Análisis de Genomas Virales a través de la Plataforma Galaxy

BU-ISCIII

Unidades Centrales Científico Técnicas – SGSAFI-ISCIII

13 al 17 Noviembre 2023
3ª Edición
Programa Formación Continua, ISCIII

Learning aims and outcomes

- Understand some principles behind NGS and its applications to whole genome sequencing.
- Know the format files generated in NGS data analysis and the workflow analysis.
- Understand the uses of NGS in viral variants identification and consensus genome reconstruction.
- Learning to use galaxy platform.

Teachers

- Sara Monzón Fernández, Biotecnóloga y Bioinformática (Analista de datos). Titulado Superior Especialista OPIS. Responsable técnico BU-ISCIII

- Sarai Varona Fernández, Bioquímica y Bioinformática (Analista de Datos). Contrato Titulado Superior asociado a proyecto (2021-2024)

- Isabel Cuesta, Dra Biología, Bioinformática (Científico de Datos). Científico Titular de OPIS. Coordinador BU-ISCIII

Session 1.1 – Uso de la secuenciación masiva en virología

Isabel Cuesta

BU-ISCIII

Unidades Centrales Científico Técnicas – SGSAFI-ISCIII

13 al 17 Noviembre 2023

3ª Edición

Programa Formación Continua, ISCIII

Index

- BU-ISCIII, Bioinformatics and preparedness to be a service provider
- High throughput sequencing in clinical virology
  - Application of HTS or NGS
  - Nucleic acid amplification
  - Library preparation
  - High throughput sequencing platforms
  - Data analysis

Index

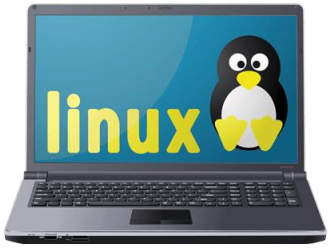
- BU-ISCIII, Bioinformatics and preparedness to be a service provider

What is Bioinformatics?

Biological Problems

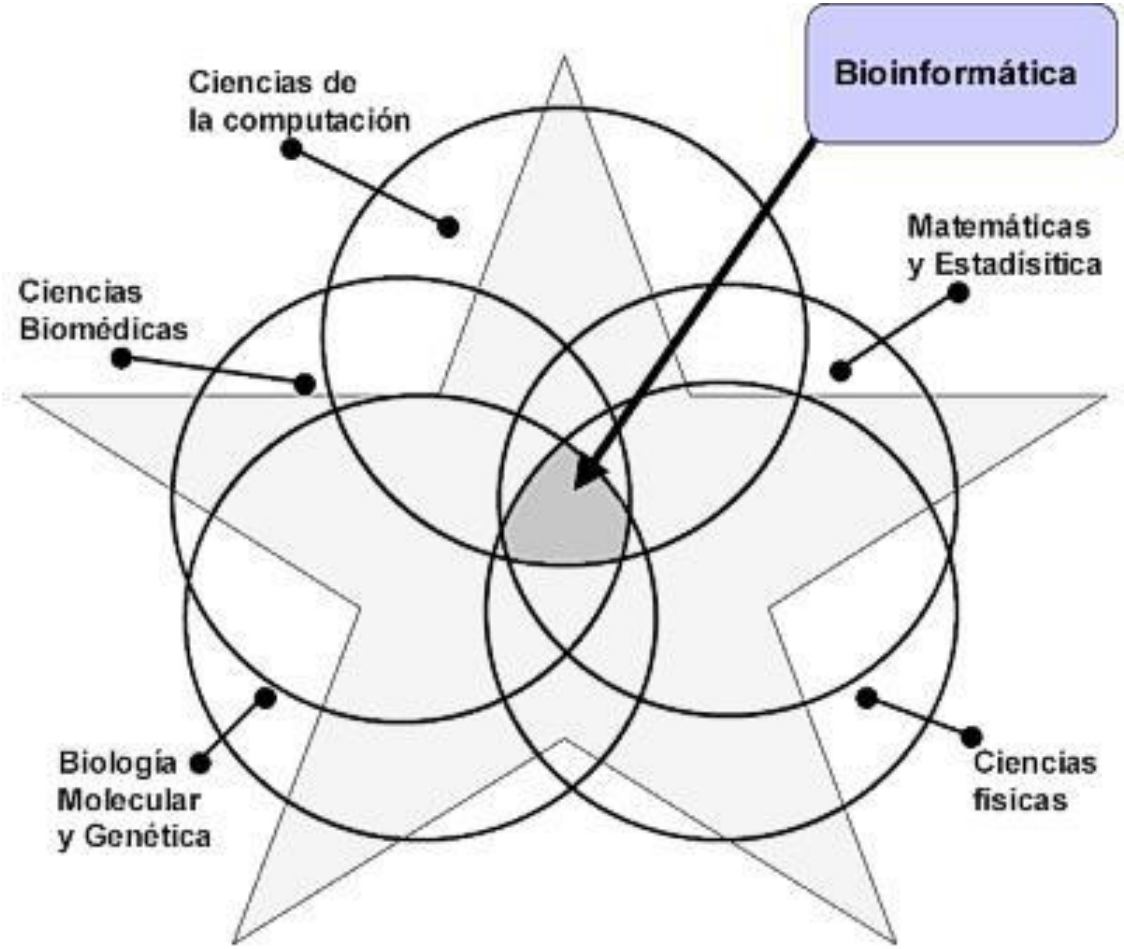
Data Processing

Computational Methods

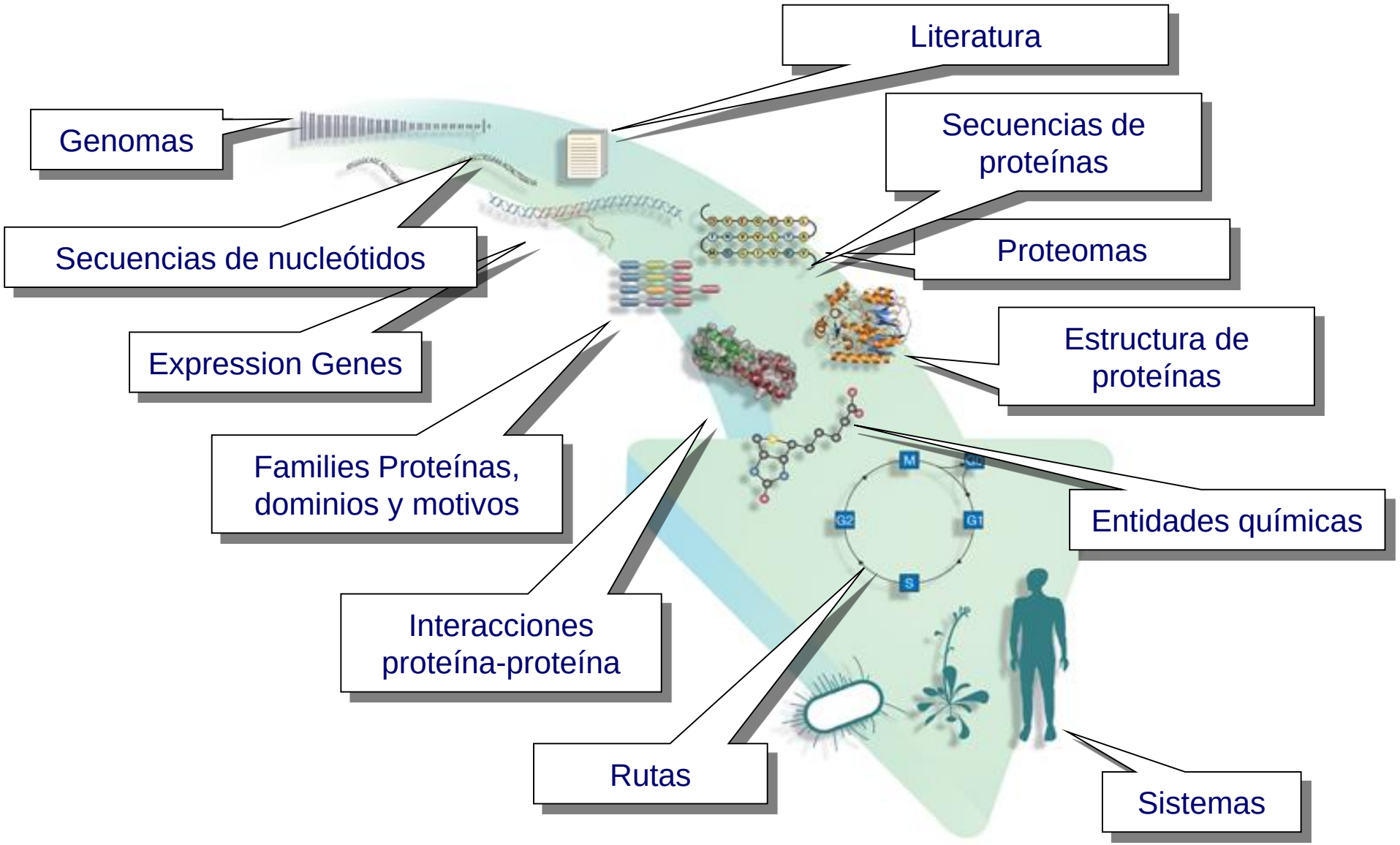


Bioinformatics is the science of storing, retrieving and analysing large amounts of biological information

Bioinformatics is a multidisciplinary field



Dimension of Bioinformatics is given by data type



Why BU-ISCIII was founded

Genomics Unit

2010 —  454

2013 —  

NextSeq500 MiSeq

2019 —  MinIon - Nanopore

2021 —  NovaSeq 6000

Bioinformatics Unit

2012

Scientific & technical Support to Researchers on HTS Data Analysis

National Microbiology Centre (CNM)

Research Institute for Rare Diseases (IIER)

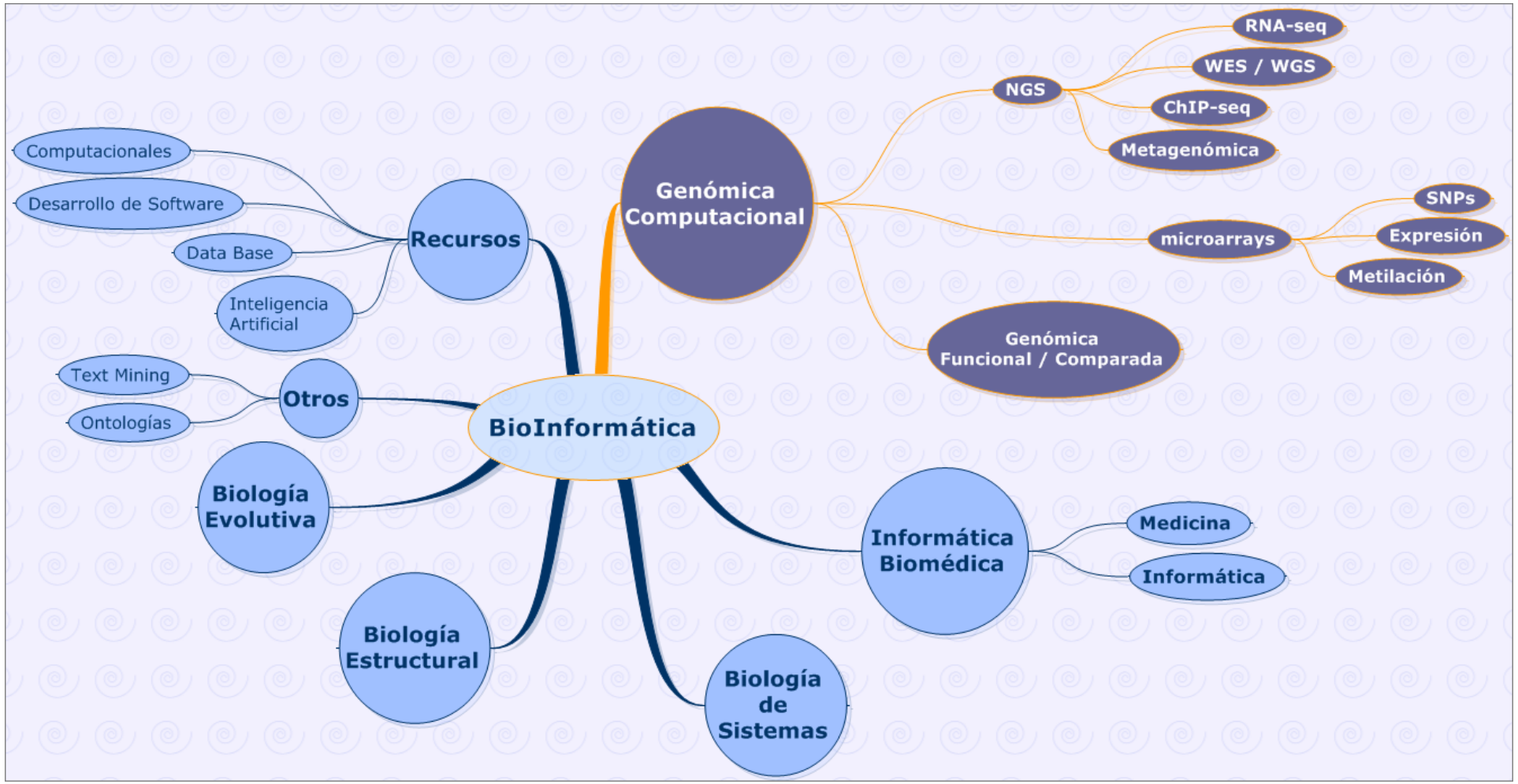
Functional Unit for Research in Chronic Disease

Network of Biological Alerts

National Centre of Tropical Medicine

National Environment Health Centre

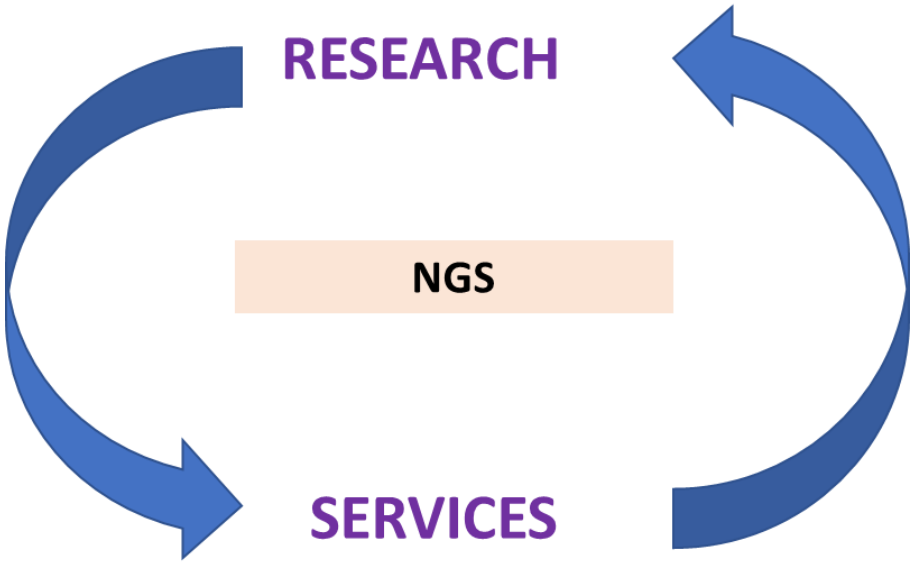
BU-ISCIII Mission - Activities



Bioinformatics Unit Activities

- Identify biological problems (PI / Groups) that could be target of NGS
- Early adopters: establish collaboration with.
- Be strategic providing transversal solutions → reusable tools

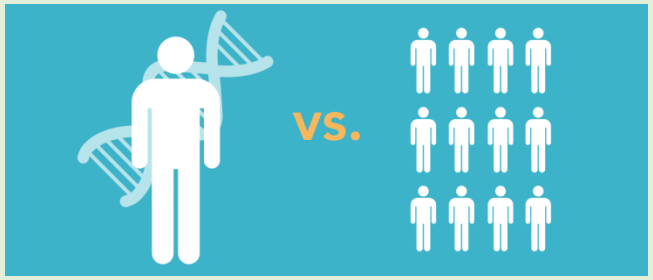
Provide scientific and technical solutions for using NGS in the diagnostic routine or research activity from different ISCIII labs

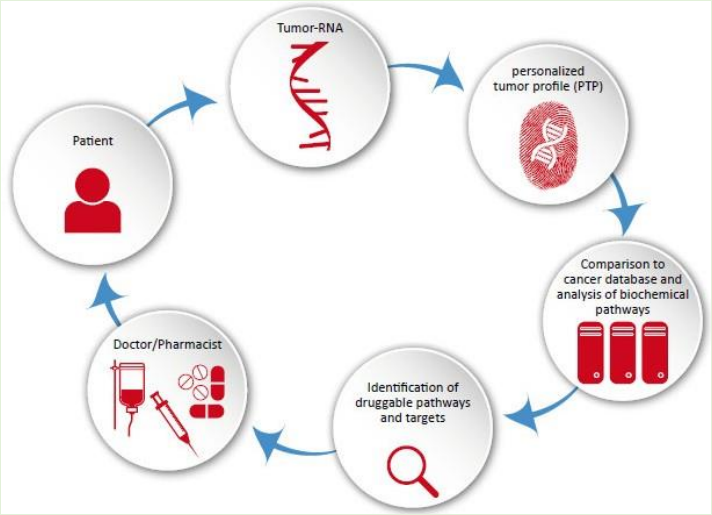


Clinical Bioinformatics - Precision Medicine

IIER

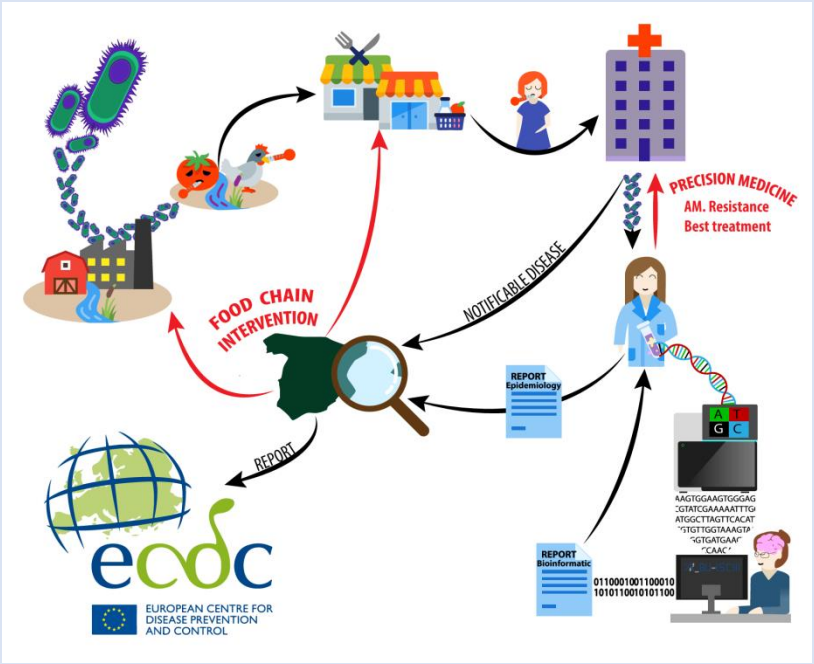
Tumour or Rare Diseases



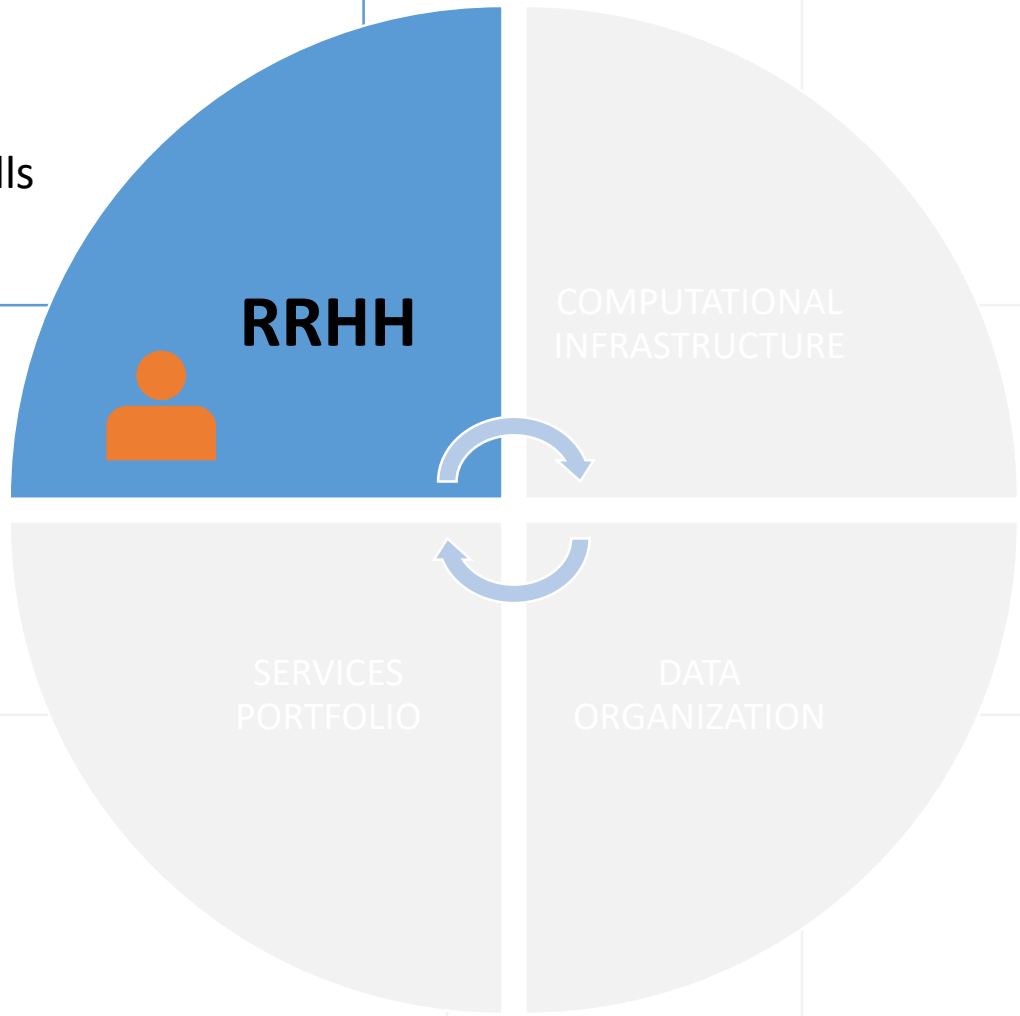


CNM

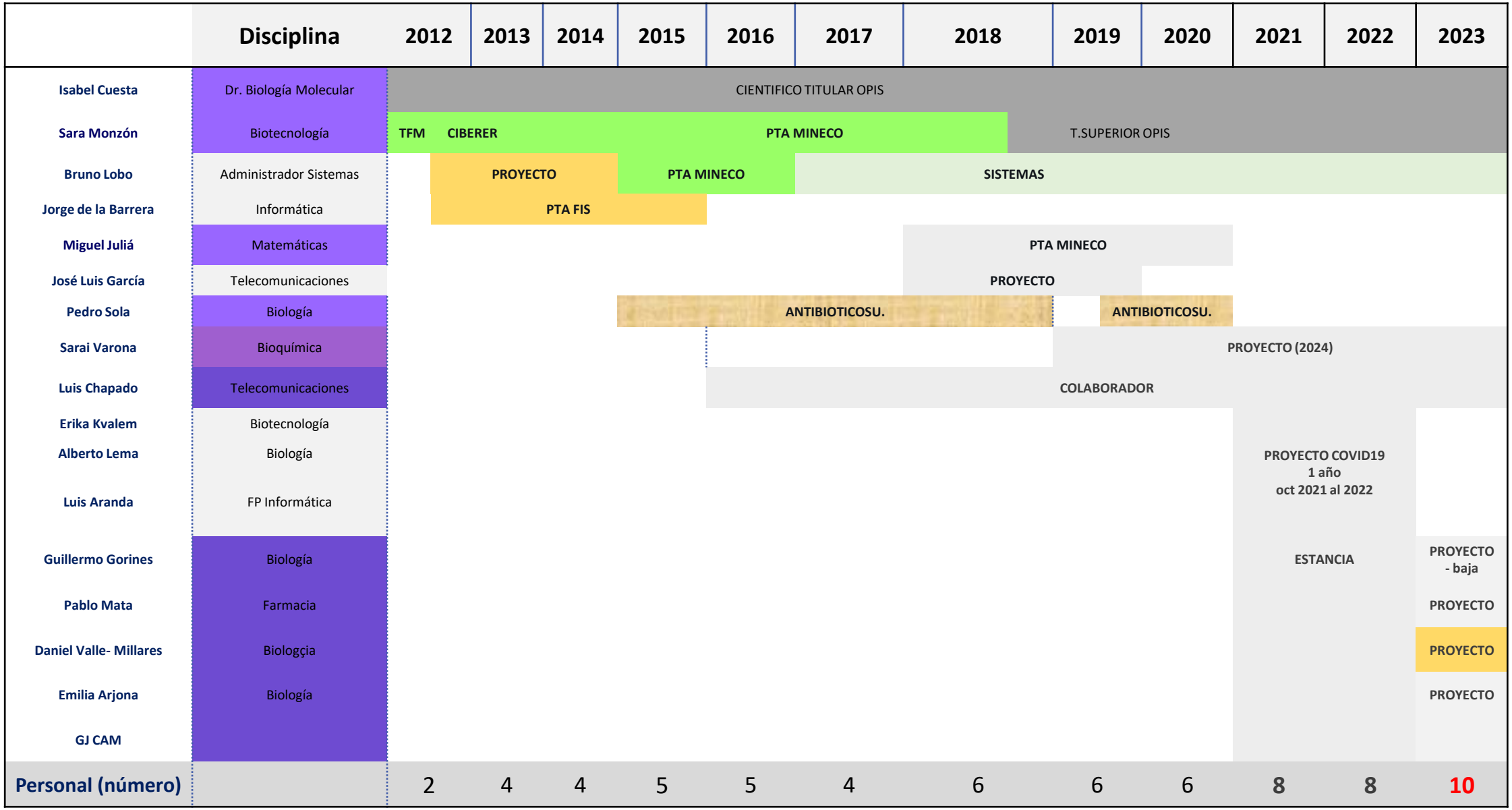
Pathogen associated



- TEAM: Bioinformatician & IT
- SOURCE OF FUNDING:
  - Research Project
  - National or International Calls
  - Permanent position – Civil Servant

