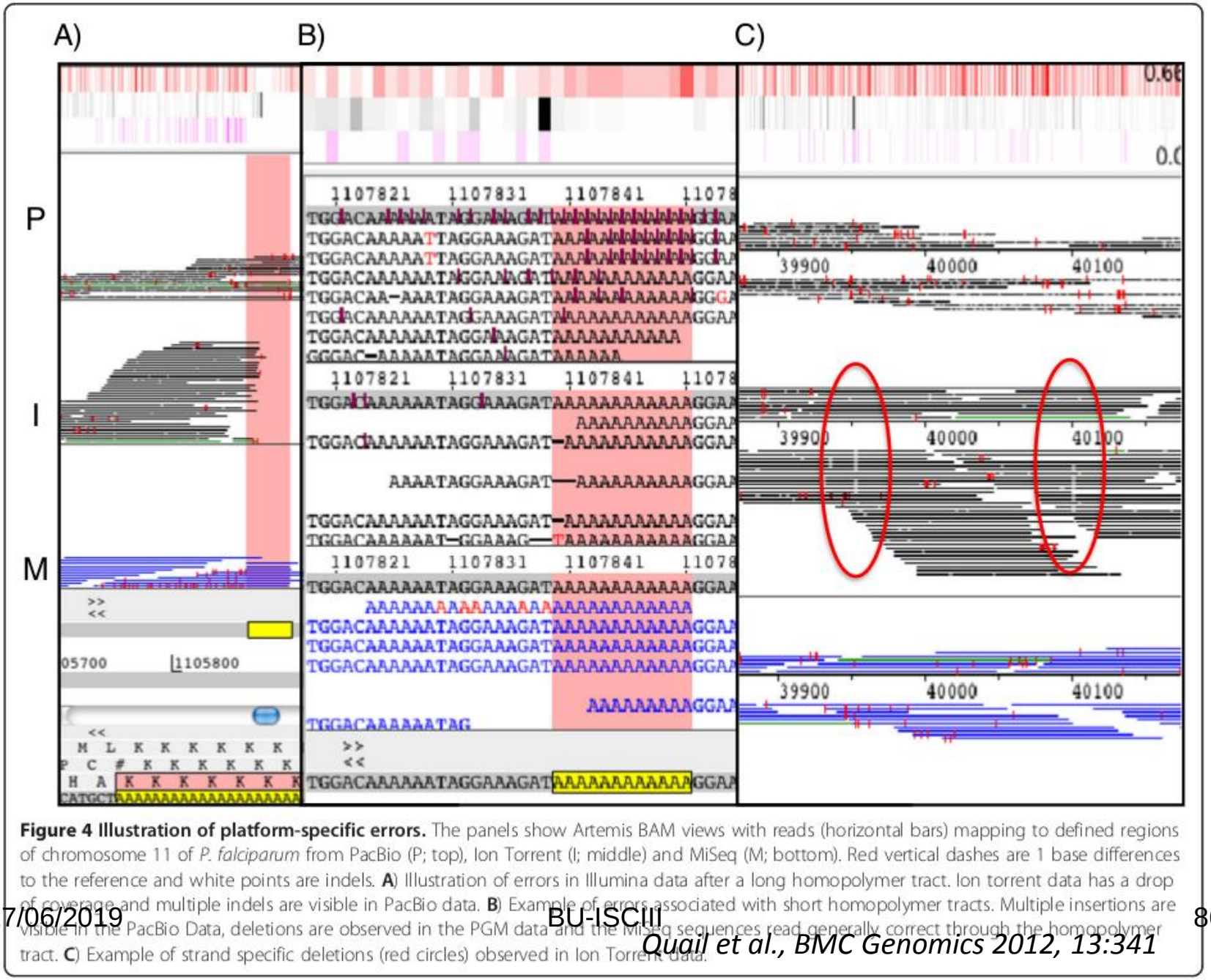



**Figure 4 Illustration of platform-specific errors.** The panels show Artemis BAM views with reads (horizontal bars) mapping to defined regions of chromosome 11 of *P. falciparum* from PacBio (P; top), Ion Torrent (I; middle) and MiSeq (M; bottom). Red vertical dashes are 1 base differences to the reference and white points are indels. **A**) Illustration of errors in Illumina data after a long homopolymer tract. Ion torrent data has a drop of coverage and multiple indels are visible in PacBio data. **B**) Example of errors associated with short homopolymer tracts. Multiple insertions are visible in the PacBio Data, deletions are observed in the PGM data and the MiSeq sequences read generally correct through the homopolymer tract. **C**) Example of strand specific deletions (red circles) observed in Ion Torrent data.

*Quail et al., BMC Genomics 2012, 13:341*

# Conclusiones

***A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers***
***Quail et al., BMC Genomics 2012, 13:341***

- Ion Torrent no es recomendable para secuenciar genomas con bajo contenido en GC
- Pac Bio:
  - Requiere elevada cantidad de DNA (no amplificación)
  - No recomendable para aplicaciones de counting (RNAseq, Chipseq, exoma)
  - No valido para identificar SNPs
  - Necesario adaptar software para aprovechar la longitud de lectura en el assembly.
- Illumina, preparación de librería adecuada a amplicones para generar correctamente los clusters
- Aplicaciones dependientes de plataforma

# Conclusiones

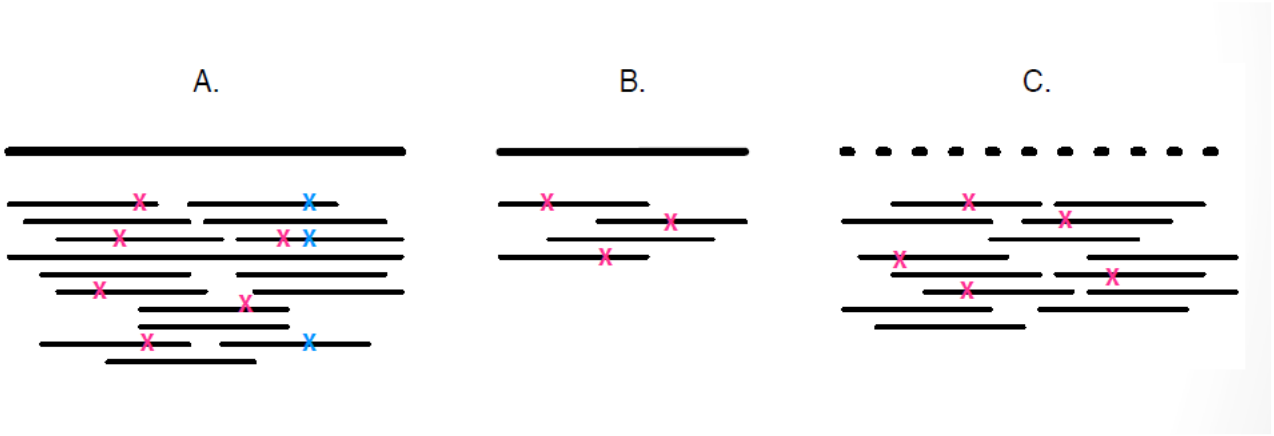
***A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers***
***Quail et al., BMC Genomics 2012, 13:341***

- Conclusiones obtenidas con reactivos y plataformas a 2011
- Hay errores intrínsecos a la plataforma
- Otros errores se solucionaran con la actualización de las plataformas