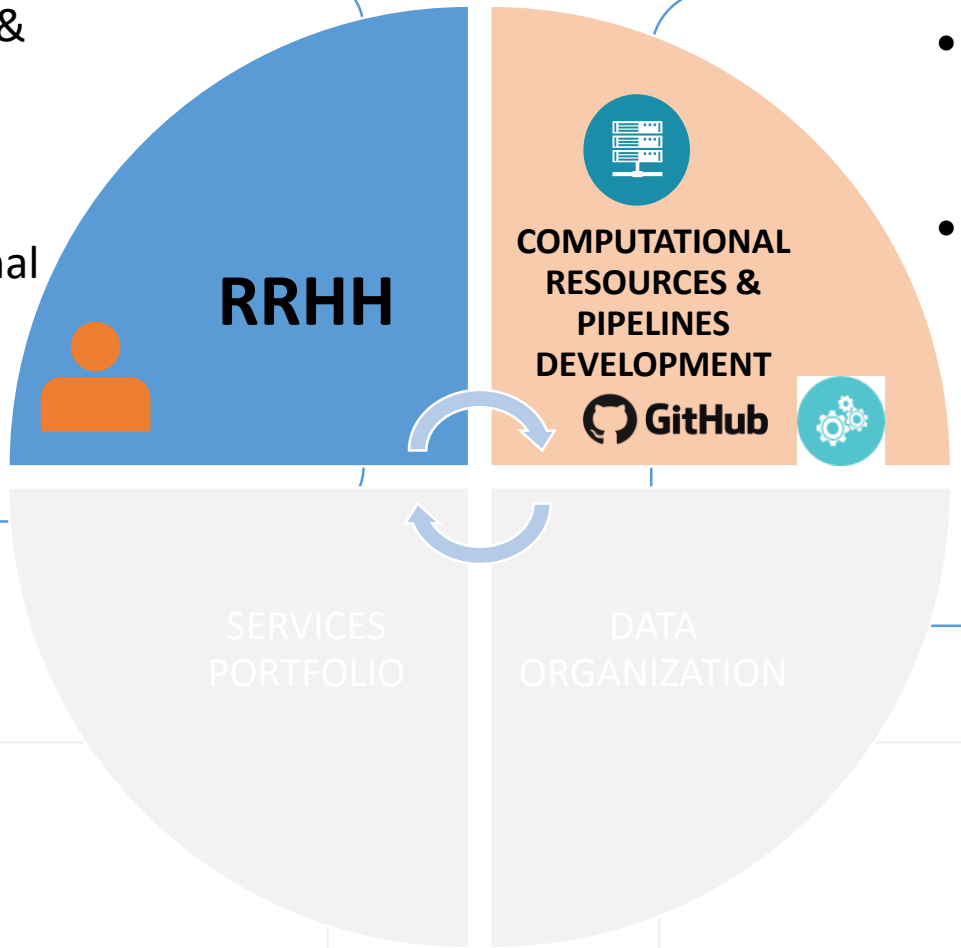


Human resources



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

- TEAM: Bioinformatician & IT
- SOURCE OF FUNDING:
  - Research Project
  - National or International Calls
  - Stable position – Civil Servant



- INFRASTRUCTURE
  - HPC
  - Workstations
- REPOSITORY:
  - GitHub
  - Benchmarking
  - Validation

Computational Resources

- IT support: establish agreement with IT department including permission for using Linux.

Workstations (5), 4cores, 64Gb, 8TB
Server, 4-quad, 120Gb, 16TB

Data Centre (CPD-ISCIII)

HPC 320 cores, 8TB RAM, 10Gbps.
2 flexible and scalable storages, NetApp, 70 TB and 250TB

- Reproducibility of in-silico pipelines analysis

nextflow

Singularity containers
Admin support & environment independency
Sharing code easier

GitHub

https://github.com/BU-ISCIII

Bioinformatic Analysis: Software validation: ECDC EQAs

Table 5. Results of allele-based cluster analysis

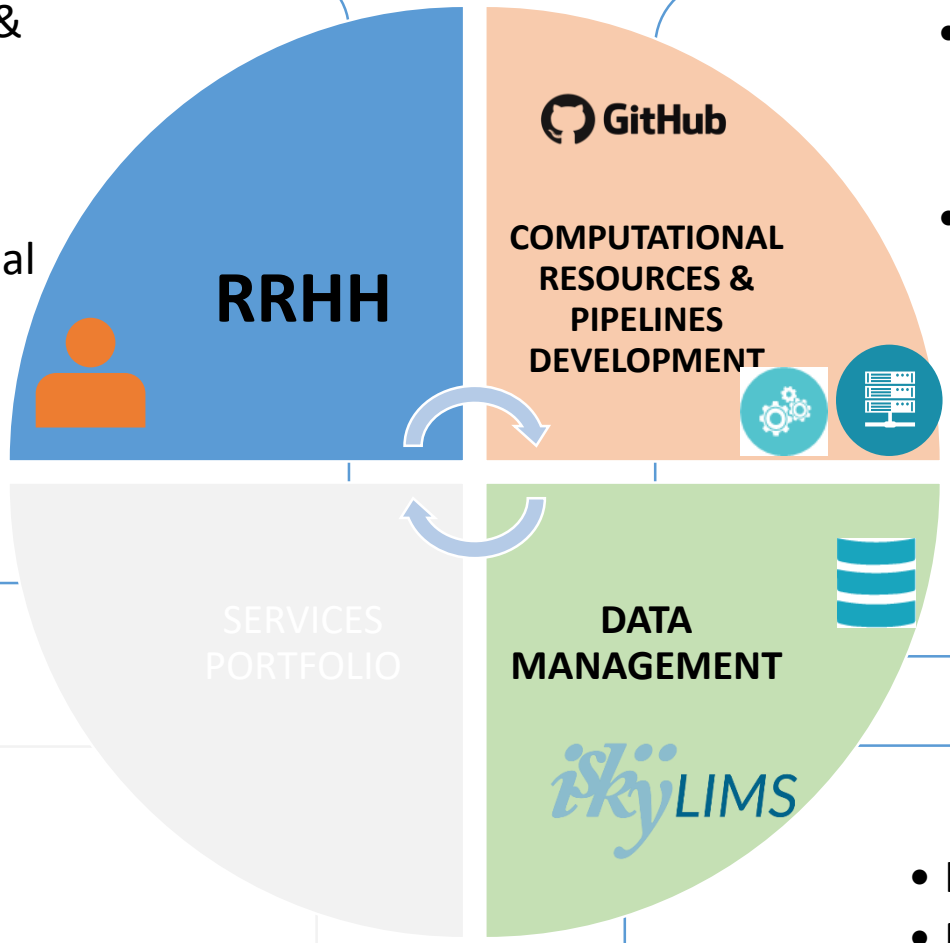
Lab ID	Allele based analysis					
	Approach	Allelic calling method	Assembler	Scheme	Difference within cluster	Difference outside cluster
EQA provider	BioNumerics	Assembly- and mapping-based	SPAdes	Applied Math (cgMLST/Pasteur)	0-3	24-1112
19	BioNumerics	Assembly- and mapping-based	SPAdes	Applied Math (cgMLST/Pasteur)	0-3	25-1120
35	SeqPhere	Assembly-based only	Velvet	Ruppitsch (cgMLST)	0-2	16-1065
70	SeqPhere	Assembly-based only	Velvet	Ruppitsch (cgMLST)	0-2	16-1062
105*	SeqPhere	Assembly-based only	SPAdes v 3.80	Ruppitsch (cgMLST)	0-1#	23-812
129	SeqPhere	Assembly-based only	Velvet			
135	SeqPhere	Assembly-based only	CLC Genomic Workbench 1(			
141	SeqPhere	Assembly-based only	SPAdes 3.9.0			
142	Inhouse	Assembly-based only	SPAdes			
144	SeqPhere	Assembly-based only	Velvet			

Fifth external quality assessment scheme for Listeria monocytogenes typing

Table 4. Results of SNP-based cluster analysis

Lab ID	SNP-based						
	Approach	Reference	Read mapper	Variant caller	Assembler	Distance within cluster	Distance outside cluster
Provider	Reference-based	ST6 (REF4)	BWA	GATK		0-3	38-71
19*	Reference-based	ST6 ID 2362	BWA	GATK		0-4	43-81
56	Assembly-based			ksnp3	SPAdes	0-57#	561-591 (6109)
105	Reference-based	ST6 J1817	Bowtie2	VARSCAN 2		0-2#	22-42 (1049)
108	Reference-based	In-house strain resp ST	CLC assembly cell v4.4.2	CLC assembly cell v4.4.2		0-2	37-72
142*	Reference-based	*Listeria* EGDe (cc9)	CLC Bio	CLC Bio		0-1219	1223-2814 (8138)
146	Reference-based	ST6 ref. CP006046 ST1 ref. F2365 ST213/ST382 no ref.	BWA	In-house		0-358	

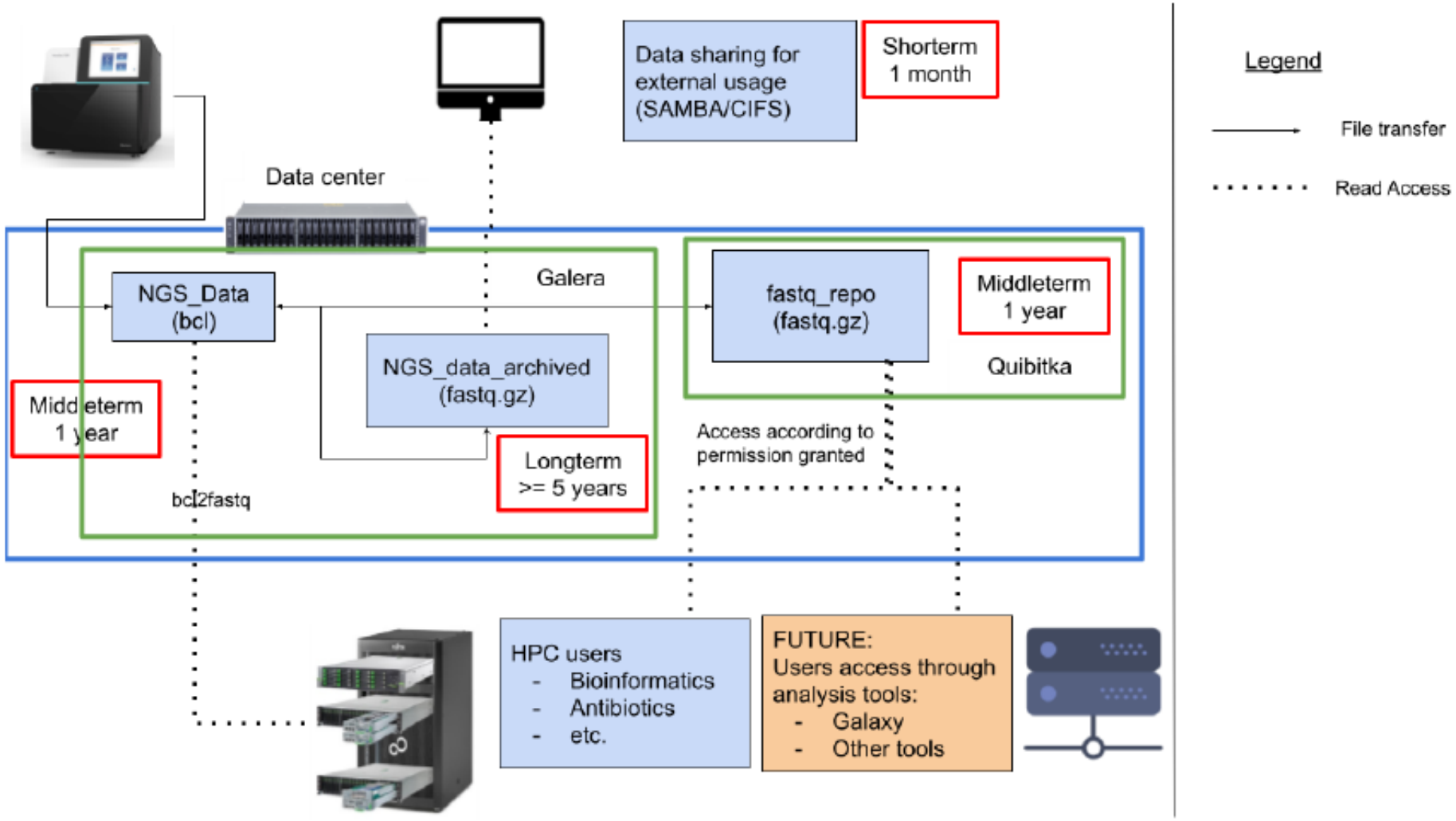
- TEAM: Bioinformatician & IT
- SOURCE OF FUNDING:
  - Research Project
  - National or International Calls
  - Permanent position – Civil Servant

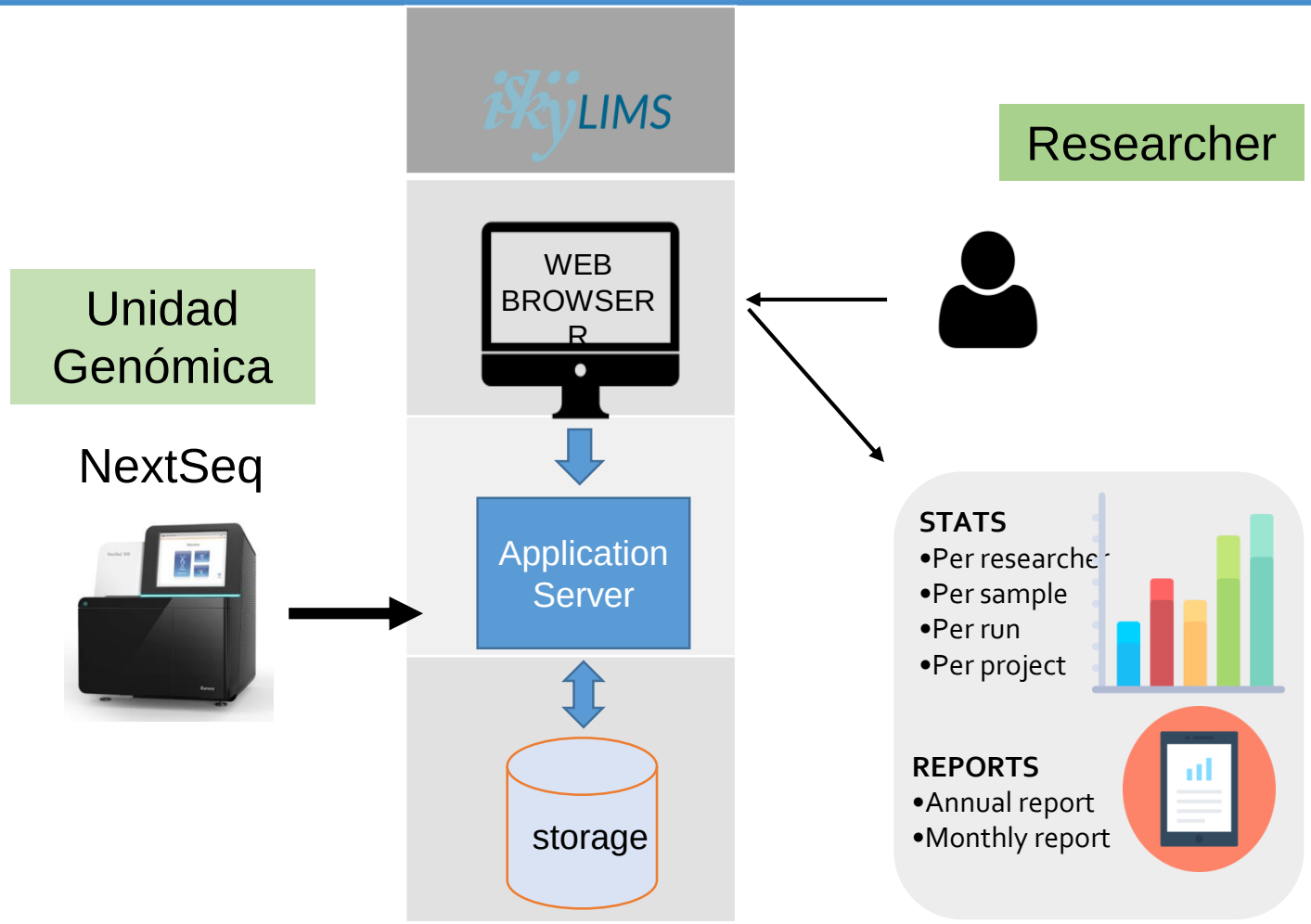


- INFRASTRUCTURE
  - HPC
  - Workstations
- REPOSITORY:
  - GitHub
  - Benchmarking
  - Validation

- INFRASTRUCTURE
- DATA WORKFLOW
- LIMS

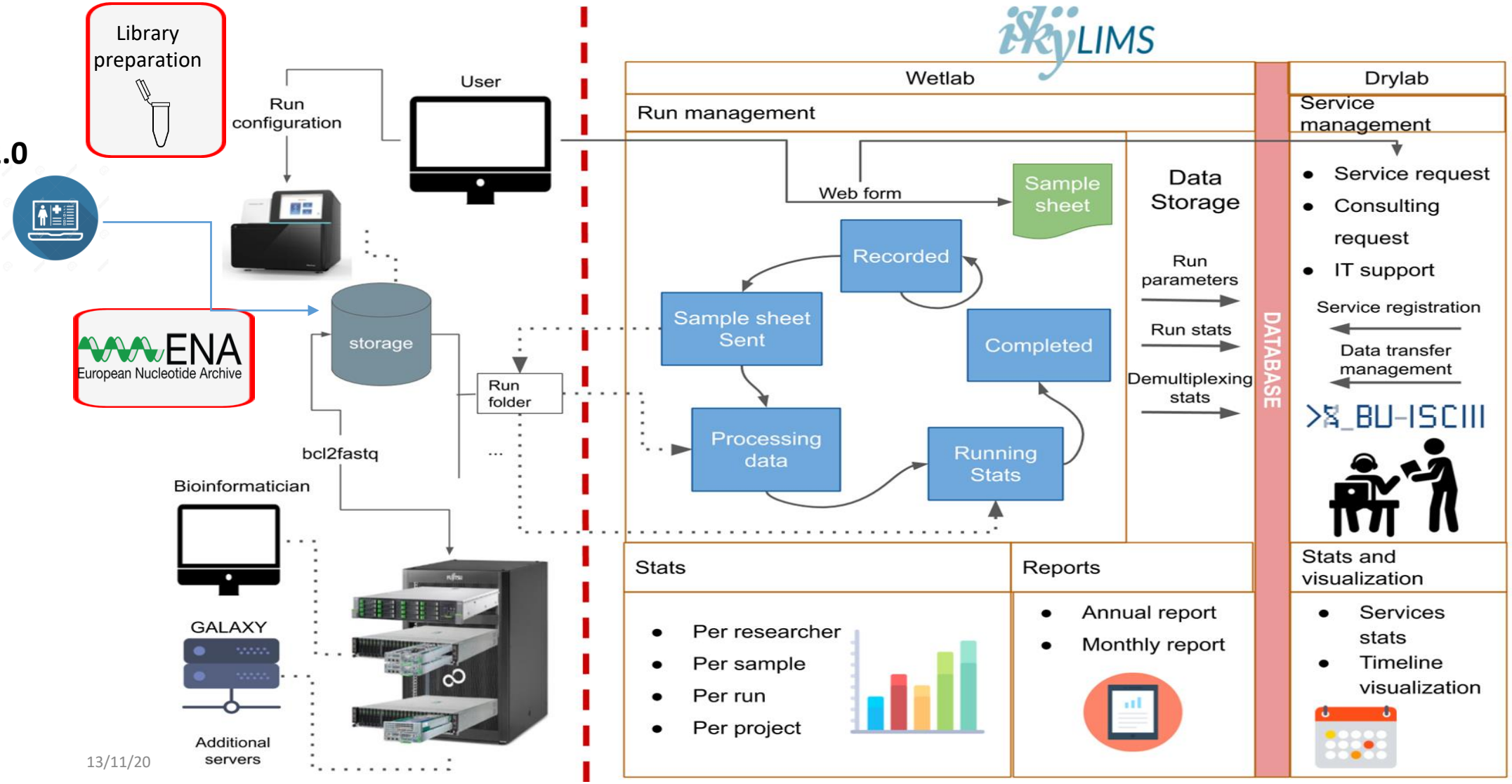
Infrastructure and data management




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

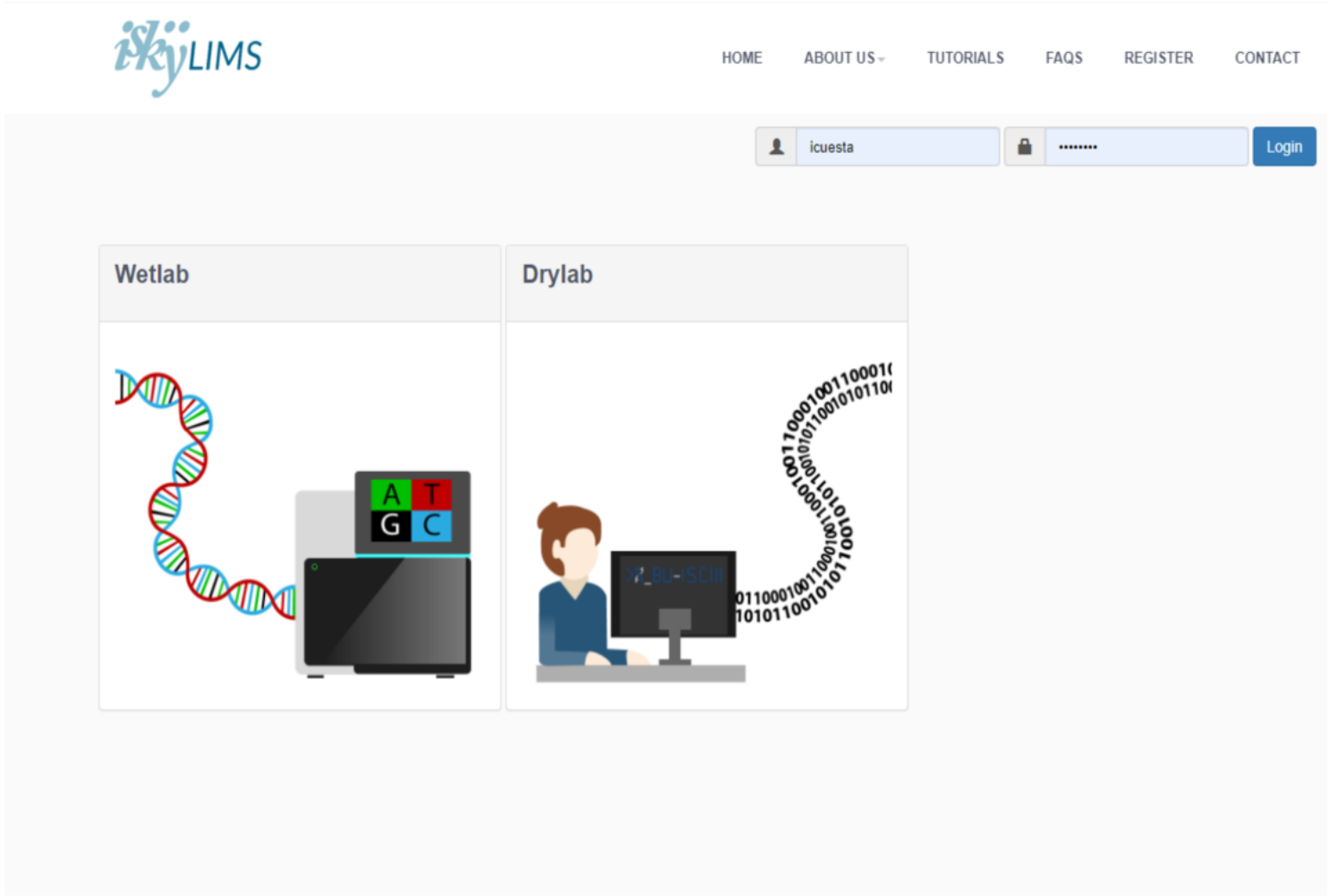
Infrastructure and data management: LIMS