# Algunos conceptos en secuenciación

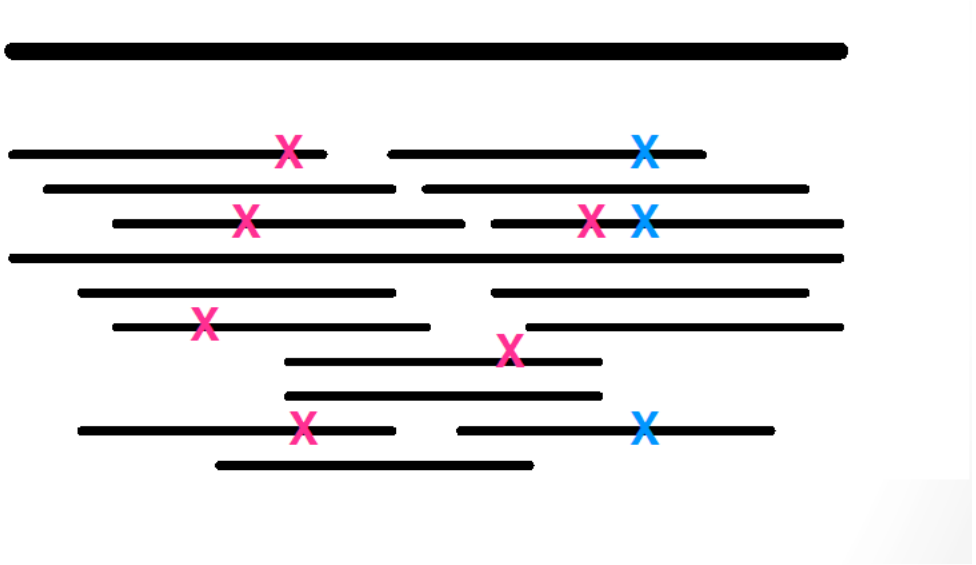
# Básicamente tres problemas

Resecuenciación, Conteo y ensamblado



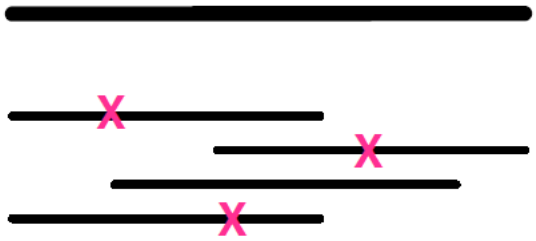
# Resecuenciación

Conocemos el genoma, genoma de referencia, y queremos identificar variaciones (azul), en un background de errores (rosa)



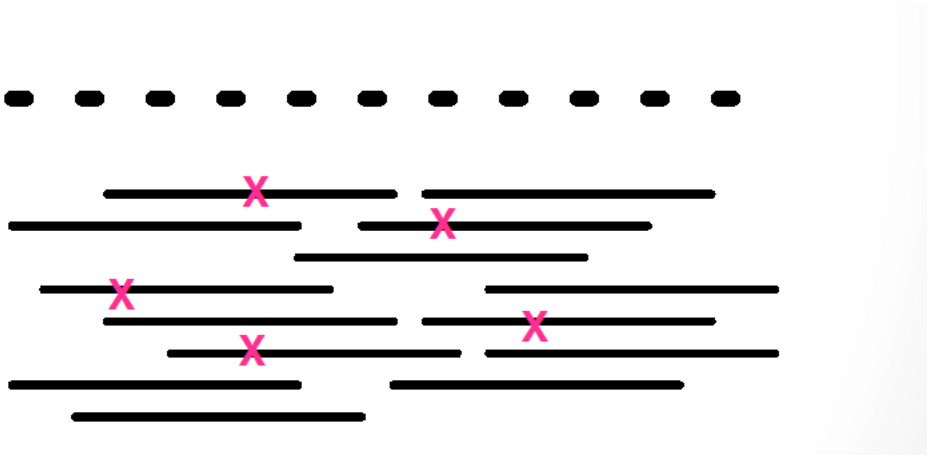
# Conteo

Número de lecturas de un gen (amplicón) o mRNA (RNAseq). Equivalente a expresión en Microarrays.