v2.0



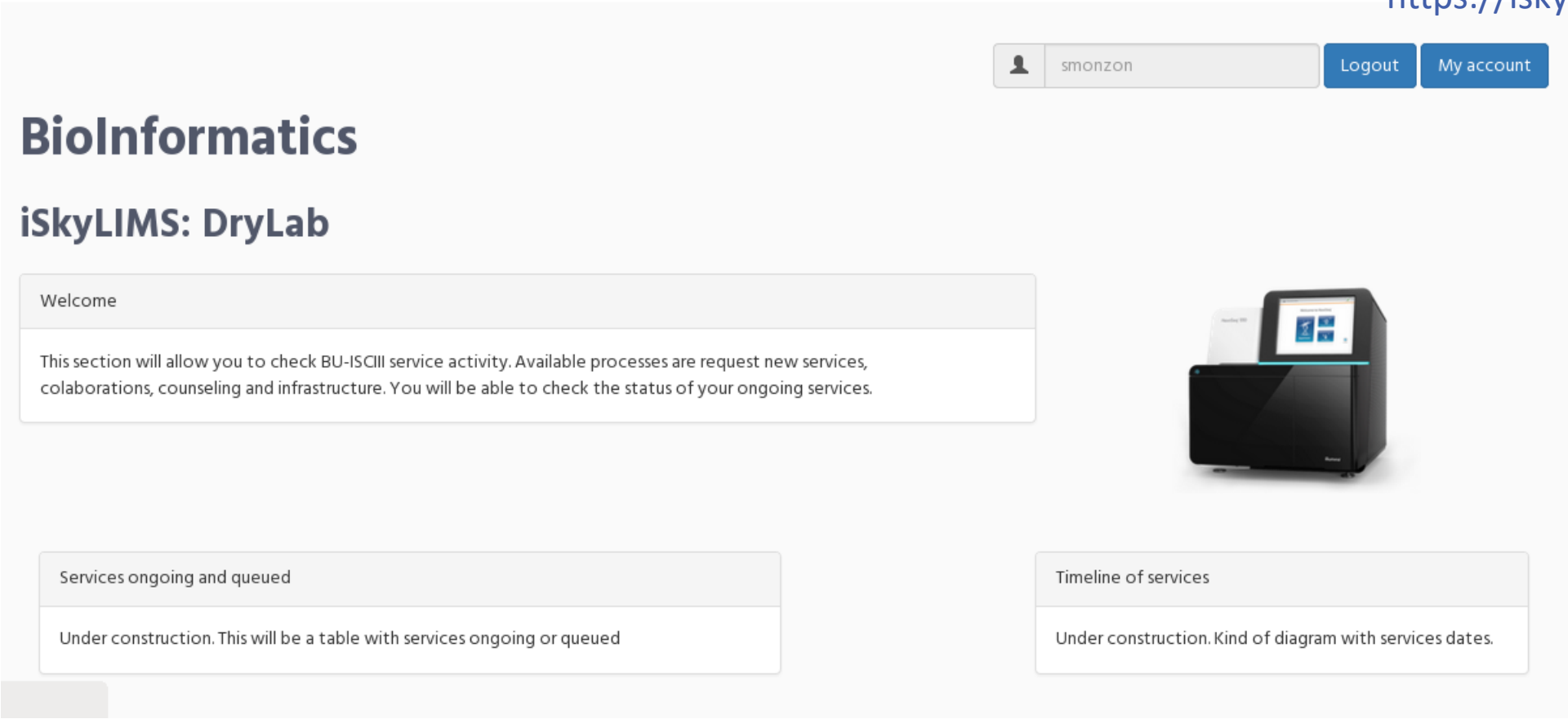
SERVICIOS DE LA UNIDAD

https://iskylims.isciii.es/

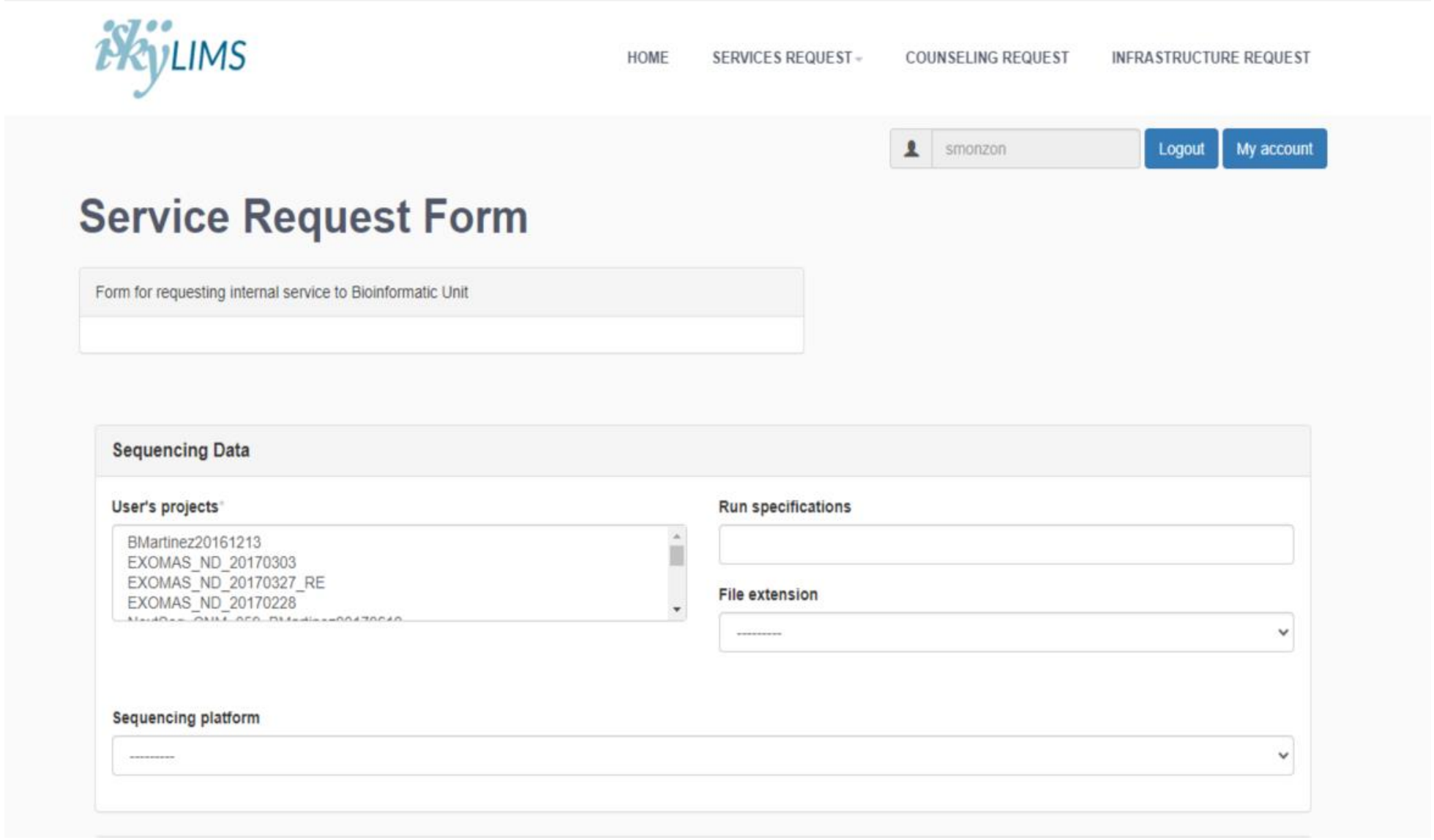


SERVICIOS DE LA UNIDAD

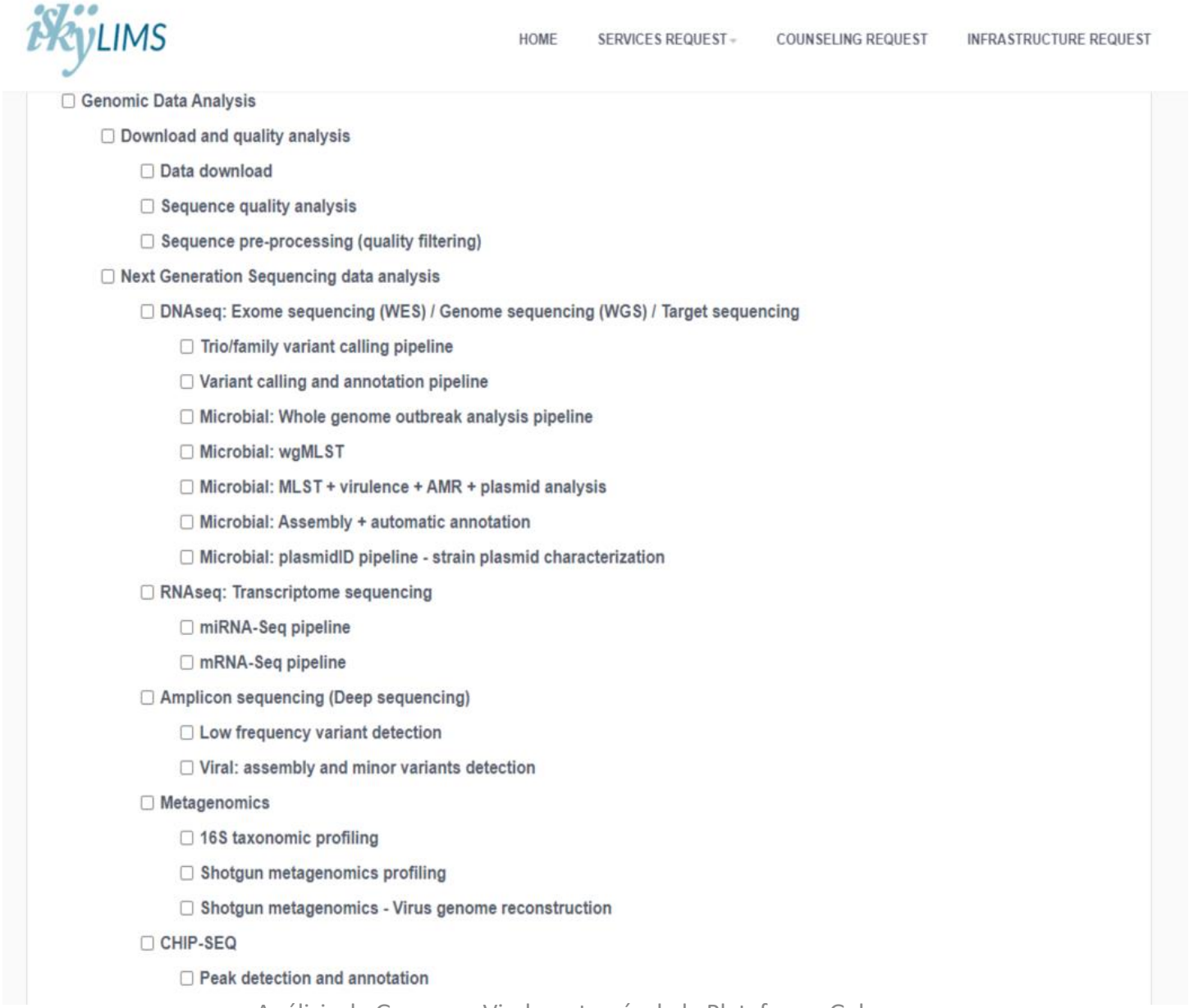
https://iskylims.isciii.es/



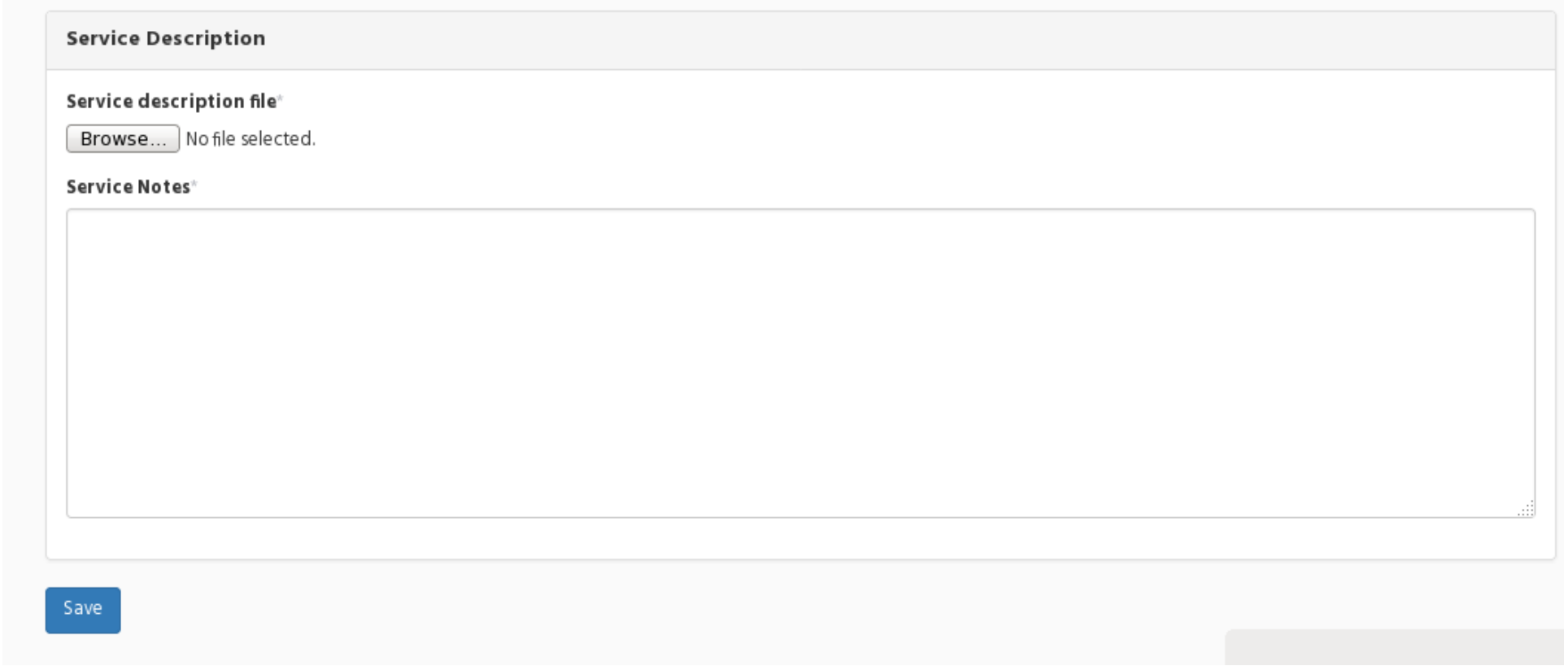
SERVICES REQUEST



SERVICES REQUEST



SERVICES REQUEST



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,...**

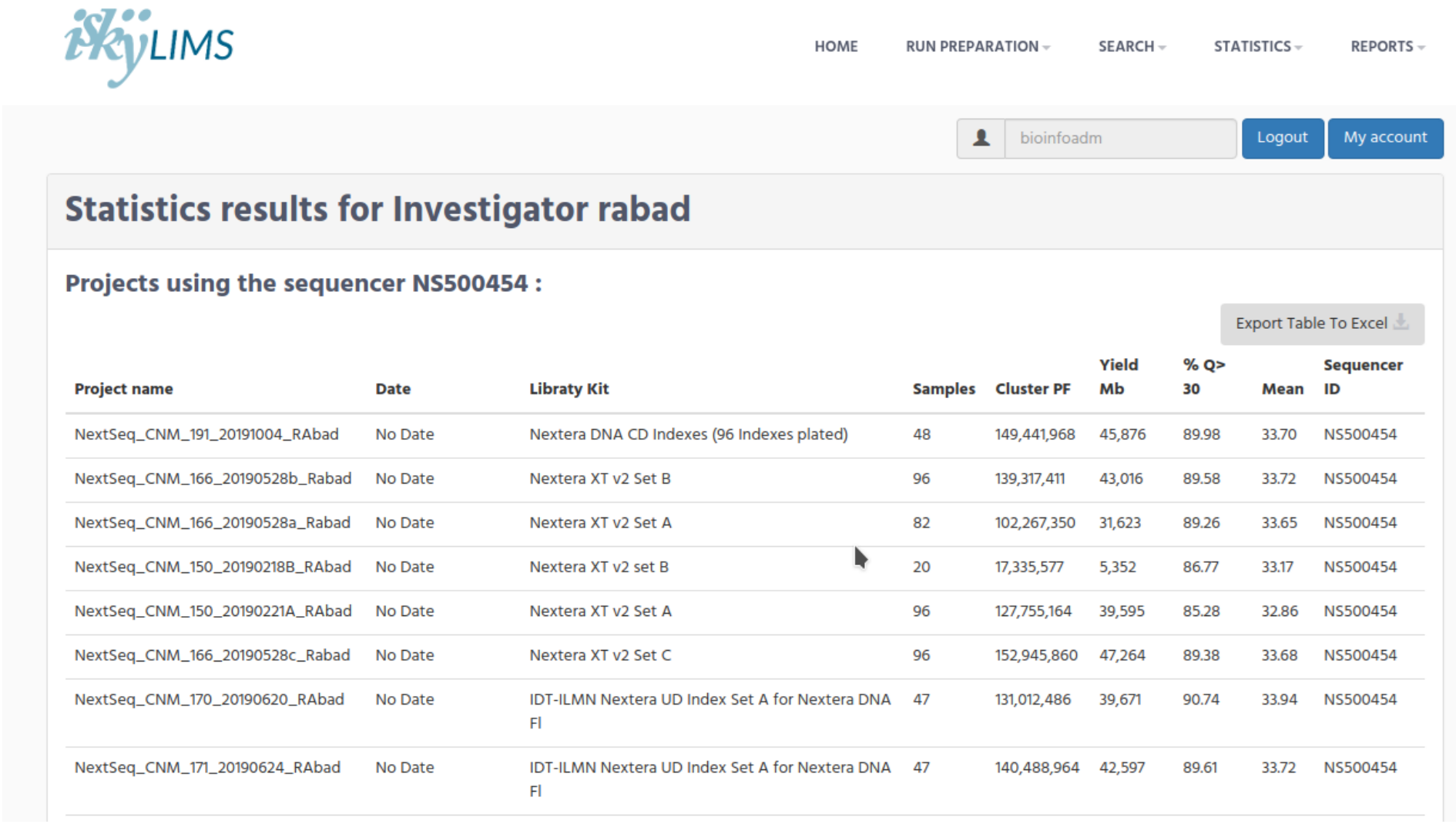
Service Description

Service description file*

Browse... No file selected.

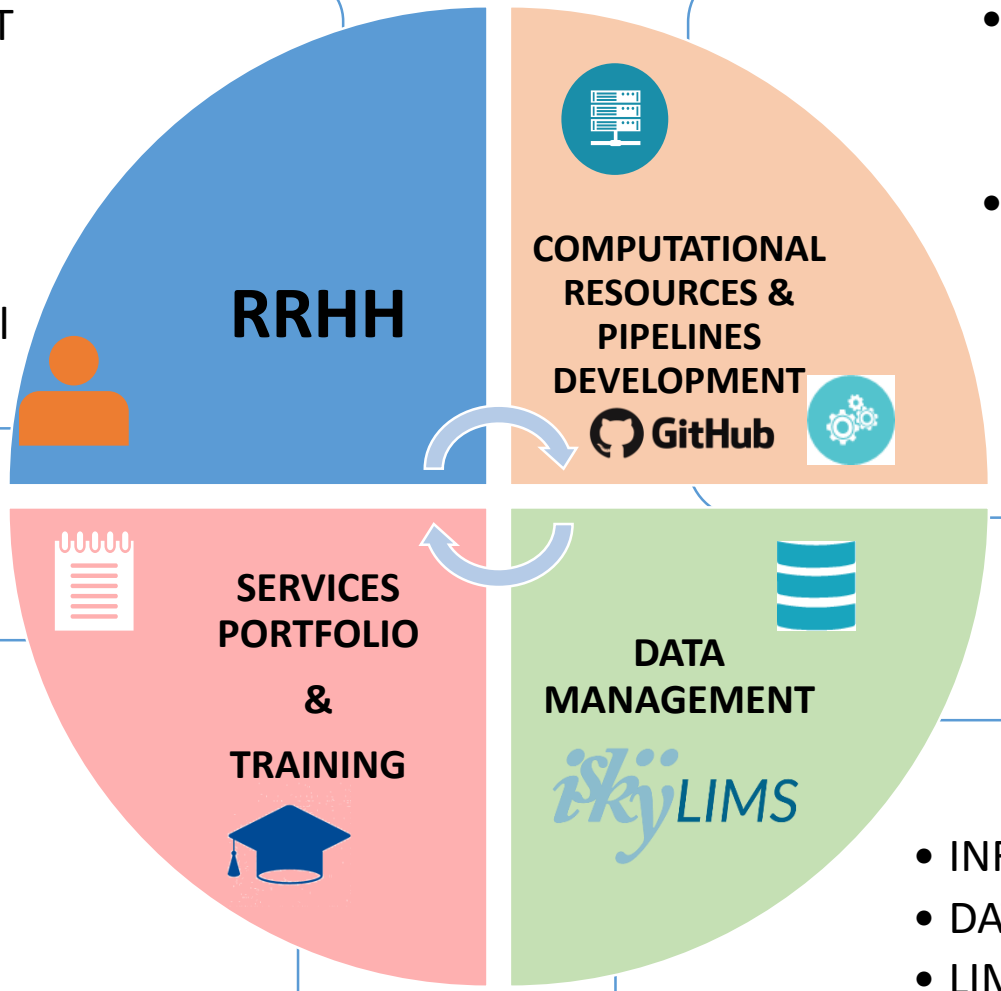
Service Notes*

Infrastructure and data management



Project name	Date	Libraty Kit	Samples	Cluster PF	Yield Mb	% Q> 30	Mean	Sequencer ID
NextSeq_CNM_191_20191004_RAbad	No Date	Nextera DNA CD Indexes (96 Indexes plated)	48	149,441,968	45,876	89.98	33.70	NS500454
NextSeq_CNM_166_20190528b_Rabad	No Date	Nextera XT v2 Set B	96	139,317,411	43,016	89.58	33.72	NS500454
NextSeq_CNM_166_20190528a_Rabad	No Date	Nextera XT v2 Set A	82	102,267,350	31,623	89.26	33.65	NS500454
NextSeq_CNM_150_20190218B_RAbad	No Date	Nextera XT v2 set B	20	17,335,577	5,352	86.77	33.17	NS500454
NextSeq_CNM_150_20190221A_RAbad	No Date	Nextera XT v2 Set A	96	127,755,164	39,595	85.28	32.86	NS500454
NextSeq_CNM_166_20190528c_Rabad	No Date	Nextera XT v2 Set C	96	152,945,860	47,264	89.38	33.68	NS500454
NextSeq_CNM_170_20190620_RAbad	No Date	IDT-ILMN Nextera UD Index Set A for Nextera DNA Fl	47	131,012,486	39,671	90.74	33.94	NS500454
NextSeq_CNM_171_20190624_RAbad	No Date	IDT-ILMN Nextera UD Index Set A for Nextera DNA Fl	47	140,488,964	42,597	89.61	33.72	NS500454

- TEAM: Bioinformatician & IT
- SOURCE OF FUNDING:
  - Research Project
  - National or International Calls
  - Permanent position – Civil Servant



- INFRASTRUCTURE
  - HPC
  - Workstations
- REPOSITORY:
  - GitHub
  - Benchmarking
  - Validation

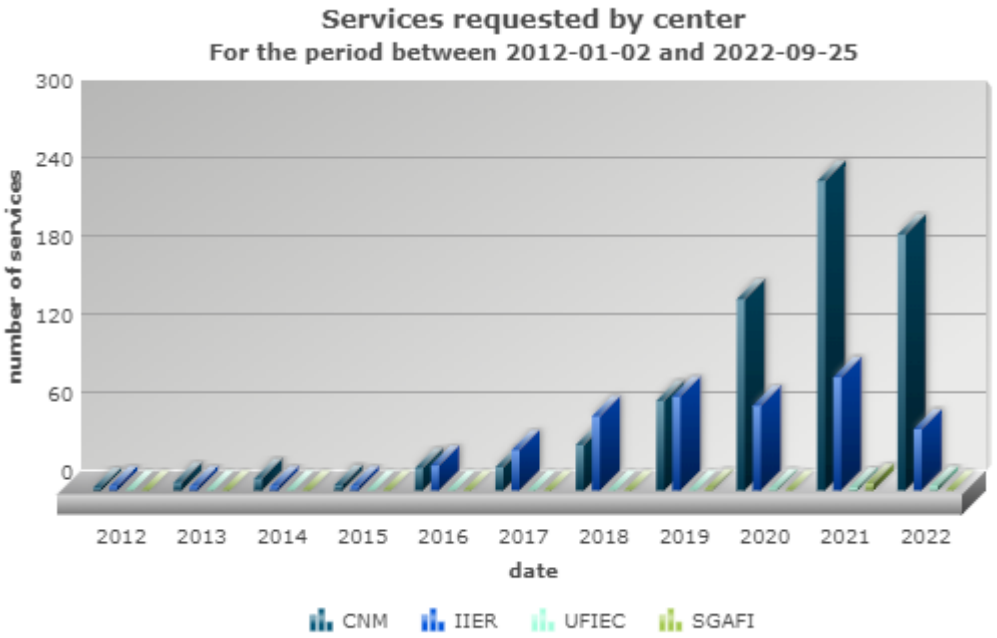
- COURSES, TFM, TFG
- DATA ANALYSIS
  - DNAseq
  - RNAseq
  - Metagenomics

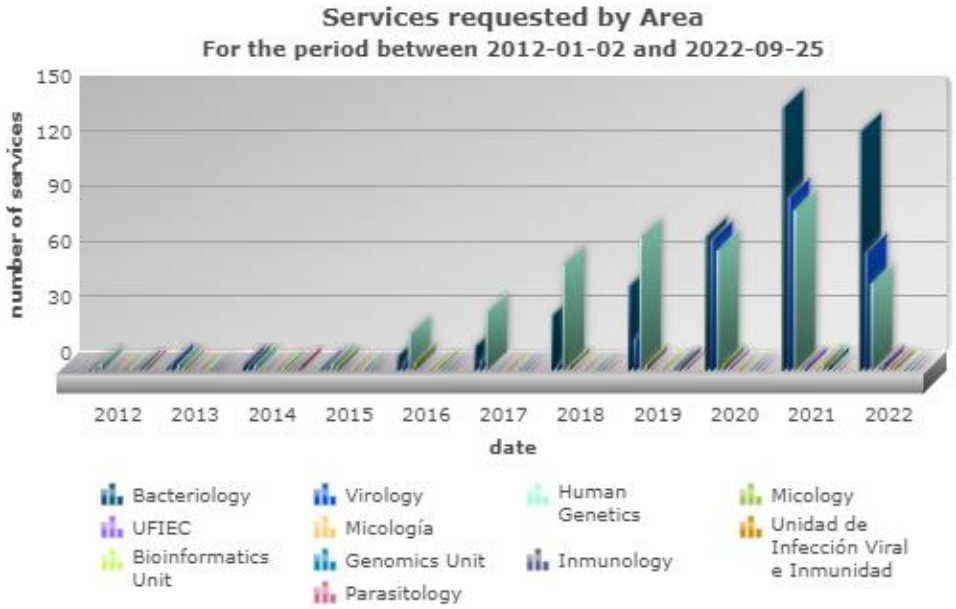
- INFRASTRUCTURE
- DATA WORKFLOW
- LIMS

Services Portfolio

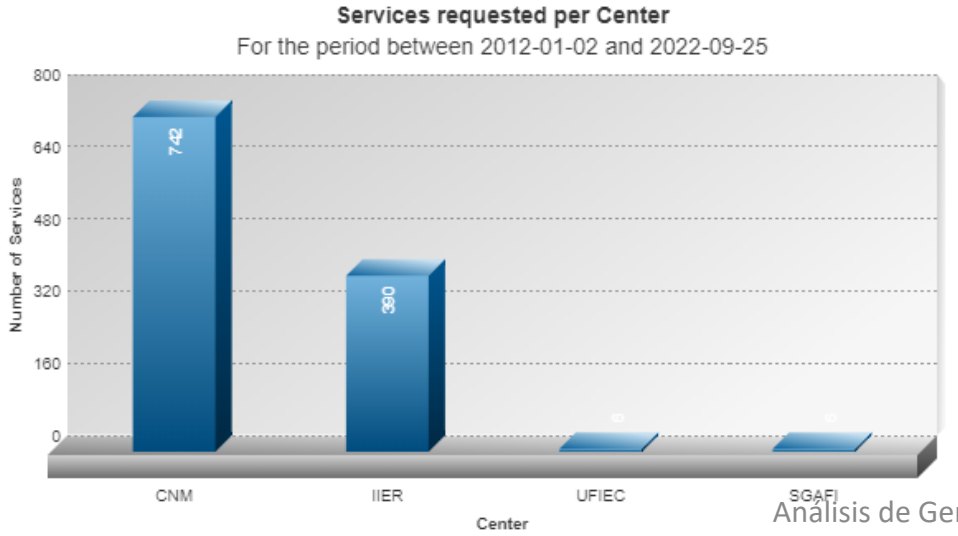
		QC	Assembly	Reference based Mapping	Variant calling	Annotation	Pipelines
DNAseq	**HUMAN**						
	WES Target –Panels	Report html		(Bam file)	(Vcf file)	Desease model (Vcf file annotated)	.Trio / family .Tumor .Pampu caller
	MICROBIAL						
	WGS Amplicon	Report html	*De novo* / Reference (fasta file)	MLST, 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
	Shotgun			Genome Ref Seq		Pathogen / Genome coverage	PikaVirus

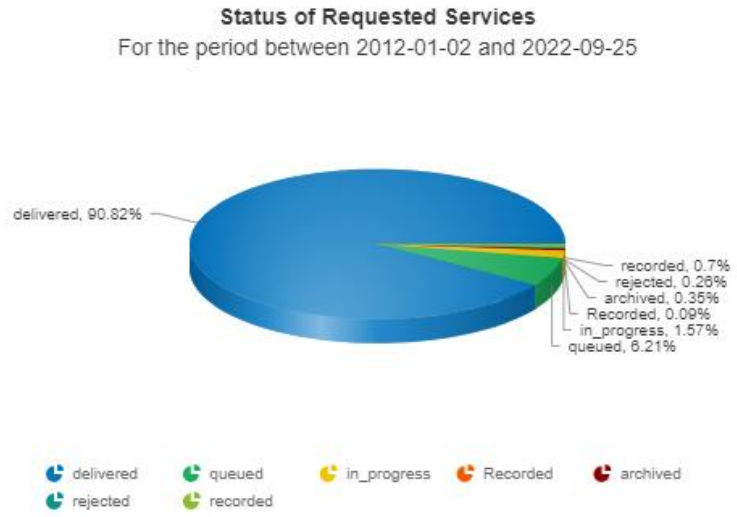
Number of services: 2012 – 2022





10 AÑOS





Training

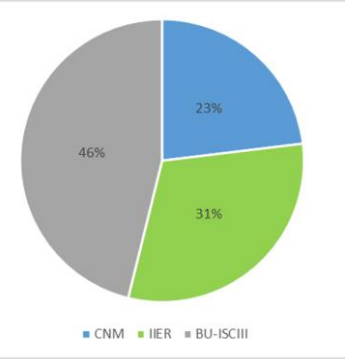
Courses

ISCIII

Introduction to massive sequencing data analysis, 2013-2021 (8 editions)

Secuenciación de genomas bacterianos: herramientas y aplicaciones, 2018-2021 (3 editions)

Análisis de genomas virales a través de la plataforma Galaxy, 2021 (1 edition)

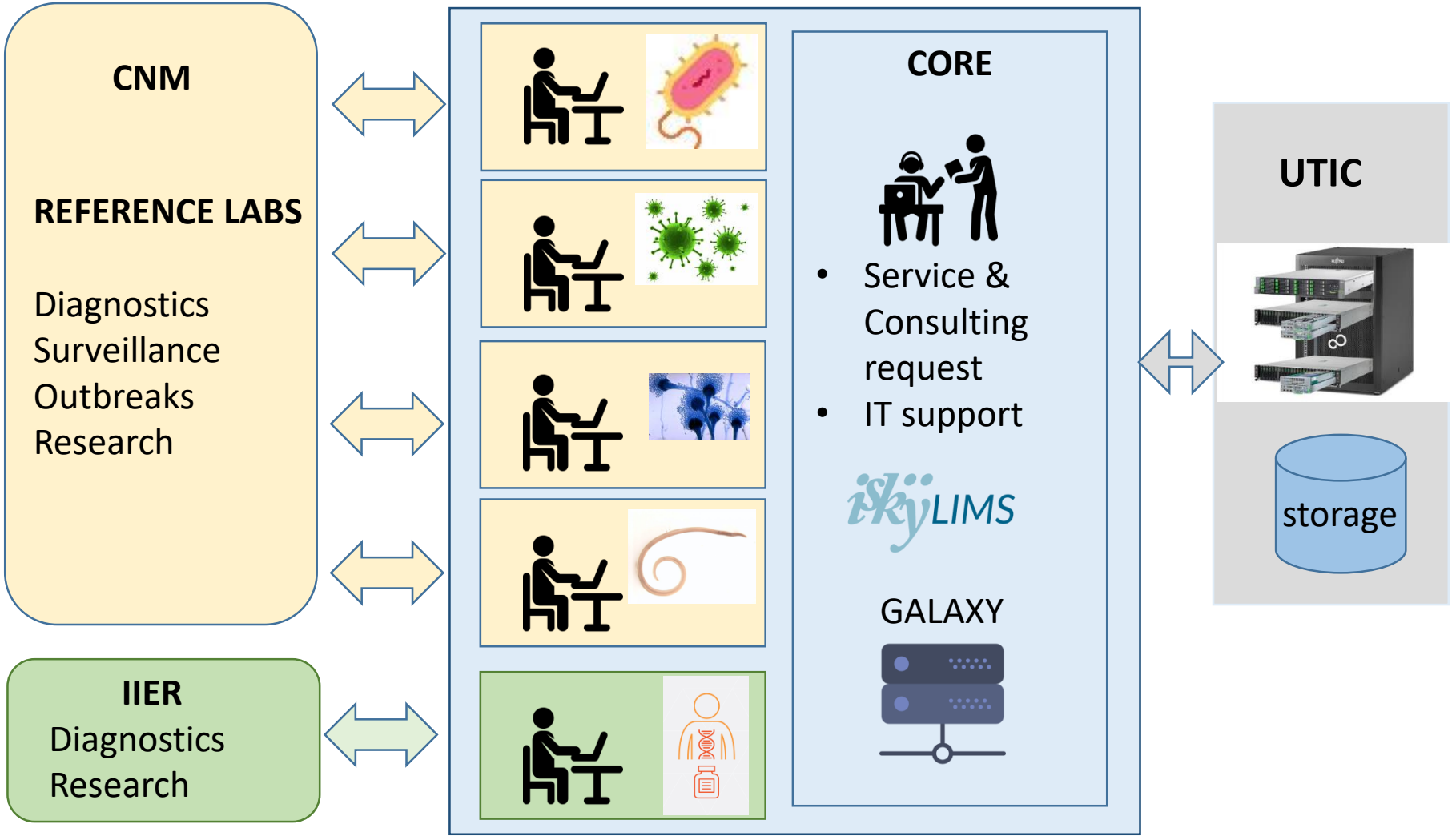


Master & Grade Students

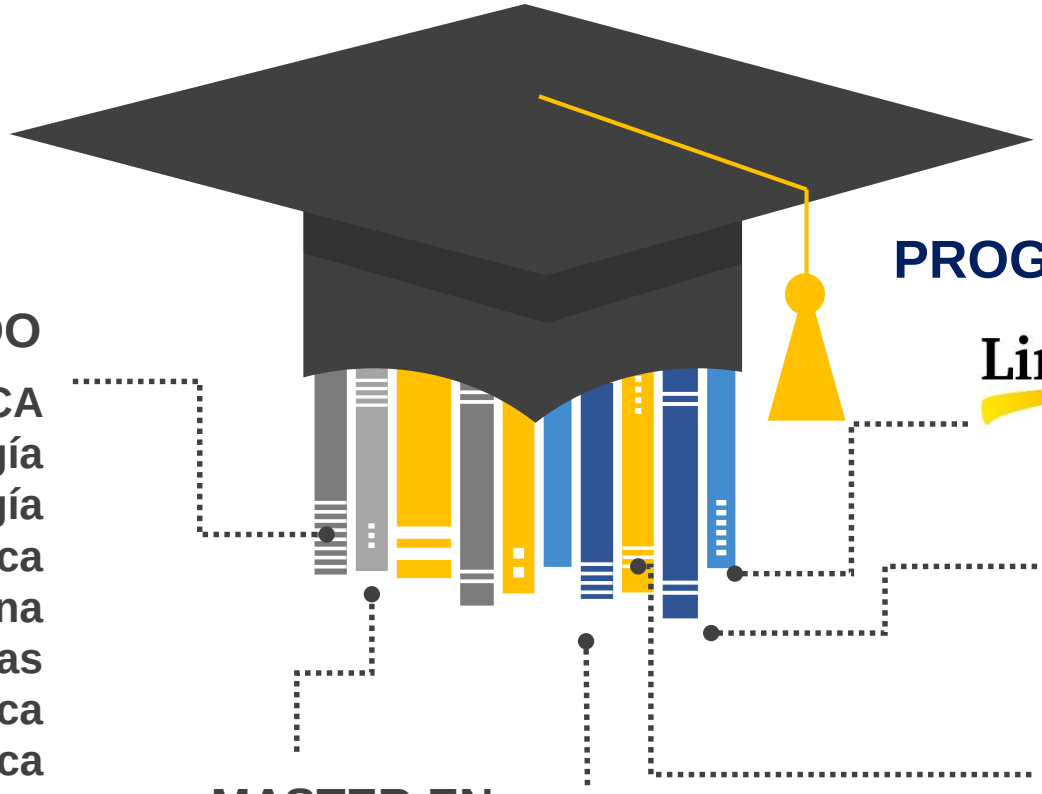
- Bioinformatica y Biología Computacional ENS-ISCIII
- Bioinformatica UAM
- Genética y Biología Molecular UAM
- Microbiología aplicada a la salud pública e investigación en enfermedades infecciosas, U. Alcala de Henares
- Sciences in Omics Data Analysis, Universidad de VIC, U. Central de Cataluña
- Complutense University

Hospitals Students

Roadmap: BU-ISCIII Model



FORMACIÓN EN BIOINFORMÁTICA



Universidad Barcelona.

GRADO

BIOINFORMÁTICA
Biología
Biotecnología
Bioquímica
Medicina
Matemáticas
Química
Fisica
Informática
Telecomunicaciones

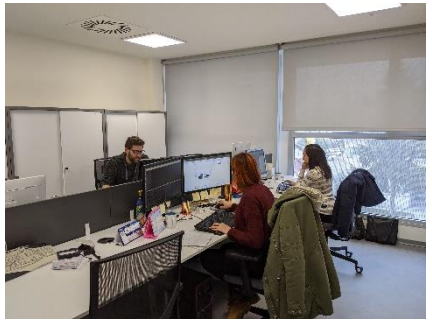
MASTER EN BIOINFORMÁTICA

DOCTORADO

BIOLOGÍA – ANÁLISIS DE DATOS

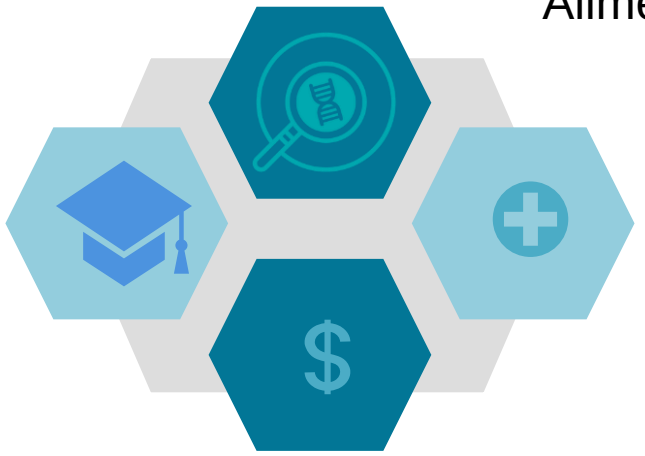
PROGRAMACIÓN

¿Dónde trabaja un Bioinformático?