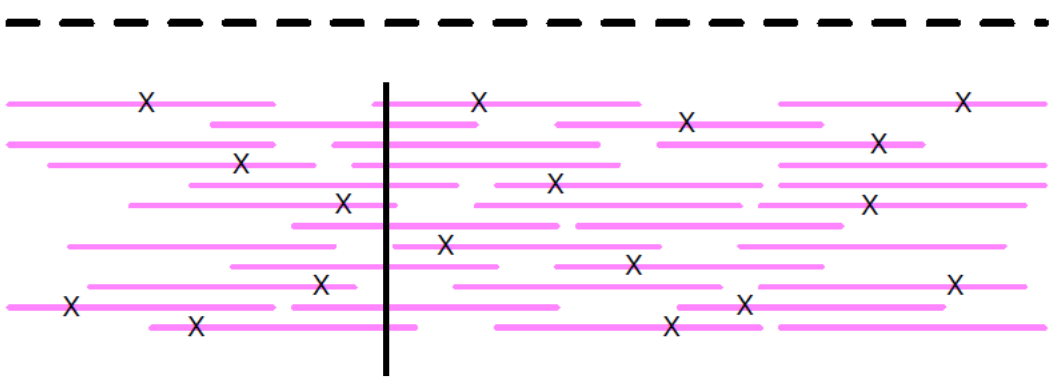
# Ensamblado

No hay genoma de referencia y lo construimos de novo



# Cobertura (depth of covarage)

Número medio de lecturas por base. i.e. 10x



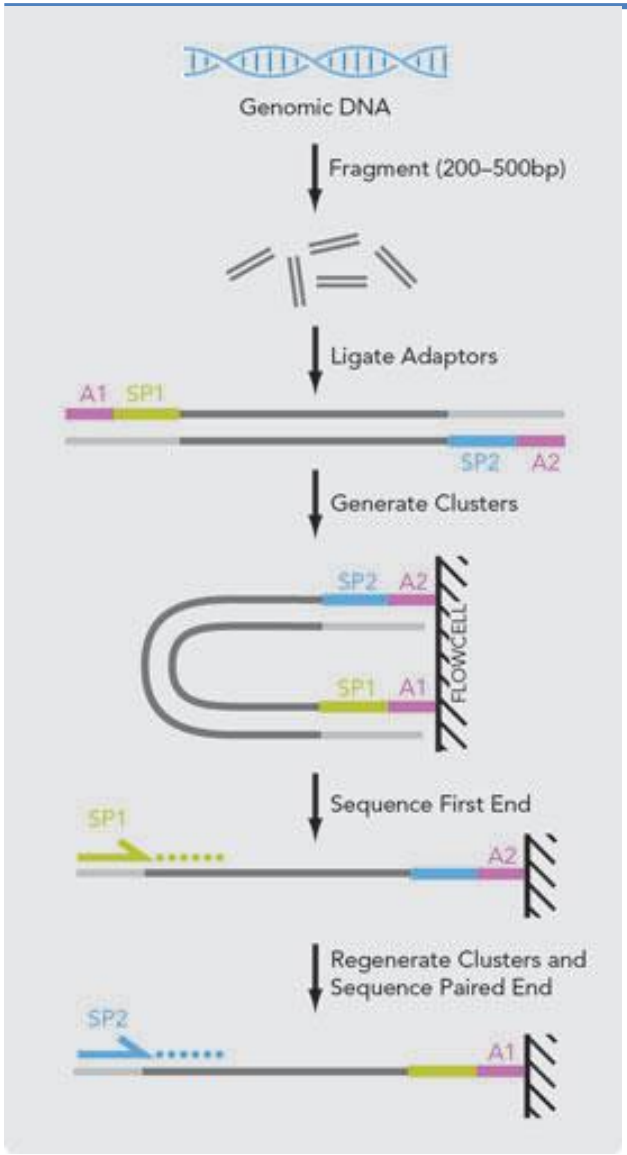
# Sequencing coverage

as the average number of reads that align known reference bases

Number of reads x read length / target size

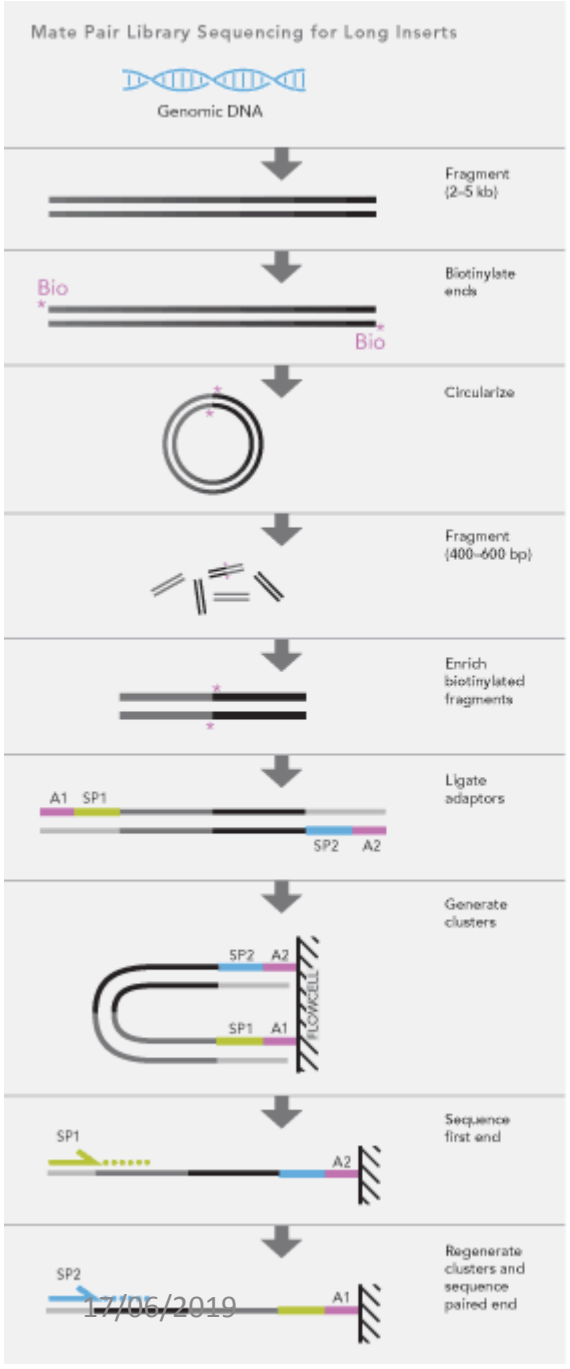
assuming that reads are randomly distributed across the genome.

# Que es Pair-end?




**Secuenciación de un fragmento (bp)**

**Modificación de single-read DNA,**
**Leyendo por ambos extremos, forward y reverse**




Mate Pair library preparation is designed to generate short fragments that consist of two segments that originally had a separation of several kilobases in the genome. Fragments of sample genomic DNA are end-biotinylated to tag the eventual mate pair segments.
Self-circularization and refragmentation of these large fragments generates a population of small fragments, some of which contain both mate pair segments with no intervening sequence. These Mate Pair fragments are enriched using their biotin tag. Mate Pairs are sequenced using a similar two-adapter strategy as described for paired-end sequencing.