CENTRO DE INVESTIGACIÓN

Biomedicina
Agricultura
Alimentación



UNIVERSIDAD

Biociencias
Informática

HOSPITAL

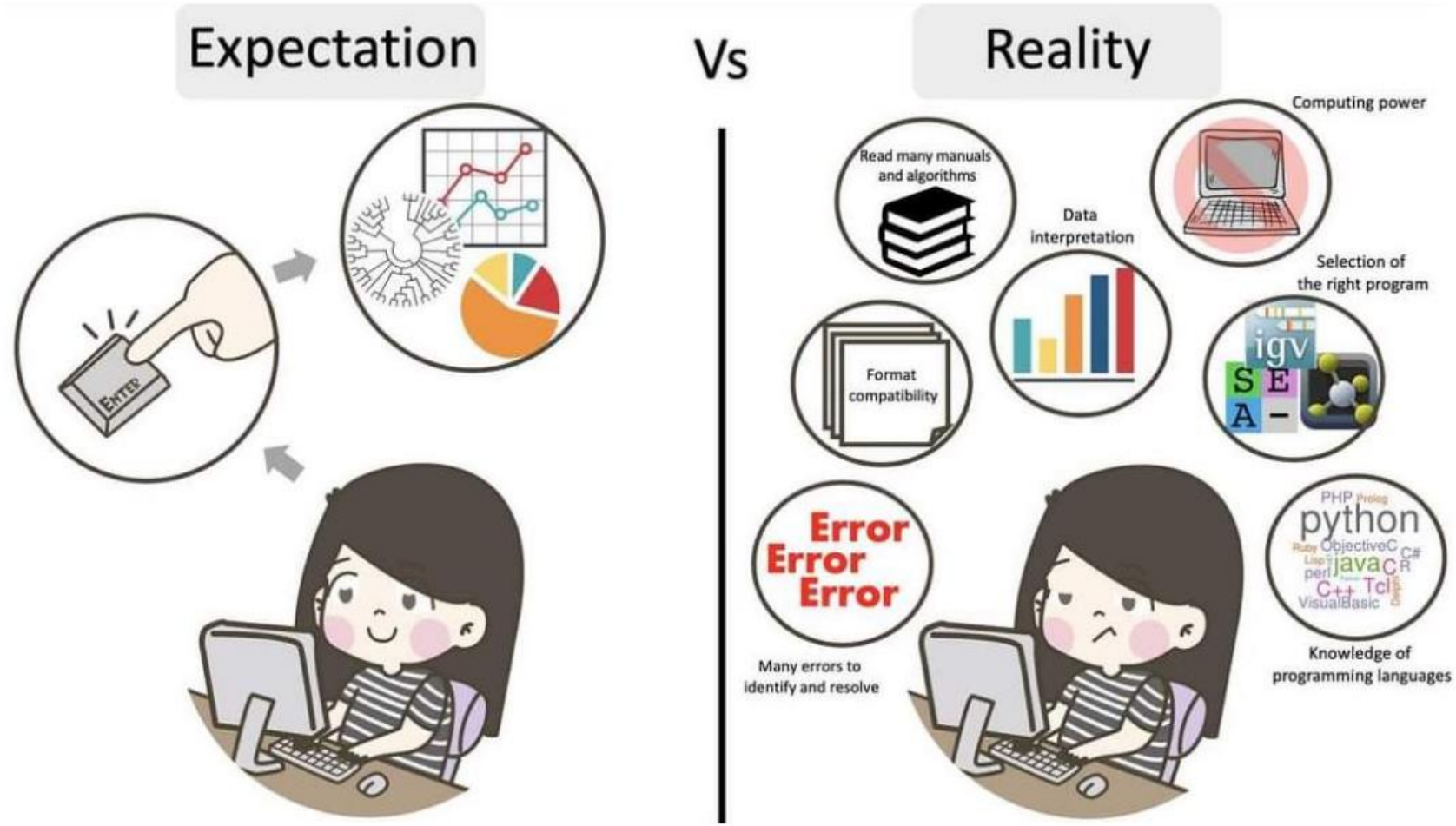
BIOINFORMÁTICO CLÍNICO
Genética
Oncología
Cardiología

EMPRESA

Bioinformática
Genética
Genómica

The truth about bioinformatics

.image-100[

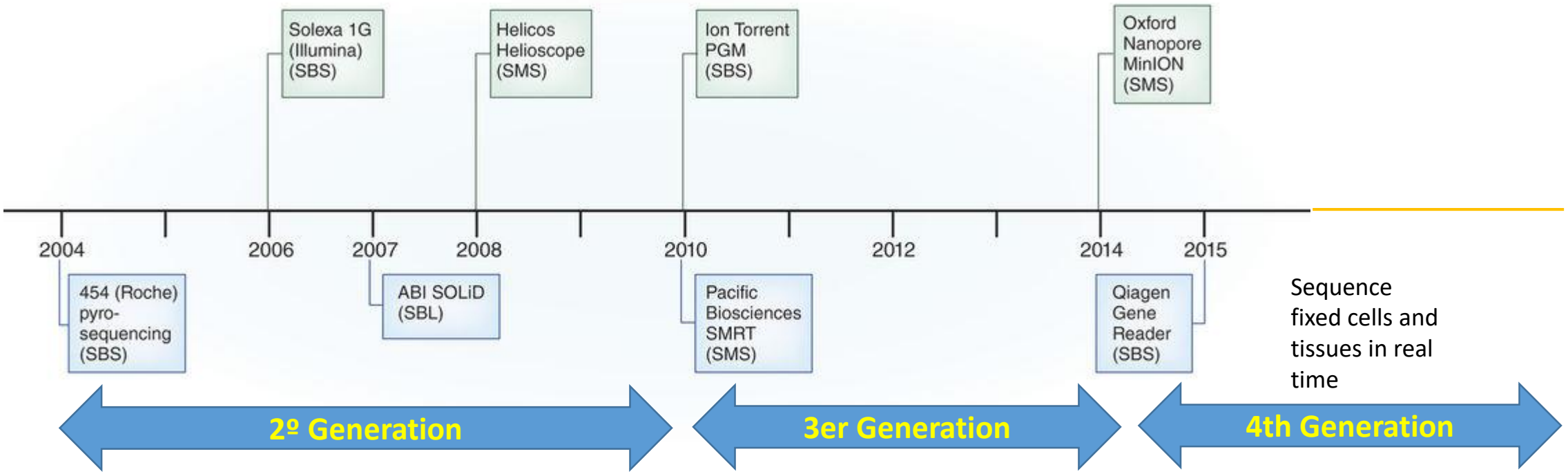


https://training.galaxyproject.org/training-material/topics/assembly/tutorials/get-started-genome-assembly/slides-plain.html

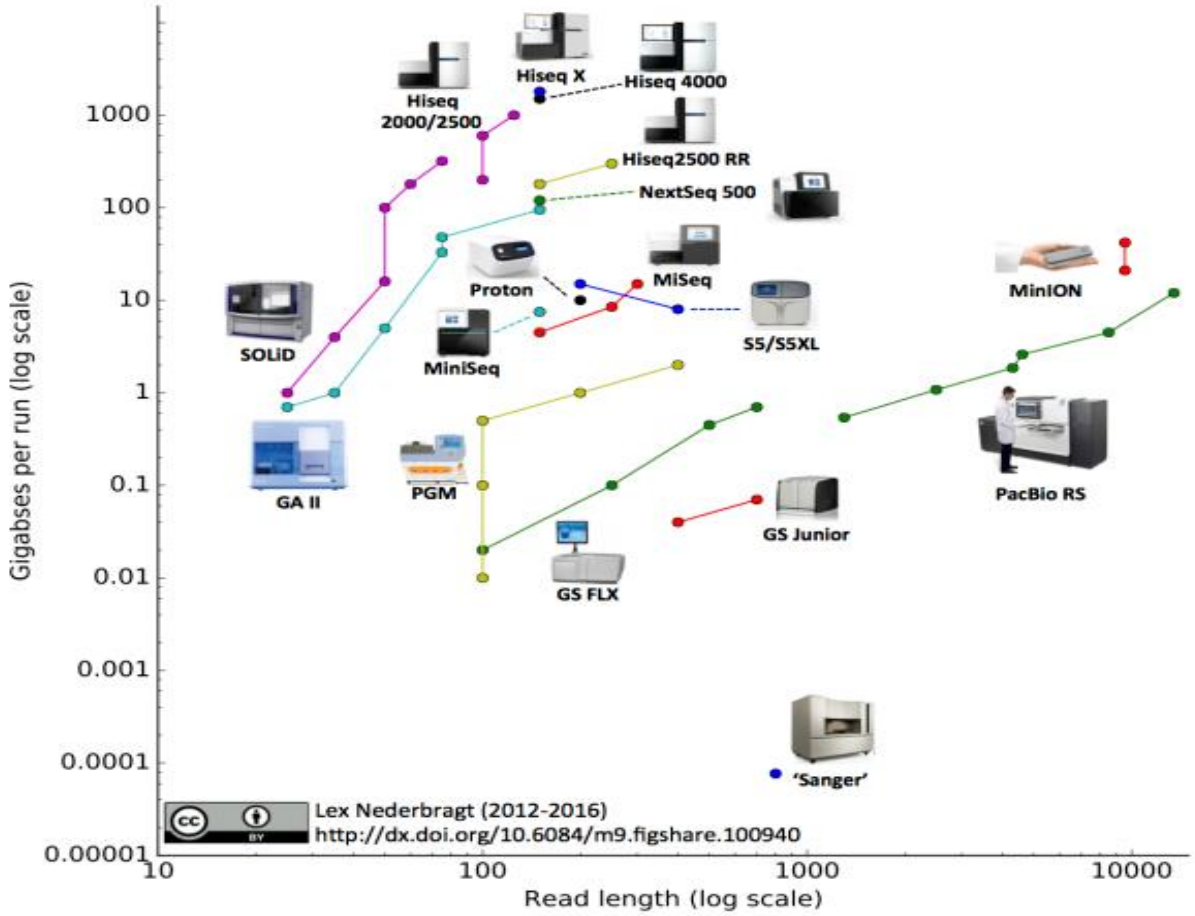
Index

- **High throughput sequencing in clinical virology, Applications**

NGS Platforms - Timelime

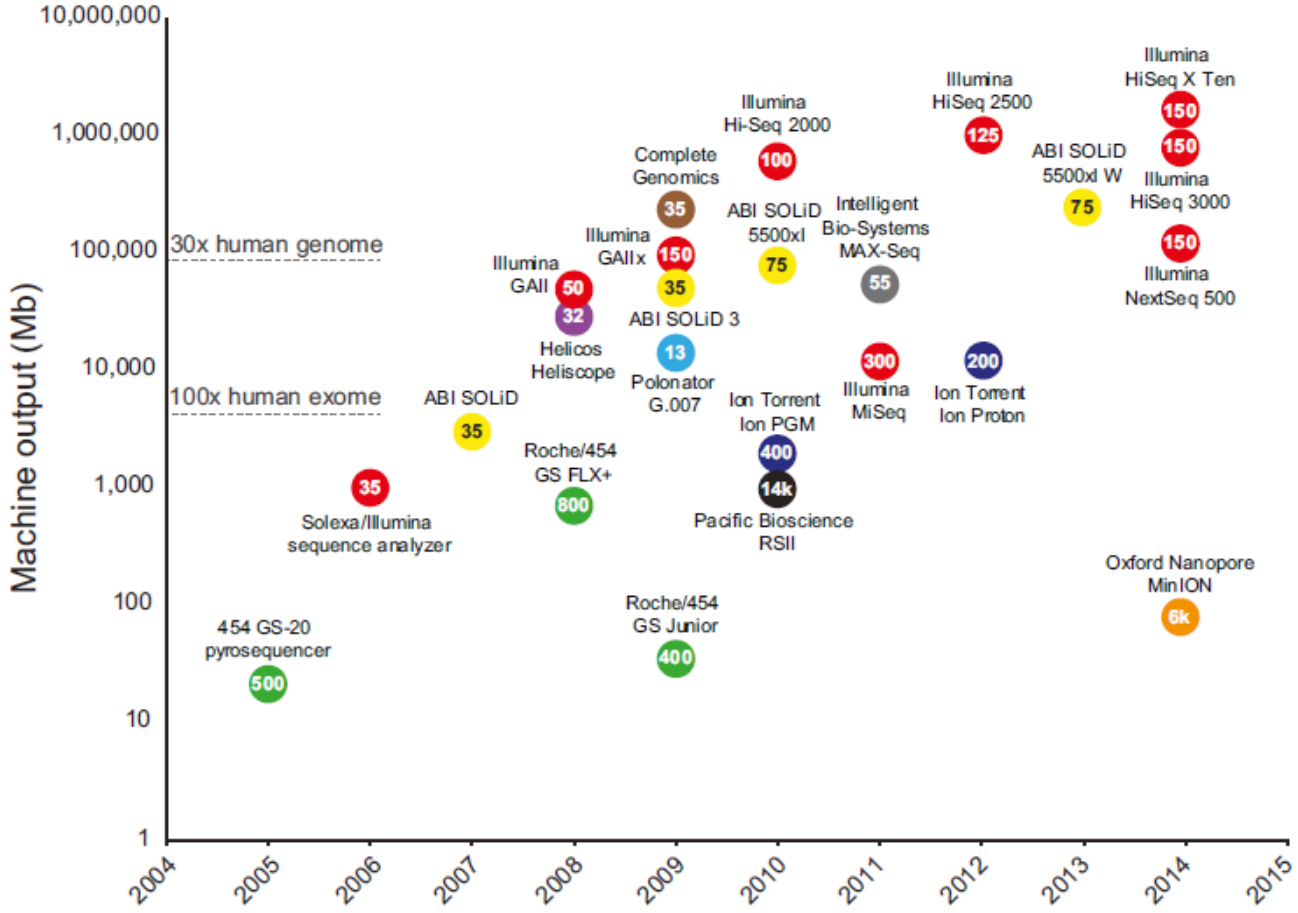


High-Throughput Sequencing Technologies



https://flxlexblog.wordpress.com/

High-Throughput Sequencing Technologies

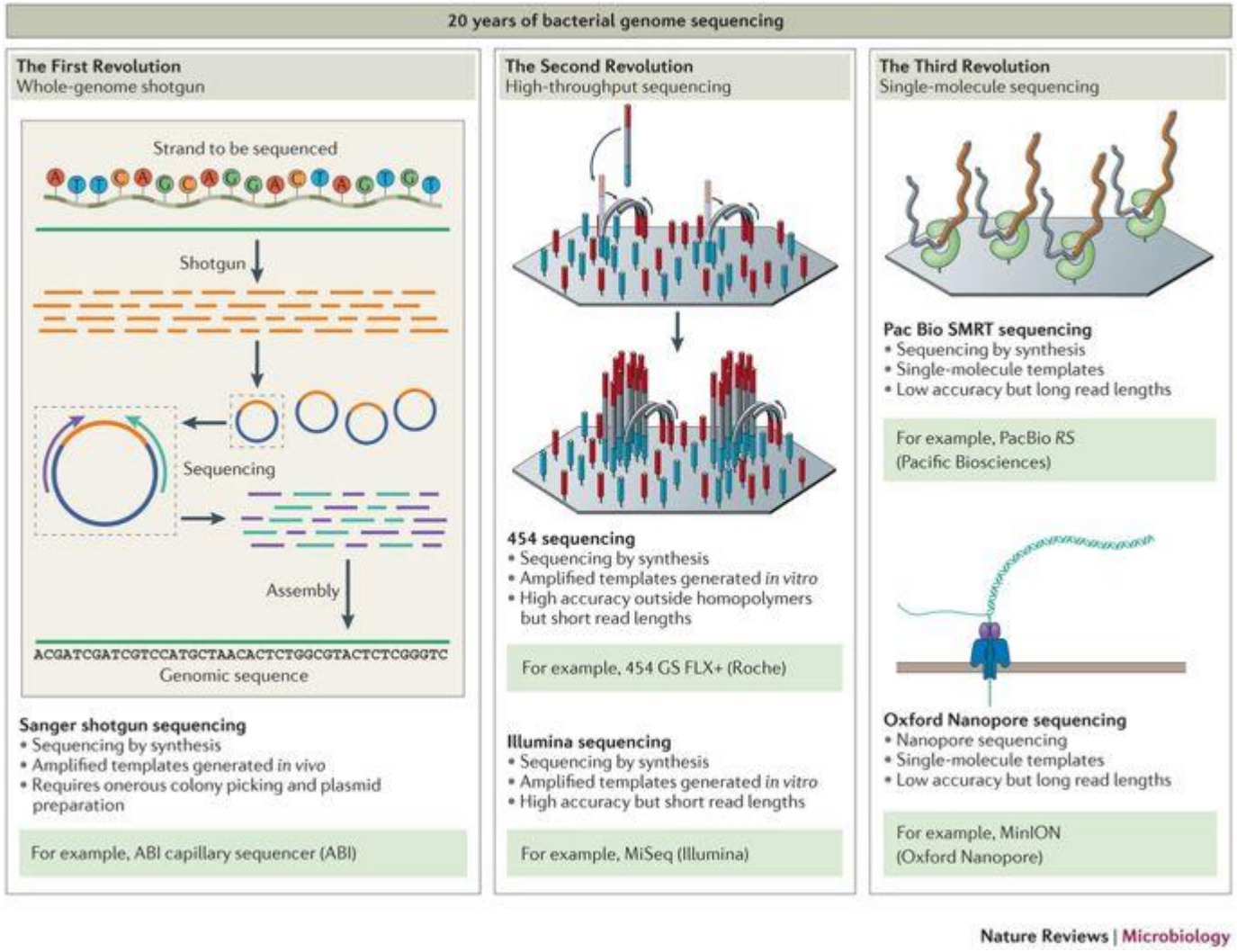


Numbers inside data points denote current read lengths. Sequencing platforms are color coded.

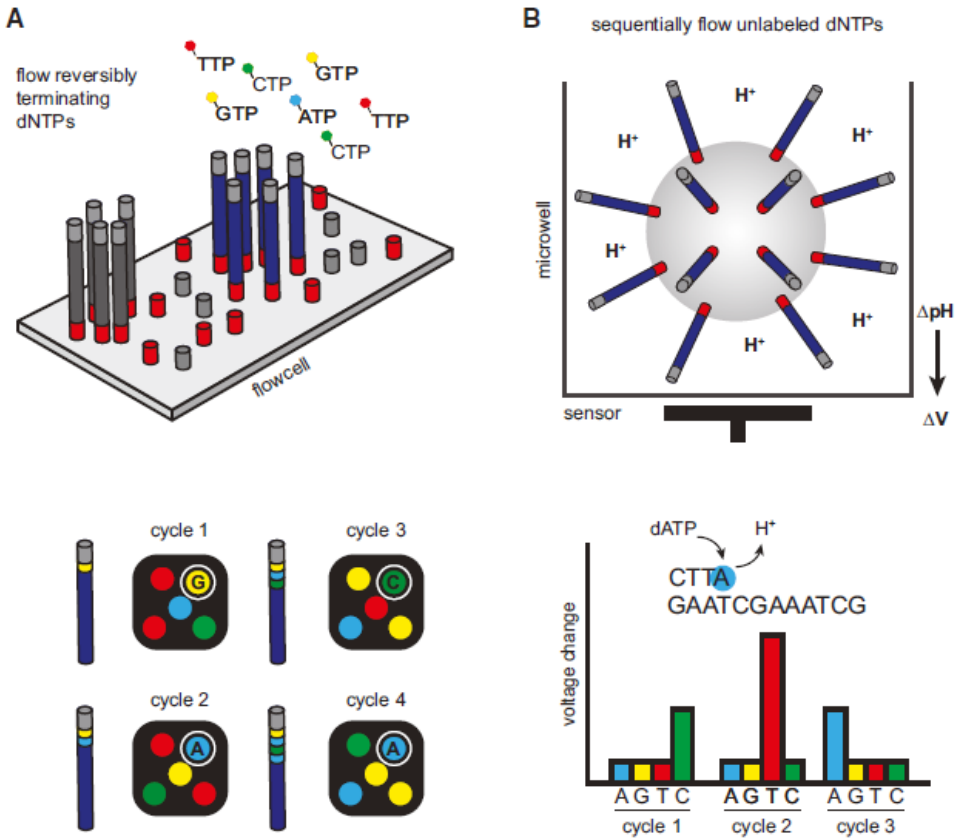
Reuter et al., Mol Cell 2015

High-Throughput Sequencing Technologies

The three revolutions in sequencing technology that have transformed the landscape of bacterial genome sequencing



The Second-generation Sequencing Technologies



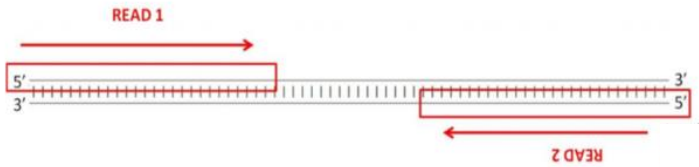
Clonal Amplification-Based Sequencing Platforms

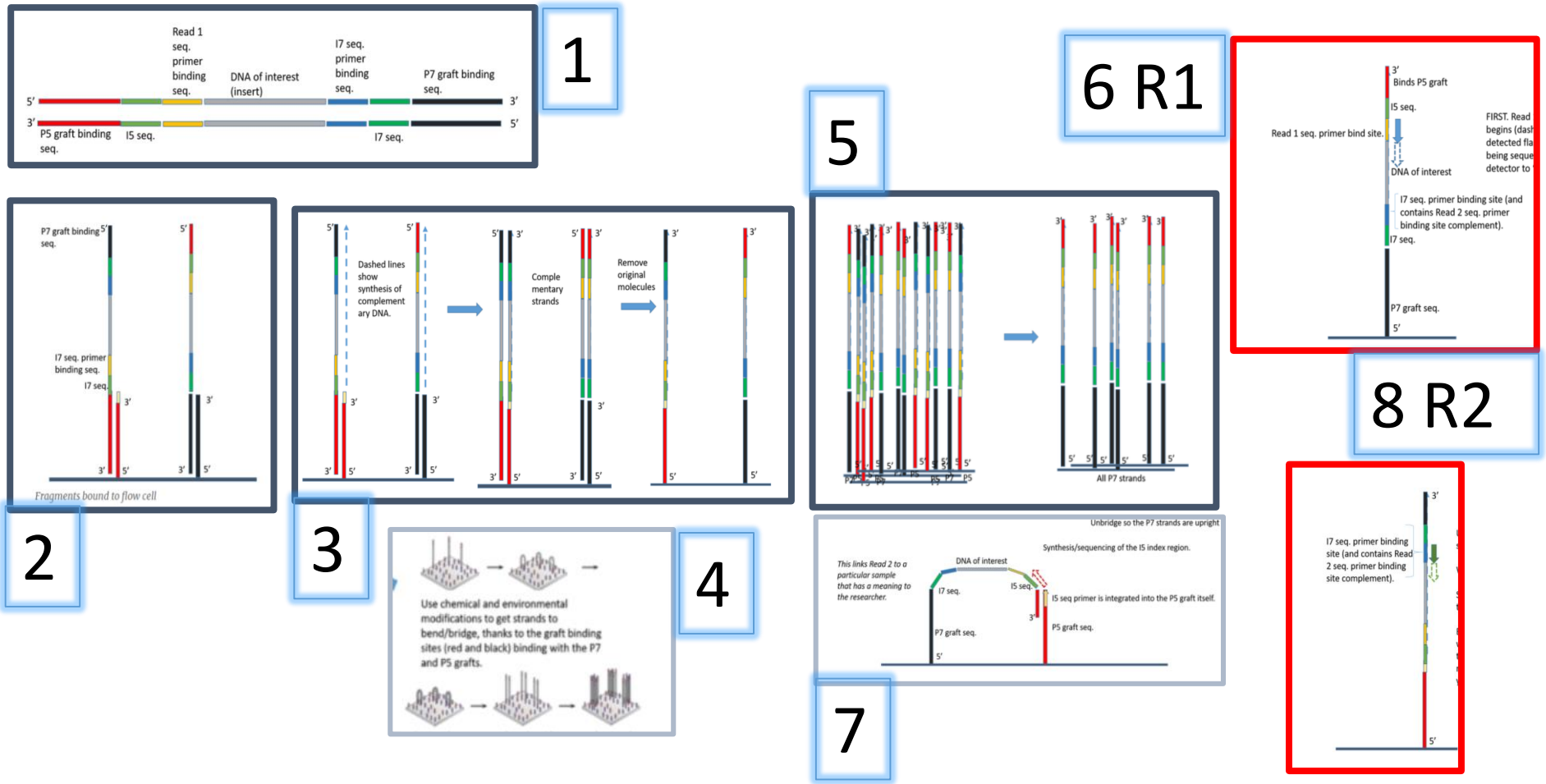
(A) Illumina's four-color reversible termination sequencing method.

(B) Ion Torrent's semiconductor sequencing method.

Reuter et al., Mol Cell 2015

Illumina sequencing





https://kscbioinformatics.wordpress.com/2017/02/13/illumina-sequencing-for-dummies-samples-are-sequenced/

Illumina Benchtop Sequencers

Pervez et al., BioMed Research International 2022

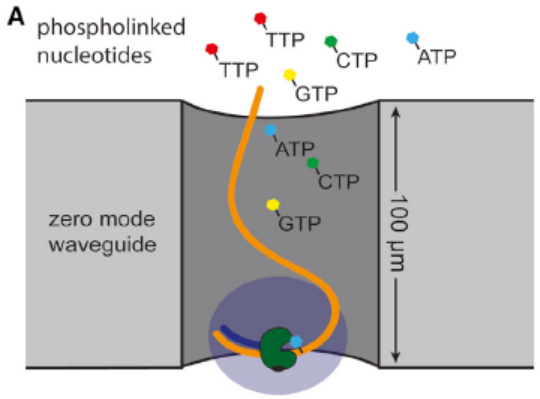
Methods/applications	iSeq 100	MiniSeq	MiSeq series	NextSeq 550 series	Next Seq 1000 & 2000
Ideal for	Every size lab	TG sequencing	Long read applications	Exome and transcriptome sequencing	miRNA and sRNA analysis
Major applications	sWGS (microbes) and TGS	iSeq 100+TG EP and 16S MS	iSeq 100+16S MGS	iSeq 100+TCS	sWGS (microbes), ES, SC profiling, TS, miRNA, and sRNA analysis
Max. data quality	>85% > Q30	>85% > Q30	>90% > Q30	>80% > Q30	>90% > Q30
Run time	9.5–19 h	4–24 hours	4–55 hours	12–30 hours	11-48 hours
Maximum output	1.2 Gb	7.5 Gb	15 Gb	120 Gb	330 Gb*
Maximum reads per run	4 million	25 million	25 million	400 million	1.1 billion
Maximum read length	2 × 150 bp	2 × 150 bp	2 × 300 bp	2 × 150 bp	2 × 150 bp

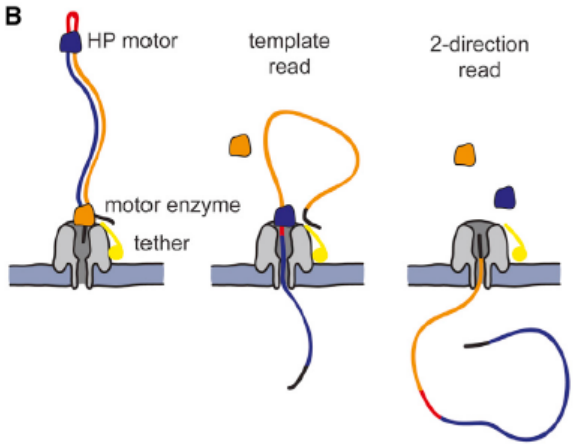
Illumina Production Scale Sequencers

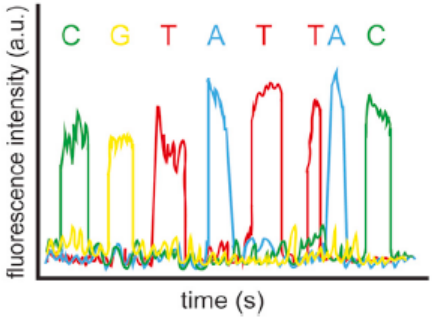
Pervez et al., BioMed Research International 2022

Methods/applications	NextSeq 550	NextSeq 550Dx	NextSeq 1000 & 2000	NovaSeq 6000
Ideal for	Research	Research+in vitro diagnostic	Targeted sequencing	Long read applications
Major applications	sWGS (microbes), TGS, and TCS	NextSeq 550+clinical NGS applications	NextSeq 550 series+SCP	NextSeq 550 series+NextSeq 1000 & 2000+lWGS
Max. data quality	>80% > Q30	>75% > Q30	>90% > Q30	>90% > Q30
Run time	12-30 hours	35 hours	11-48 hours	13-44 hours
Maximum output	120 Gb	90 Gb	360 Gb	6000 Gb
Maximum reads per run	400 million	300 million	1.2 billion	20 billion
Maximum read length	2 × 150 bp	2 × 150 bp	2 × 150 bp	2 × 250 bp

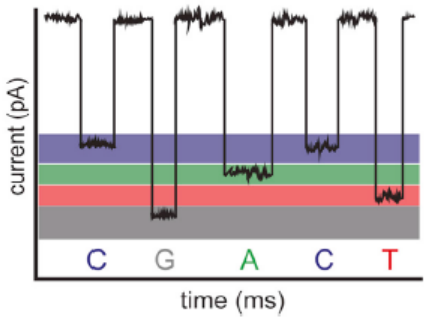
The Third-generation Sequencing Technologies







Pacific Bioscience's SMRT sequencing



Oxford Nanopore's sequencing strategy

Reuter et al., Mol Cell 2015

Single Molecule Sequencing Platforms

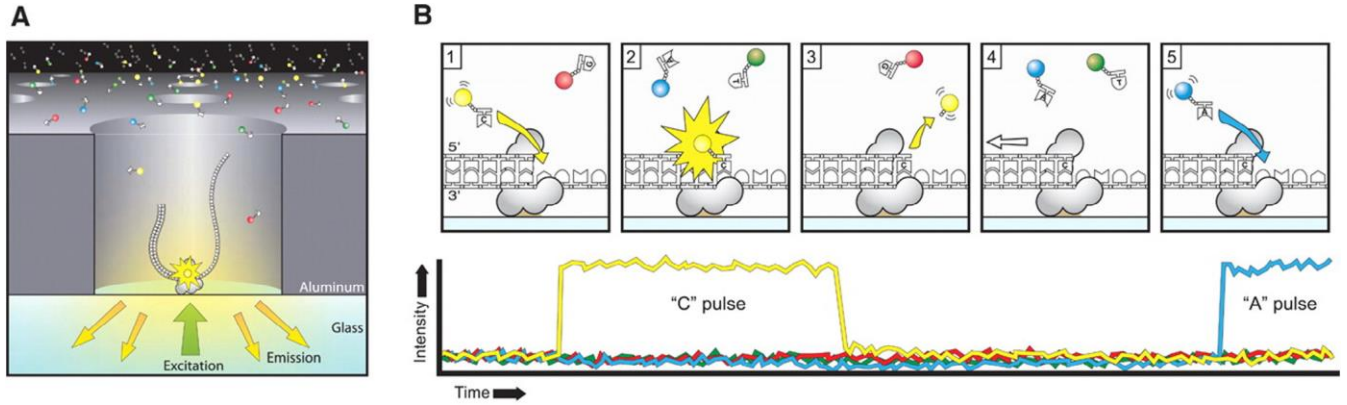
PacBio sequencing and its applications

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

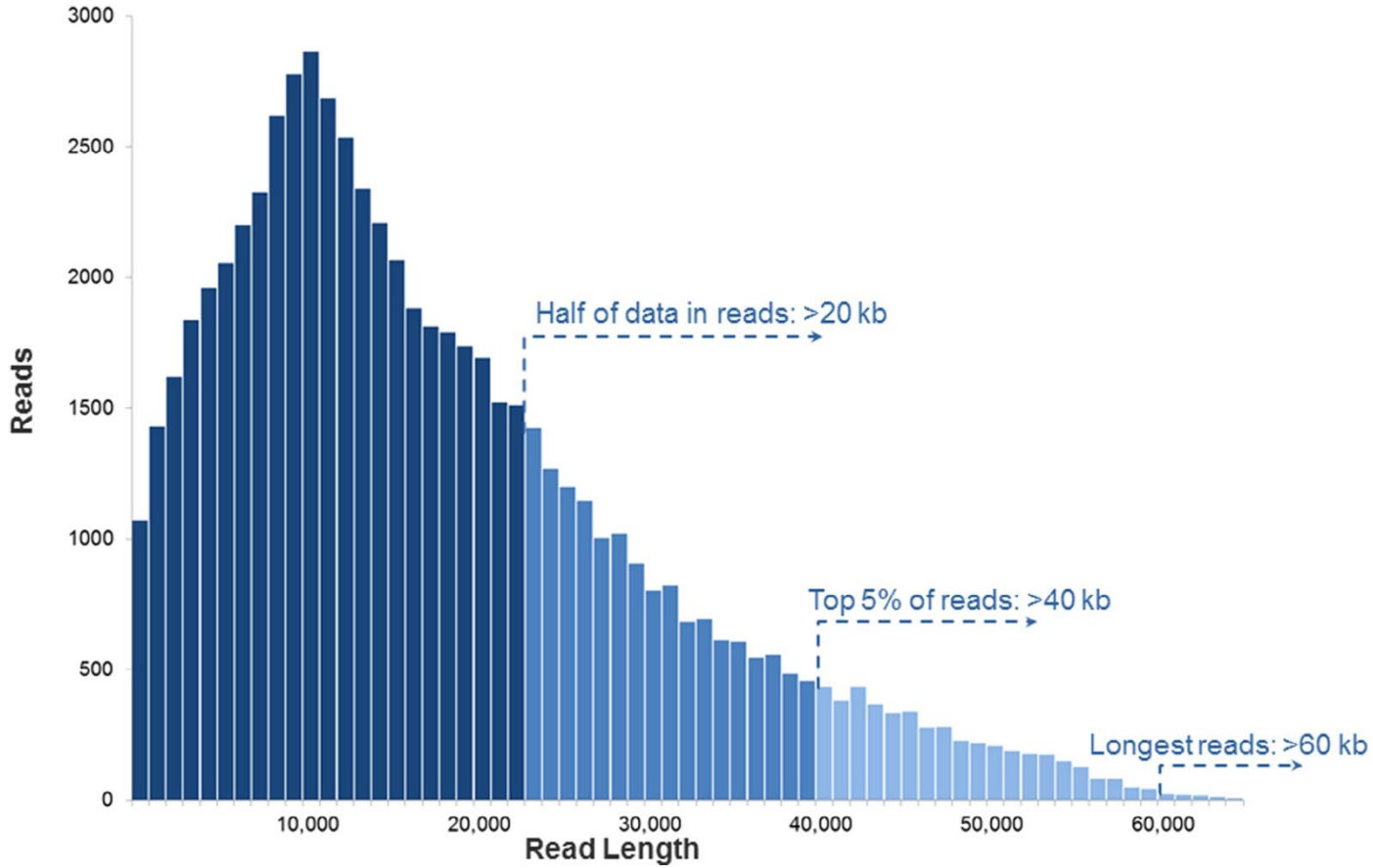
A single SMRT cell: this contains 150000 ZMWs (zero-mode waveguide). A SMRTbell diffuses into a ZMW.
Approx 35000 -75000 ZMWs produce a read in a run lasting 0,5-4h resulting in 0,5-1Gb.



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	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

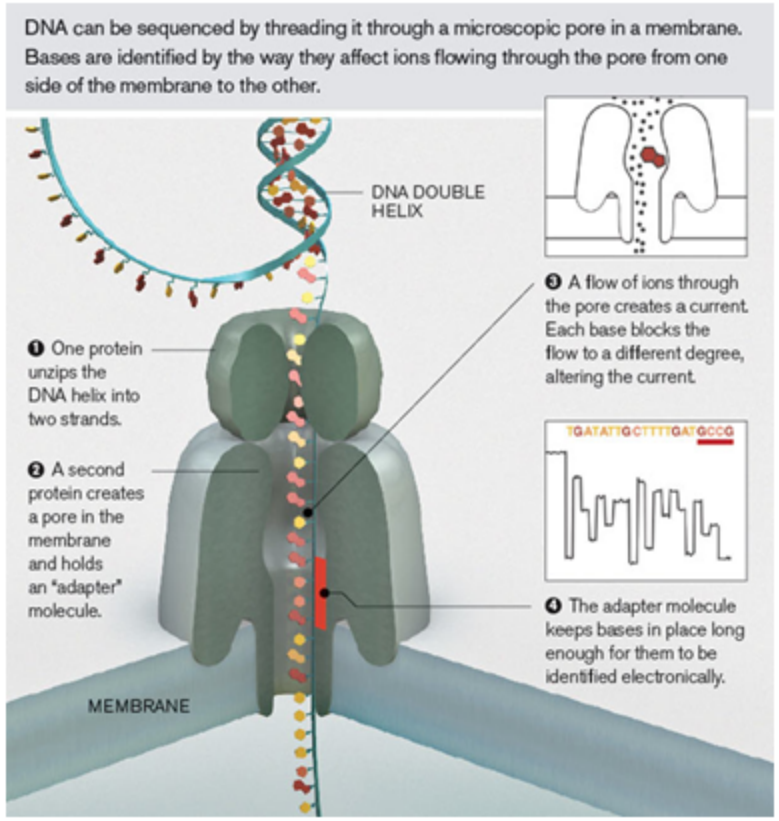
Produced highly-contiguous assemblies of bacterial and eukaryotic genomes

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

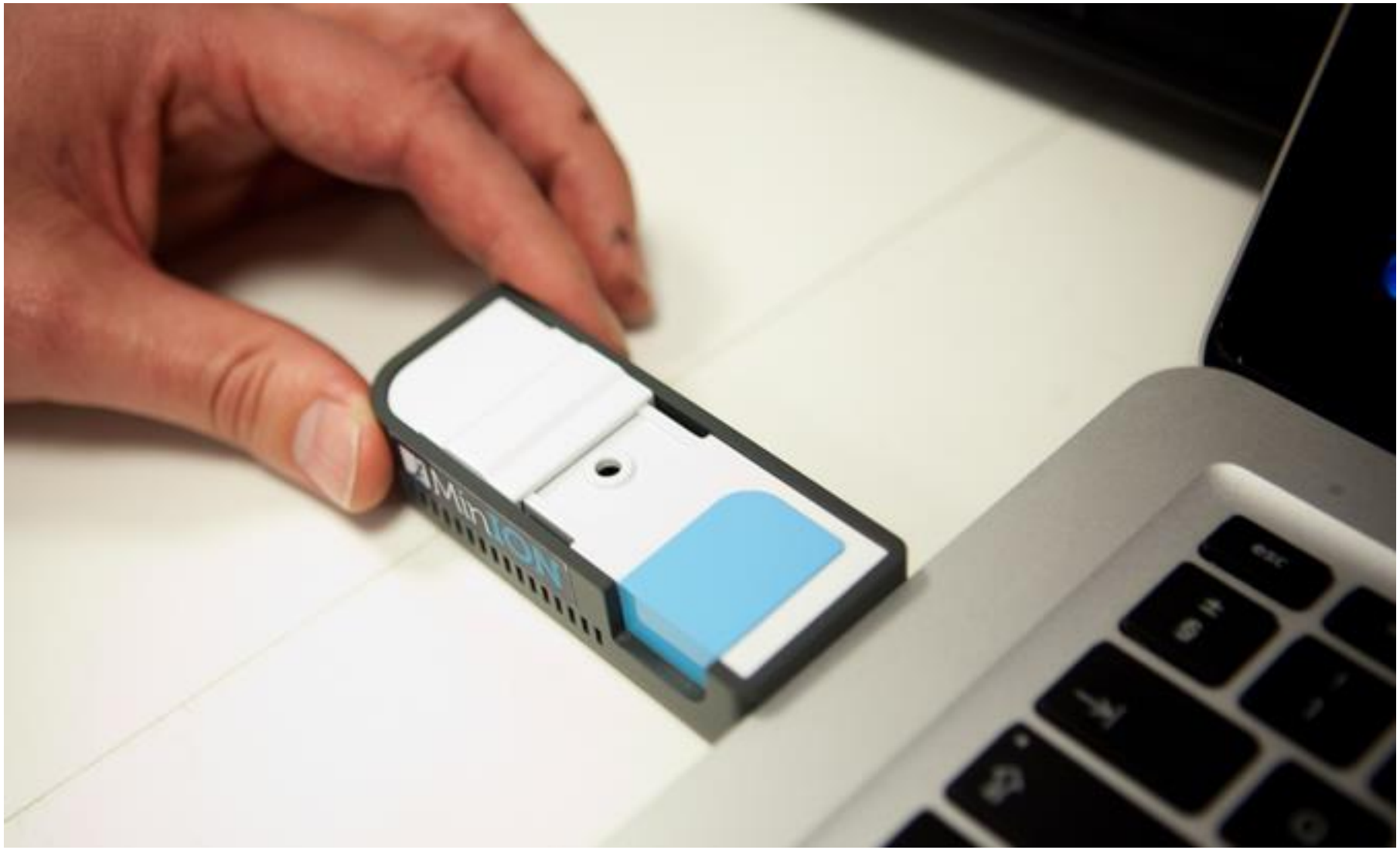


Strand sequencing' is a technique that passes intact DNA polymers through a protein nanopore, sequencing in real time as the DNA translocates the pore.

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



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

Flow cell name	Flongle	MinION Flow Cell	PromethION Flow Cell
Number of channels	126	512	2,675
Theoretical maximum output*	2.8 Gbases	50 Gbases	290 Gbases

Device name	MinION	MinION Mk1C	GridION	PromethION 2 Solo	PromethION 2	PromethION 24	PromethION 48
Flow cell compatibility	Flongle, MinION			PromethION			
Number of flow cells that can be run	1	1	5	2	2	24	48

Fig. 2 The flow cells and devices for nanopore sequencing. The Flongle (**a**) consists of two parts, a reusable adapter, and a single-use flow cell. It has the same footprint as the MinION Flow Cell (**b**) meaning both can be run on the MinION (**d**), MinION Mk1C (**e**), or GridION (**f**) devices. Any combination of Flongle or MinION can be run on the GridION device. The PromethION Flow Cell (**c**) is compatible with all PromethION devices (**g**–**j**). With capacity for different numbers of flow cells, total device yields vary in line with the number of flow cells they can run. Where multiple flow cells can be run, all are individually controllable, meaning no requirement exists to run all flow cells at once and as a result samples can be run on demand. *Theoretical maximum output when flow cell or device is run 72 h (16 h for Flongle) at 420 bases/second. For devices, this is when all flow cells are run at once and the highest yielding flow cell option is chosen. Outputs may vary according to library type, run conditions, etc.

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.

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	8.2×10^7	2–4 h	0.1	[15,25]
454 (Roche) GS FLX+	2nd	700	1	1×10^6	23 h	8.57	[14,17,27]
Illumina HiSeq 2500 (High Output)	2nd	2×125	0.1	8×10^9 (paired)	7–60 h	0.03	[9,16,26]
Illumina HiSeq 2500 (Rapid Run)	2nd	2×250	0.1	1.2×10^9 (paired)	1–6 days	0.04	[9,16,26]
SOLiD 5500×1	2nd	2×60	5	8×10^8	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]

Comparison of various high-performing sequencing instruments

Pervez et al., BioMed Research International 2022

Manufacturer	Read length	Data output	Max. run time (hours)	Chemistry	Key applications**
Illumina (NovaSeq 6000)	300 PE	6 Tb (6000 Gb)	44	Sequencing by synthesis	SS-WGS and TGS, TGEP, 16sMGS, WES, SCP, LS-WGS, CA, MS, MGP, CFS, LBA
Thermo Fisher Scientific Ion Torrent (Ion GeneStudio S5 Prime)	600 SE	50 Gb	12	Sequencing by synthesis	WGS, WES, TGS
GenapSys (16 chips)	150 SE	2 Gb	24	Sequencing by synthesis	TS, SS-WGS, GEV, 16S rRNA sequencing, sRNA sequencing, TSCAS
QIAGEN (GeneReader)	100 SE	Not available	Not available	Sequencing by synthesis	Cancer research and identifying mutations
BGI/Complete Genomics	400 SE	6 Tb (6000 Gb)	40	DNA nanoball	Small and large WGS, WES and TGS
PacBio (HiFi Reads)	25 Kb	66.5 Gb	30	Real-time sequencing	DN sequencing, FT, identifying ASI, mutations, and EPM
Nanopore (PromethION)	4 Mb	14 Tb (14000 Gb)	72	Real-time sequencing	SV, GS, phasing, DNA and RNA base modifications, FT, and isoform detection

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

Advantages & disadvantages of sequencing platforms

Pervez et al., BioMed Research International 2022

Sequencing generation	Advantages	Disadvantages
First generation	High accuracy Helps in validating findings of NGS	High cost Low throughput
Second generation	High throughput Low cost Have clinical applications Short run time	Short read length Difficult sample preparation PCR amplification Long run time
Third generation	No PCR amplification Require less starting material Longer read lengths Very low cost Low error rate during library preparation	High sequencing error rate 10–15% in the PacBio and 5–20% in the ONT Fresh DNA requires for ensuring quality of ultralong reads Database systems and algorithms/tools are rare for analyzing 3rd and 4th GS data
Fourth generation	Advantages of 3rdGS+ Ultrafast: scan of whole genome in 15 minutes Spatial distribution of the sequencing reads over the sample can be seen	

Advantages & disadvantages for short vs. Long read sequencing

	Advantages	Limitations
Short-read sequencing	· Higher sequence fidelity · Cheap · Can sequence fragmented DNA	· Not able to resolve structural variants, phasing alleles or distinguish highly homologous genomic regions · Unable to provide coverage of some repetitive regions
Long-read sequencing	· Able to sequence genetic regions that are difficult to characterize with short-read seq due to repeat sequences · Able to resolve structural rearrangements or homologous regions · Able to read through an entire RNA transcript to determine the specific isoform · Assists *de novo* genome assembly	· Lower per read accuracy · Bioinformatic challenges, caused by coverage biases, high error rates in base allocation, scalability and limited availability of appropriate pipelines

https://www.technologynetworks.com/genomics/articles/an-overview-of-next-generation-sequencing-346532

Advantages 3rd GS over 2nd GS

Pervez et al., BioMed Research International 2022

- Higher throughput
- Detecting haplotype directly
- Longer read lengths
- Better consensus accuracy to identify rare variants
- Whole chromosome phasing
- Small amount of sample are the salient features of the 3rd-generation sequencing which had it useful in clinical diagnostic

2nd GS and 3rd GS

Pervez et al., BioMed Research International 2022

Table 5: An overview of human genome assembly quality metrics between PacBio system, Nanopore, and Illumina [49].

	Nanopore+Illumina	PacBio HiFi sequencing
Contiguity (N50)	32.3 Mb	98.7 Mb
Correctness (quality score)	Q34	Q51
Completeness (genome size)	2.8 Gb	3.1 Gb

Table 6: Overall costs for sequencing a human genome [49].