# Que es Mate-pair?

**Secuenciación de dos fragmentos separados kb.**

**Util:**
**Secuenciación de un Genoma de novo**
**Finalizar un genoma**
**Detección de variantes estructurales**

# Coverage and Read Depth Recommendations by Sequencing Application

**Table 1: Coverage and Read Recommendations by Application**

| Category | Detection or Application | Recommended Coverage (x) or Reads (millions) | References |
|---|---|---|---|
| Whole genome sequencing | Homozygous SNVs | 15x | Bentley et al., 2008 |
| | Heterozygous SNVs | 33x | Bentley et al., 2008 |
| | INDELs | 60x | Feng et al., 2014 |
| | Genotype calls | 35x | Ajay et al., 2011 |
| | CNV | 1-8x | Xie et al., 2009; Medvedev at al., 2010 |
| Whole exome sequencing | Homozygous SNVs | 100x (3x local depth) | Clark et al., 2011; Meynert et al., 2013 |
| | Heterozygous SNVs | 100x (13x local depth) | Clark et al., 2011; Meynert et al., 2013 |
| | INDELs | not recommended | Feng et al., 2014 |
| Transcriptome Sequencing | Differential expression profiling | 10-25M | Liu Y. et al., 2014; ENCODE 2011 RNA-Seq |
| | Alternative splicing | 50-100M | Liu Y. et al., 2013; ENCODE 2011 RNA-Seq |
| | Allele specific expression | 50-100M | Liu Y. et al., 2013; ENCODE 2011 RNA-Seq |
| | De novo assembly | >100M | Liu Y. et al., 2013; ENCODE 2011 RNA-Seq |