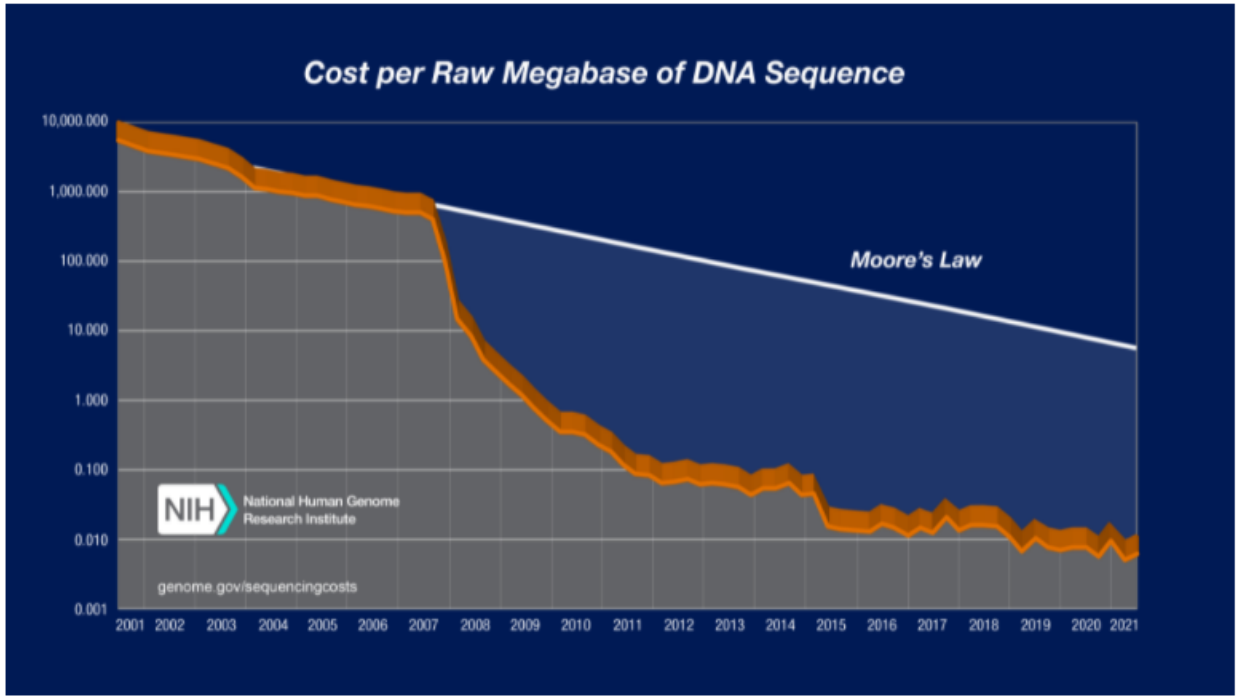
	Nanopore+Illumina (US $)	PacBio HiFi sequencing
Consumables	4800	3800
Compute	5050	3850
Data storage	5200	3900

NGS PLATFORMS, main characteristics

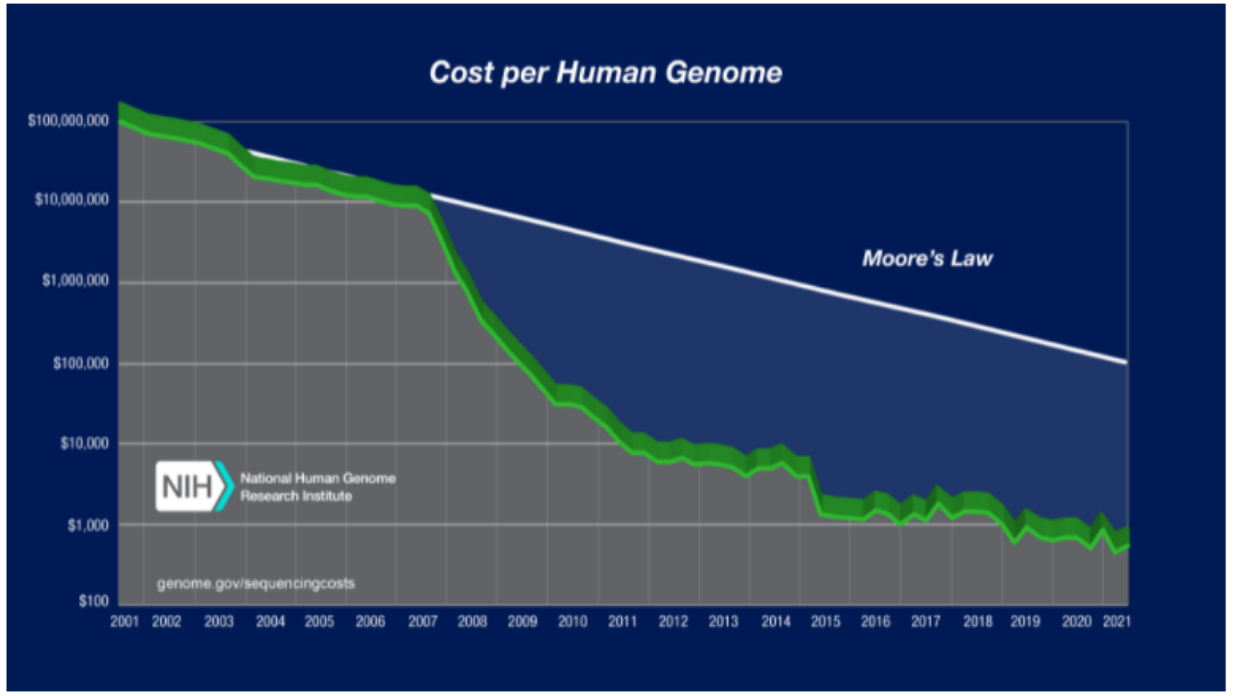
- Numero de bases que secuencia
- Numero lecturas → aplicaciones
- Longitud de las lecturas -→ importante para las aplicaciones ensamblado genomas, de illumina a PacBio

- Error de la base → Corrección con profundidad de lectura
- Formato fichero salida
- Software dedicado, universal fastq

Secuencing cost



Sequencing cost per megabase - 2021

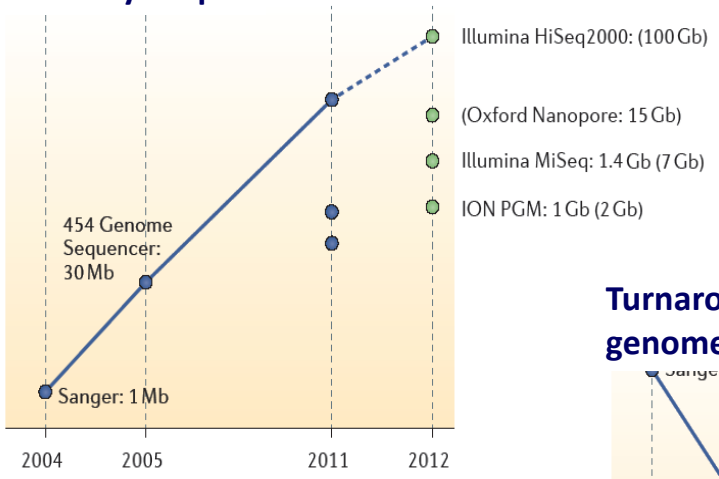


Cost per genome data - 2021

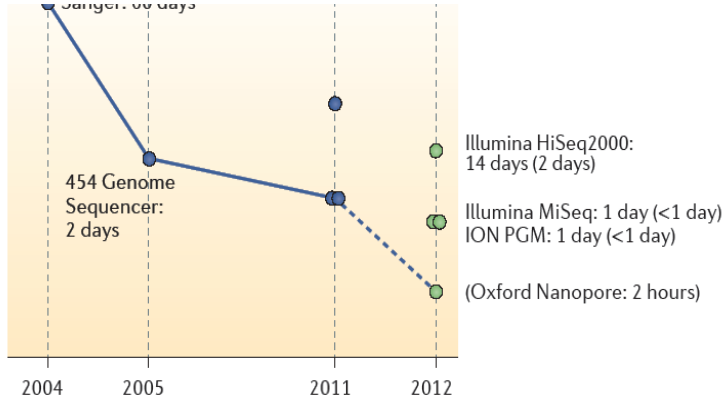
https://www.genome.gov/about-genomics/fact-sheets/DNA-Sequencing-Costs-Data

Sequencing platforms in Microbiology

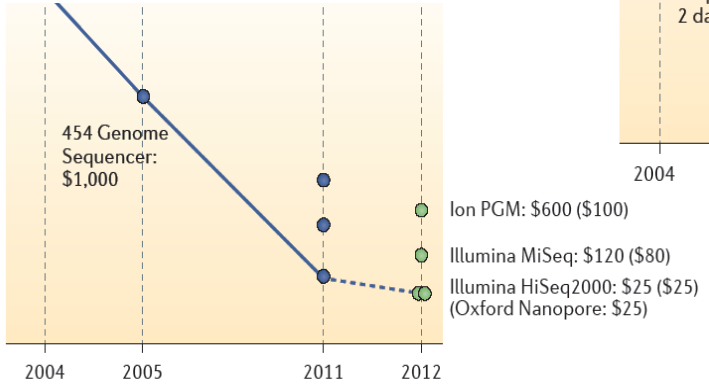
Raw daily output



Turnaround time: bacterial genome



Cost per Mb assembled sequence

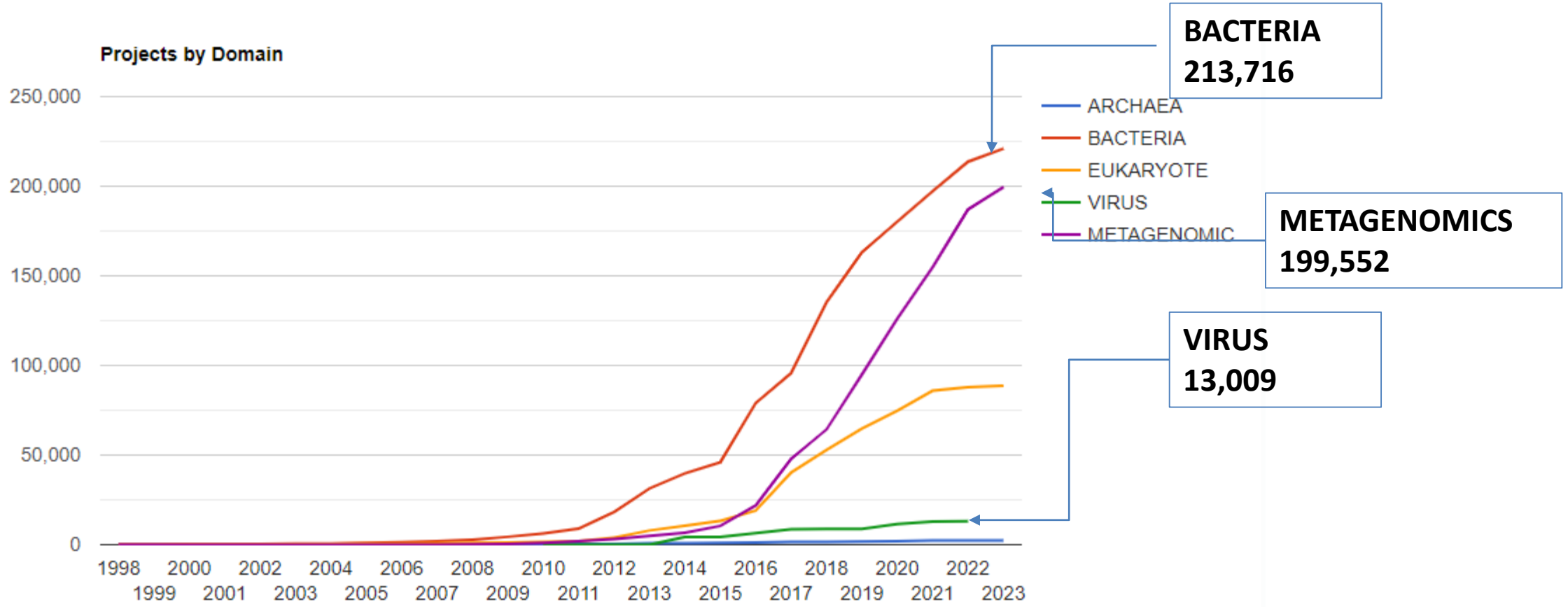


2

Sequencing projects

https://gold.jgi.doe.gov/

GOLD, Genome Online DataBase



APPLICATIONS OF NGS IN VIROLOGY

- Detección de patógenos virales no identificados en la muestra
- Identificación de virus nuevos
- Identificación de virus en muestras tumorales
- Caracterización del Viroma de un organismo
- Secuenciación del genoma viral completo
- Estudio de la Variabilidad genómica (Quasispecies)
- Monitorización de la resistencia a los Antivirales
- Vigilancia epidemiología de las infecciones virales
- Estudio de la Evolución viral
- Control de calidad de las vacunas virales vivas atenuadas

High throughput sequencing in clinical virology, Applications

- Virus discovery: Metagenomics, sequence-independent amplification
- Viral genome reconstruction
- Variants identification & quasispecies

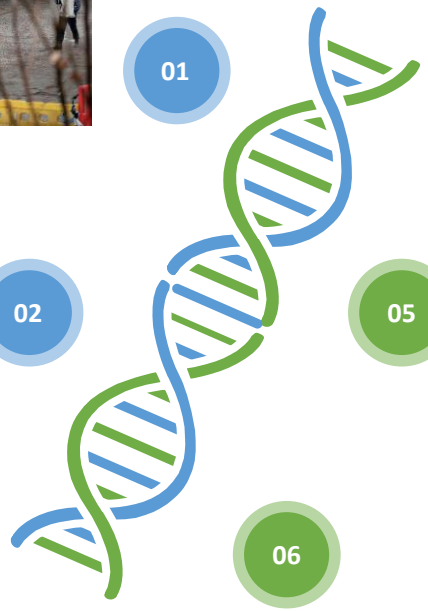
Pathogen discovery: new virus – SARS-CoV-2

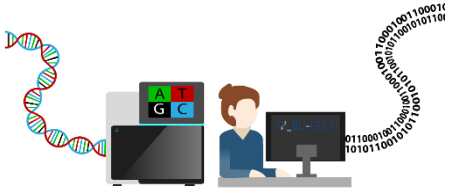
Deep Meta-Transcriptomic Sequencing

bronchoalveolar lavage fluid (BALF)

Meta-transcriptomic library

2x150 MiniSeq 56,565,928 sequences reads



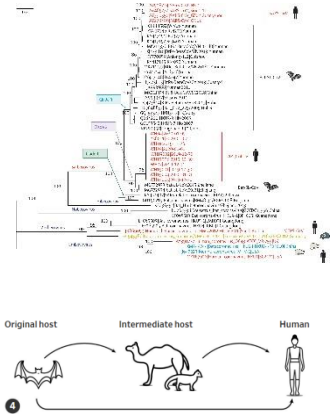


De novo-assembled - Megahit

384,096 Contigs

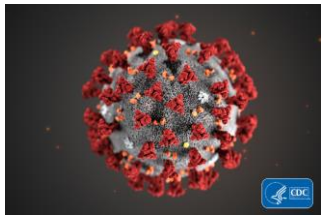
Screened for potential aetiological agents

The longest 30,474 nt



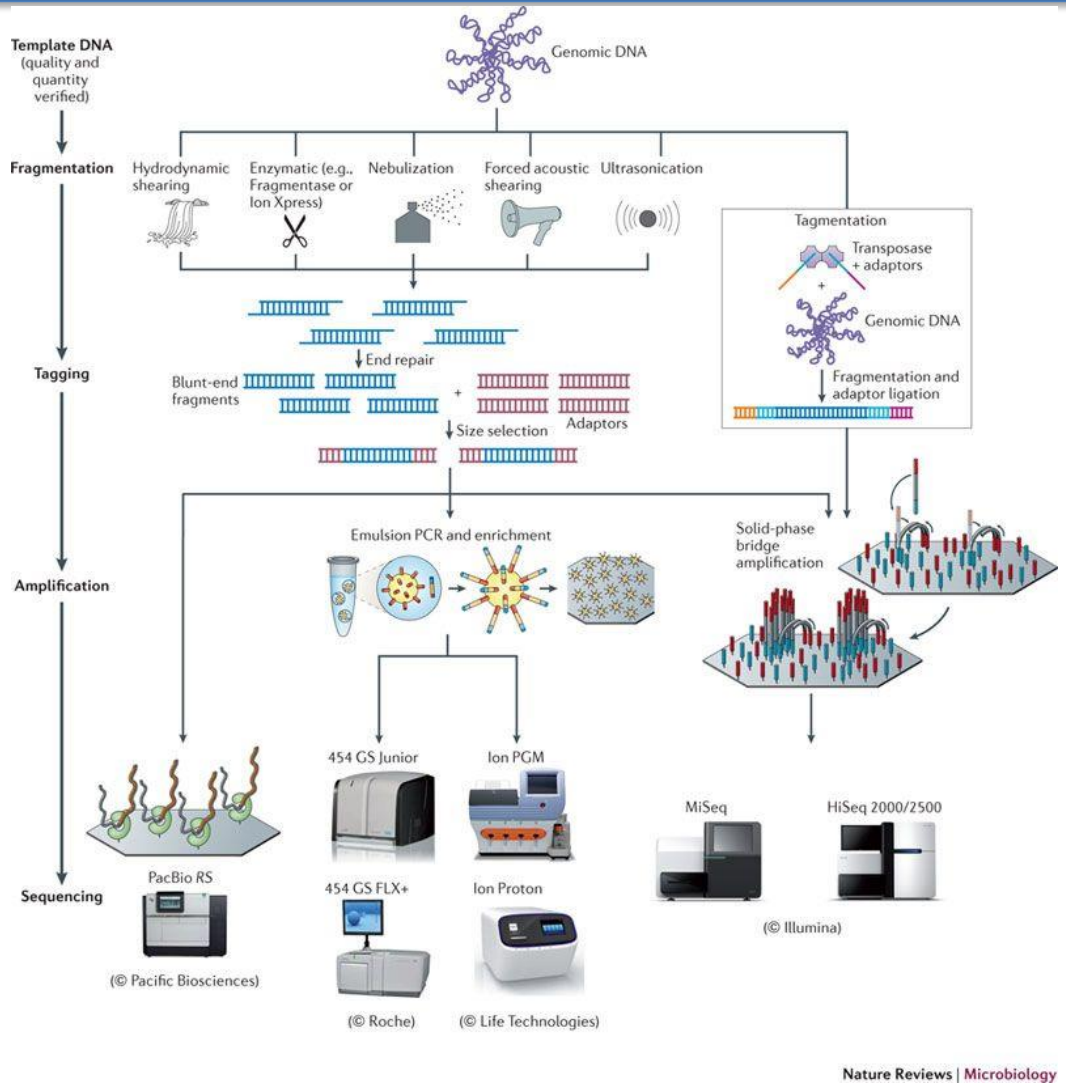
89.1% identity

Closely related to a bat SARS-like coronavirus



Wu et al., Nature 2020

High-throughput sequencing platforms



Loman et al, 2012

Main Steps of Viral Genome Sequencing by NGS or HTS

- Nucleic acid amplification
- Library preparation
- High throughput sequencing platforms
- Data analysis

General features of viral genomes

S. No.	Class	Sequenced genomes	Size (Nt)	Proteins
1	DsDNA	414	4697–335,593	6–240
2	SsDNA	230	1360–10,958	6–11
3	DsRNA	61	3090–29,174	2–13
4	SsRNA (+)	421	2343–31,357	1–11
5	SsRNA (−)	81	8910–25,142	5–6

K. V. Chaitanya, Genome and Genomics,
https://doi.org/10.1007/978-981-15-0702-1_1

Genomes of Single Stranded DNA Viruses and their Mosaicism

Table 1.2 Morphological diversity of single stranded DNA viruses

Host virus taxon	Virion morphology	Genome topology	Genome size
Microviridae	Icosahedral	Circular	4.4–6.1
Inoviridae			
Inovirus	Filamentous		5.8–12.4
Plectrovirus	Rod-shaped		4.5–8.2
Pleolipoviridae	Pleomorphic	Circular	7–10.6
Spiraviridae	Coil-shaped	Circular	24.9
Anelloviridae	Icosahedral	Circular	2–4
Bidnaviridae	Icosahedral	Linear, segmented, 6.5 per segment	
Circoviridae	Icosahedral	Circular	1.7–2.3
Geminiviridae	Icosahedral	Circular, segmented 3 per segment	
Nanoviridae	Icosahedral	Circular, segmented 0.98–1.1per segment	
Parvoviridae	Icosahedral	Linear	4–6.3

Table 1.3 Size of influenza virus segments and the proteins they encode

RNA segment	No. of nucleotides	Encoding protein	No. of amino acids
1	2341	Polymerase PB2	759
2	2341	Polymerase PB1	757
3	2233	Polymerase PA	716
4	1778	Haemagglutinin HA	566
5	1565	Nucleoprotein NP	498
6	1413	Neuraminidase NA	454
7	1027	Matrix protein M1	252
		Matrix protein M2	97
8	890	Non-structural protein NS1	230
		Non-structural protein NS2	121

K. V. Chaitanya, Genome and Genomics,
https://doi.org/10.1007/978-981-15-0702-1_1

Viral Genome Sequencing

NGS

CLINICAL SAMPLE

CULTURE

ENRICHMENT TECHNIQUES

↑↑ HOST & VIRUS reads ↓

NGS

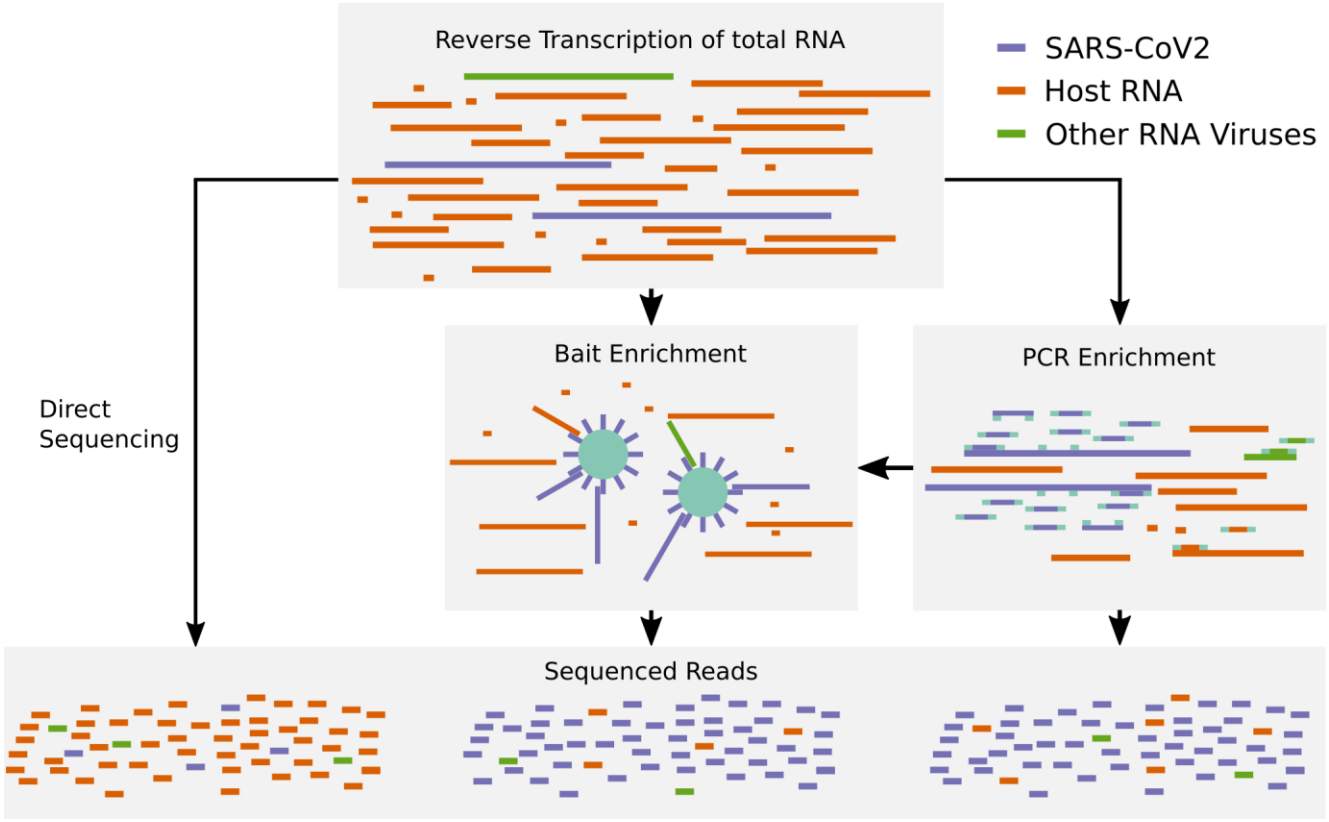
True presence of a VIRUS

↓ Host & **VIRUS** reads ↑↑

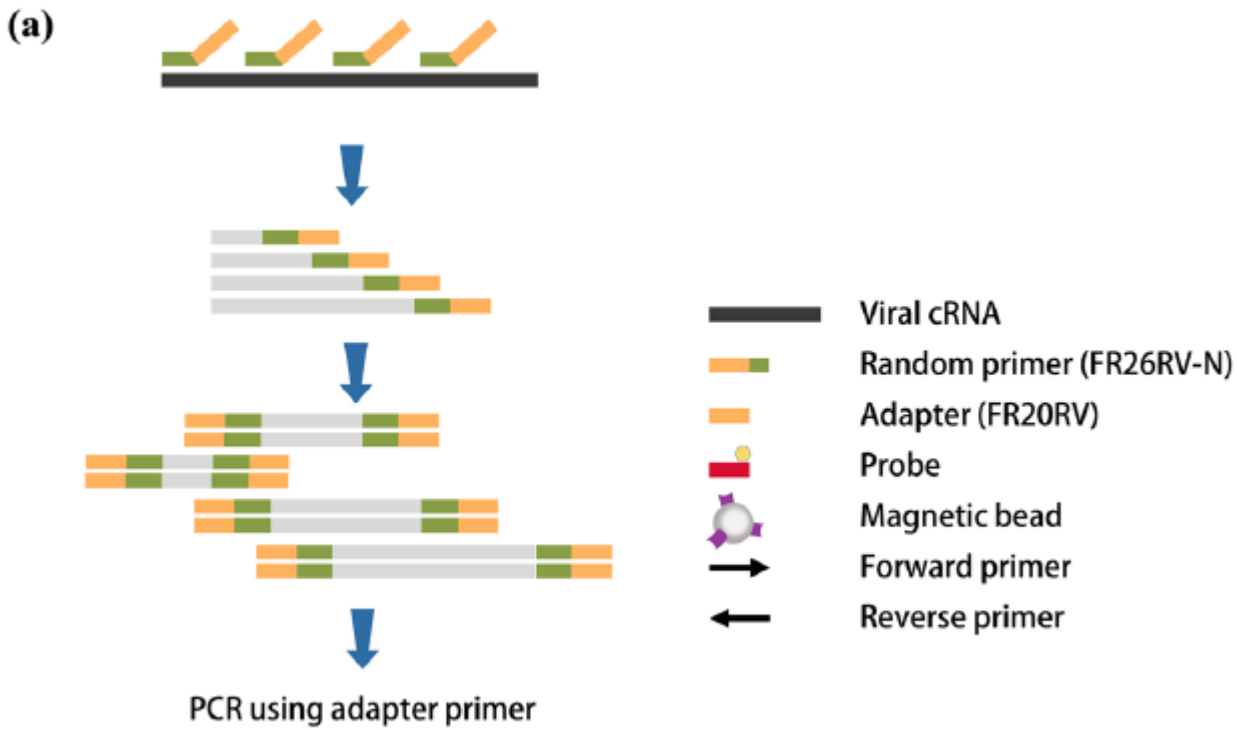
Viral Genome Coverage

NGS needs a cutoff to determine the true presence of a pathogen versus carry-over or contamination between specimens or other non-specific reads.