https://genohub.com/recommended-sequencing-coverage-by-application/

# Coverage and Read Depth Recommendations by Sequencing Application

| | | | |
|---|---|---|---|
| DNA Methylation Sequencing | CAP-Seq | >20M | Long, H.K. et al., 2013 |
| | MeDIP-Seq | 60M | Taiwo, O. et al., 2012 |
| | RRBS (Reduced Representation Bisulfite Sequencing) | 10X | ENCODE 2011 Genome |
| | Bisulfite-Seq | 5-15X; 30X | Ziller, M.J et al., 2015; Epigenomics Road Map |
| RNA-Target-Based Sequencing | CLIP-Seq | 10-40M | Cho J. et al., 2012; Eom T. et al., 2013; Sugimoto Y. et al., 2012 |
| | iCLIP | 5-15M | Sugimoto Y. et al., 2012; Rogelj B. et al., 2012 |
| | PAR-CLIP | 5-15M | Rogelj B. et al., 2012 |
| | RIP-Seq | 5-20M | Lu Z. et al., 2014 |
| Small RNA (microRNA) Sequencing | Differential Expression | ~1-2M | Metpally RPR et al., 2013; Campbell et al., 2015 |
| | Discovery | ~5-8M | Metpally RPR et al., 2013; Campbell et al., 2015 |

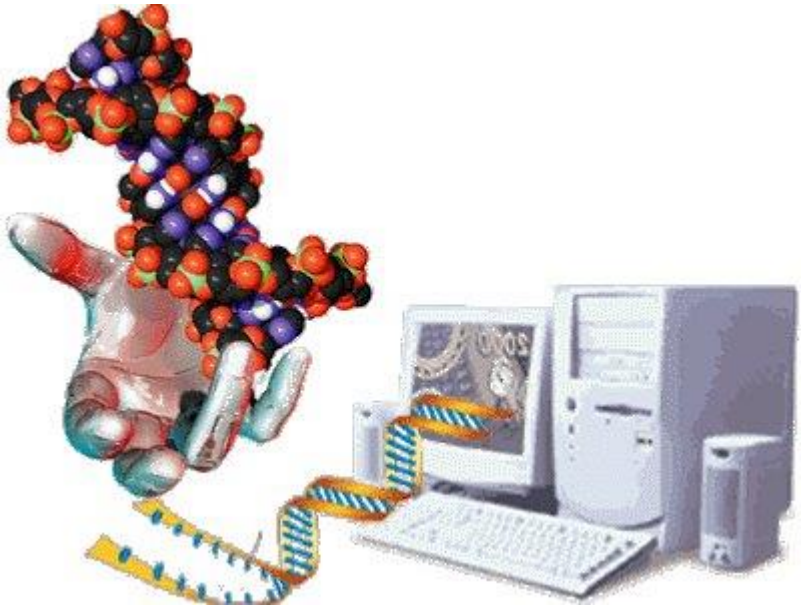
https://genohub.com/recommended-sequencing-coverage-by-application/

# Resumen

Cobertura es importante para la llamada a variantes, RNAseq. Plataforma con mayor rendimiento Illumina

La longitud de las lecturas es importante para el ensamblado PacBio y Moleculo mayor longitud de lecturas (corrección de errores con Illumina)

# Gracias por la atención
# Preguntas ???



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**isabel.cuesta@isciii.es**