Enrichment Techniques



Nucleic Acid Extraction: SISPA NGS method



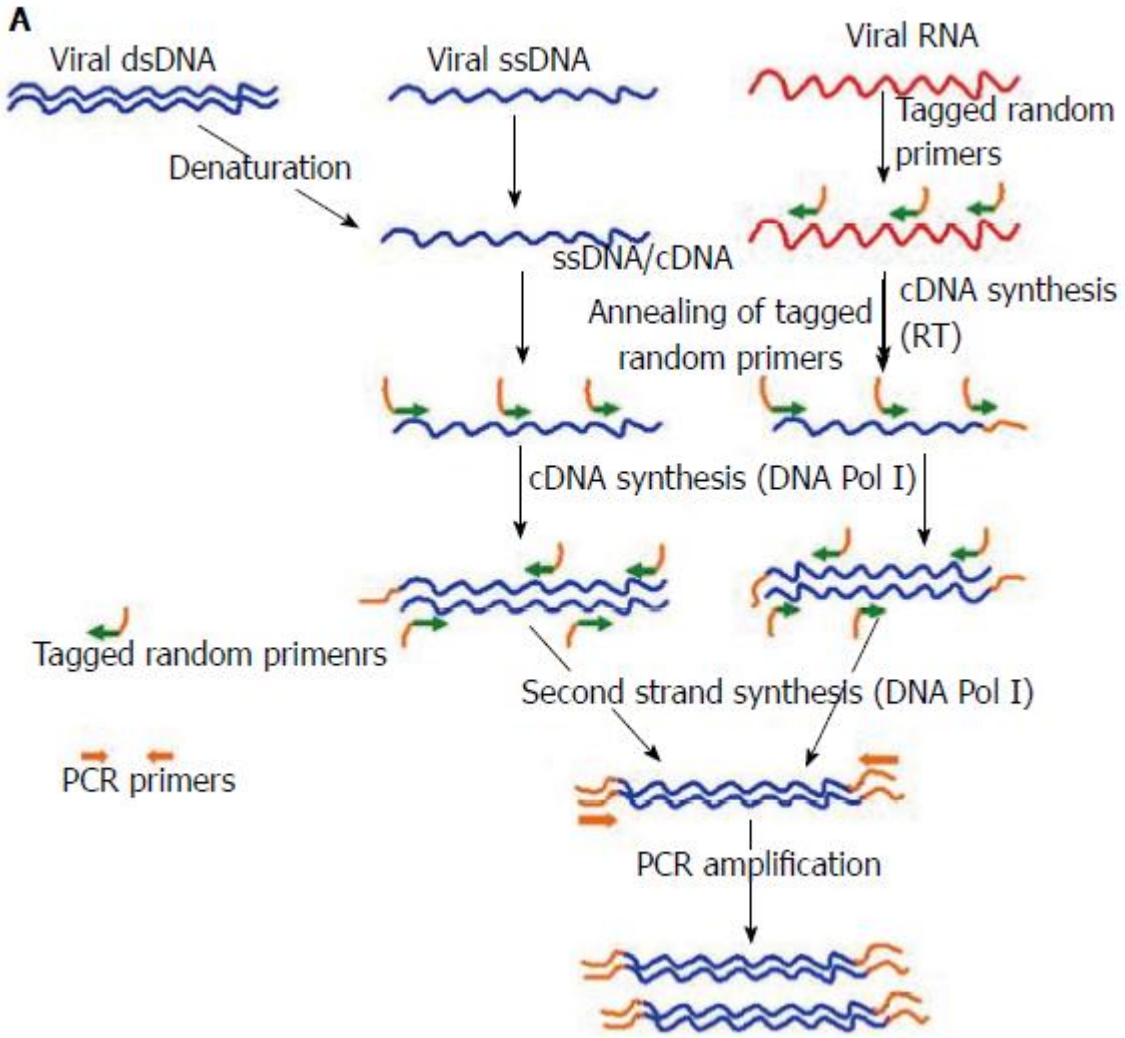
VIRUS DISCOVERY

- Nucleic acid **sequence-independent** amplification approaches
- Next-generation sequencers-based **metagenomic** approaches

RNA was reverse-transcribed using a random primer (FR26RV-N) and then cDNA was amplified using a single primer (FR20RV)

Jin Sun No et al., Scientific Reports (2019) *9:16631* | https://doi.org/10.1038/s41598-019-53043-2

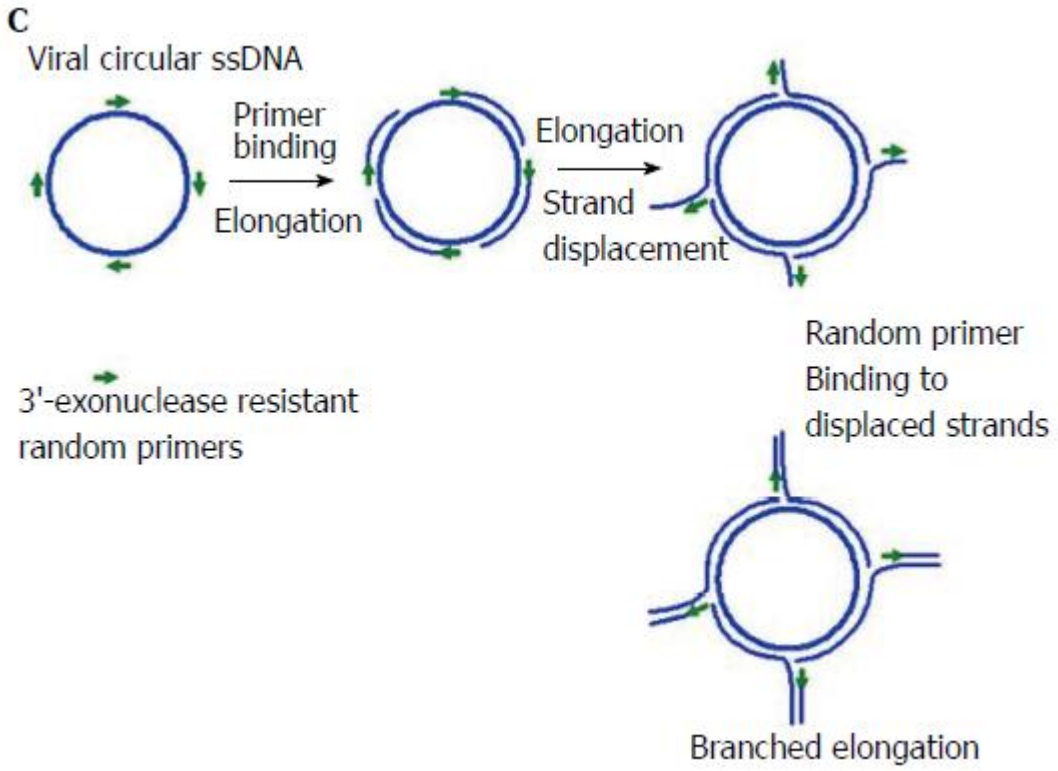
Sequence-independent single-primer amplification



Initially viral RNA and ssDNA is transcribed into complementary DNA (cDNA) using reverse transcriptase (RT) and DNA Pol I respectively, with the help of **tagged-primers having defined sequence at the 5' end while random nucleotides at the 3' end**. Subsequently, second strand synthesis is performed using DNA Pol I (Klenow) to make the cDNA double stranded (dsDNA). Now all the nuceic acids present in the reaction are dsDNA fragments have tagged sequence at their ends. Finally, **anchored dsDNA is amplified with primers annealing to the adapter specific sequences,** PCR product are checked and ready for analysis through cloning-sequencing or direct sequencing through next-generation sequencers (NGS);

Datta et al., World J Virol 2015
DOI: 10.5501/wjv.v4.i3.265

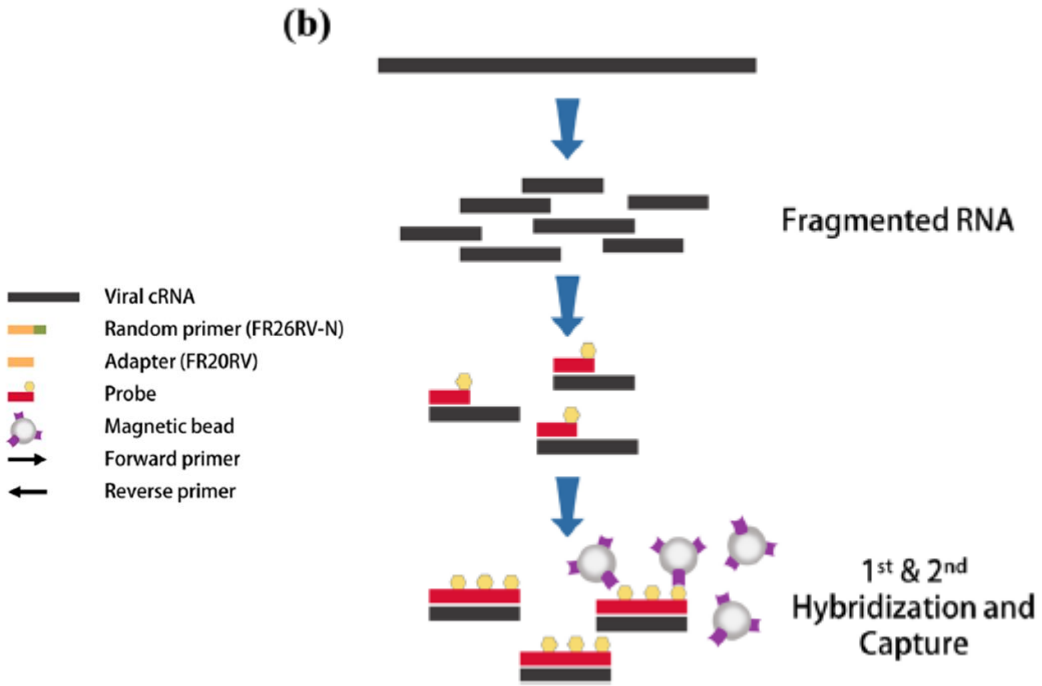
Rolling circle amplification



Amplification of multiply primed single stranded circular viral genomes. 3'-exonuclease resistant **primers randomly bind the genome and are elongated by the Phi29 polymerase**. The growing strand subsequently displaces the preceding strand of the DNA, making the strand available for binding of random primers and further elongation. This cyclic displacement and elongation leads to a highly branched structure of growing DNA, which is linear in topology.
Rolling circle amplification has the capability to specifically enrich the circular ssDNA genomes in an environment of other genetic materials, and could then be characterized by NGS.

Datta et al., World J Virol 2015
DOI: 10.5501/wjv.v4.i3.265

Nucleic Acid Extraction: Target capture NGS

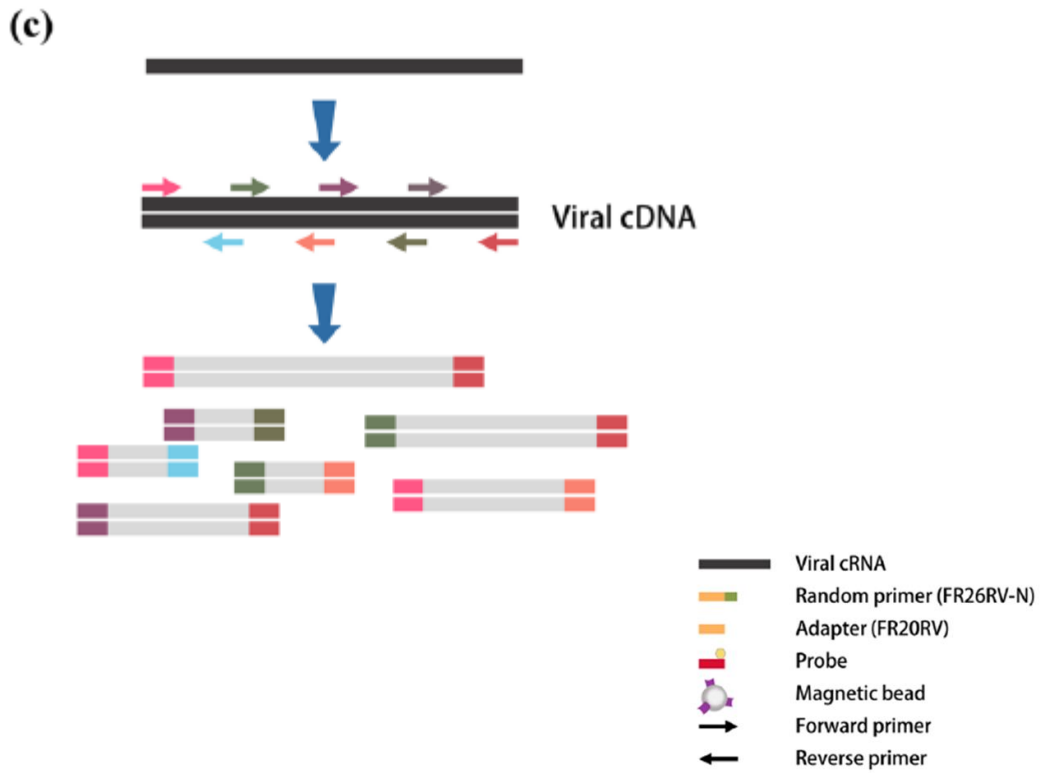


WHOLE VIRAL GENOME RECONSTRUCTION

Target capture NGS captures viral genomes by using **virus-specific probes**. RNA samples were fragmented and then cDNA was synthesized using random hexamer. To enrich the target interest, first & second hybridization and capture were performed using virus-specific probes.

Jin Sun No et al., Scientific Reports (2019) *9:16631* | https://doi.org/10.1038/s41598-019-53043-2

Nucleic Acid Extraction: Amplicon NGS



WHOLE VIRAL GENOME RECONSTRUCTION

CHARACTERIZATION OF INTRA-HOST VARIABILITY

Amplicon NGS was applied to enrich viral genomes. Viral cDNA was amplified using **VIRUS-specific multiple primer mixture**

Jin Sun No et al., Scientific Reports (2019) *9:16631* | https://doi.org/10.1038/s41598-019-53043-2

Nucleic Acid Extraction COMPARISON, Hantaan orthohantavirus genome sequencing

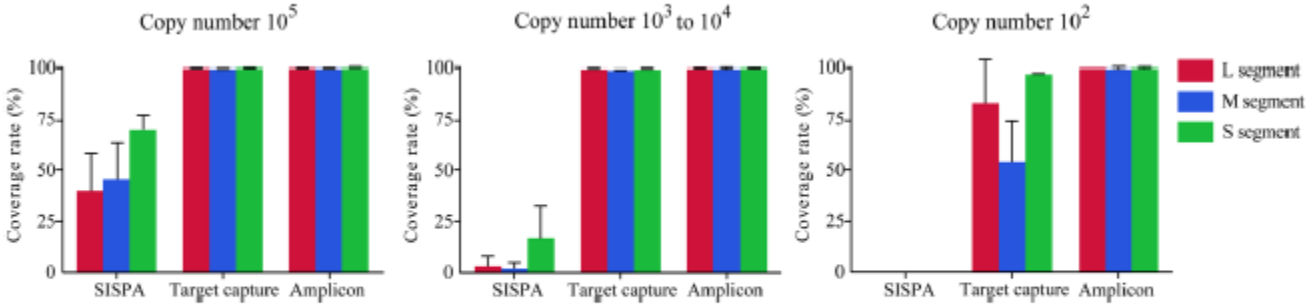


Figure 3. Coverage rate of consensus sequences for Hantaan orthohantavirus (HTNV) by viral copy number. The percentage coverage rate of consensus sequences by viral copy number among the three next-generation sequencing methods. Coverage rate was calculated by matching the consensus sequences with the sequence of HTNV 76–118 strain (GenBank accession number, NC_005222, NC_005219, NC_005218).

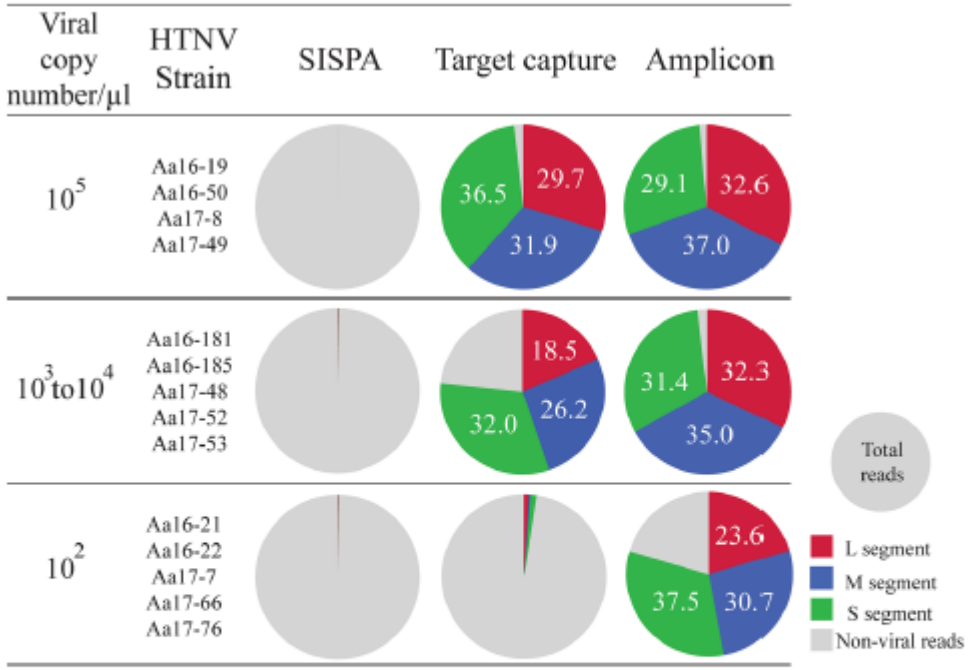
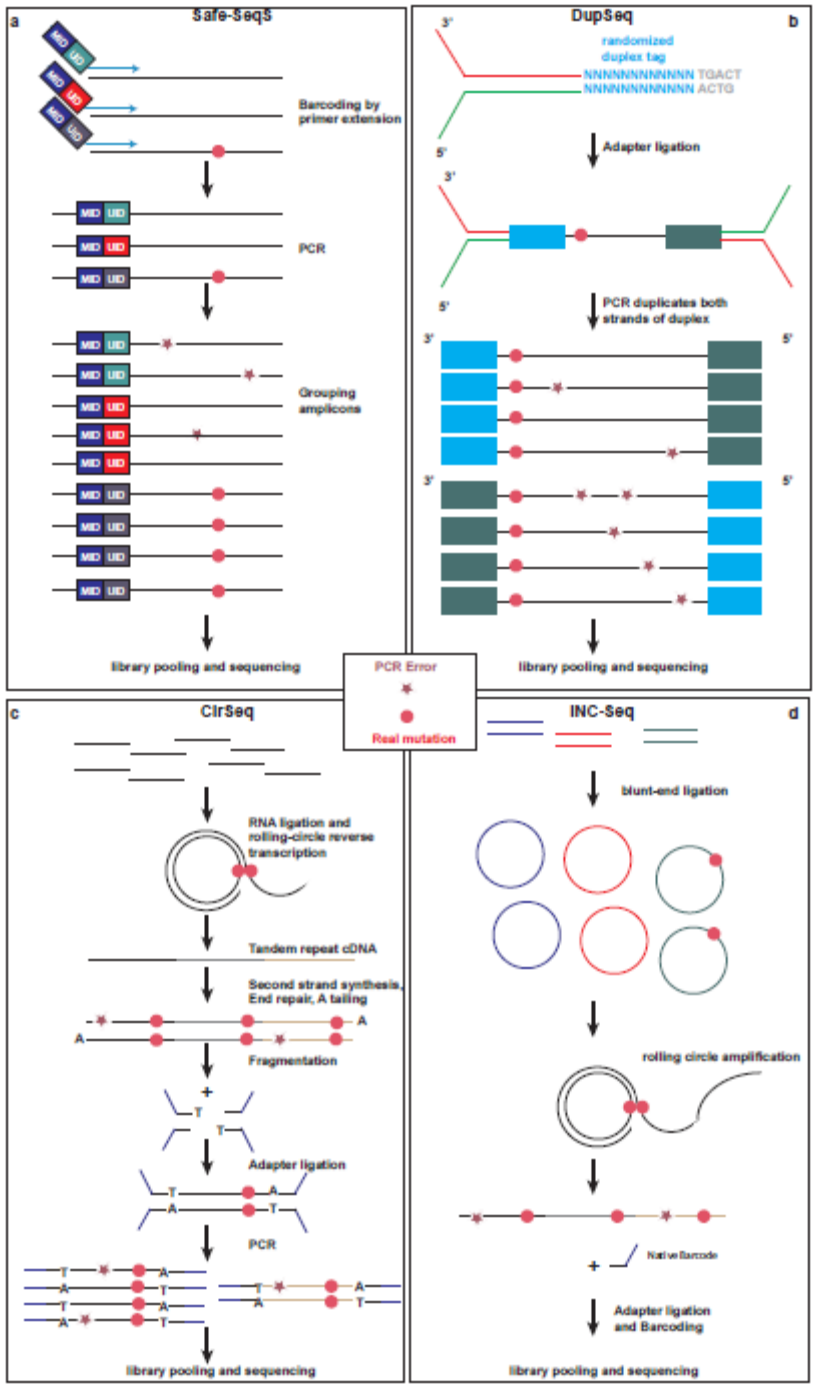


Figure 4. The composition of Hantaan orthohantavirus (HTNV) genomic reads in the total reads generated by three next-generation sequencing methods. The composition of viral reads mapped to HTNV was exhibited in the total number of reads. The total reads were produced by performing SISPA, target capture, and amplicon NGS. These reads were mapped to HTNV 76–118 tripartite genomes (GenBank accession number: L segment, NC_005222; M segment, NC_005219; S segment, NC_005218). A circle represents for total reads obtained by SISPA, target capture, and amplicon NGS methods. Red, Blue, and Green colors indicate the composition of viral reads for HTNV L, M and S segments, respectively, over the total reads. Gray color indicates non-viral reads in the total reads. The HTNV reads were shown as a percentage (%) evaluated by the ratio of viral reads over the total reads.

Jin Sun No et al., Scientific Reports (2019) *9:16631* | https://doi.org/10.1038/s41598-019-53043-2

Nucleic Acid Extraction
low frequency variant identification, quasispecies



Lu et al., Virus Research 2020

Schema main steps of clinical virus Discovery by NGS

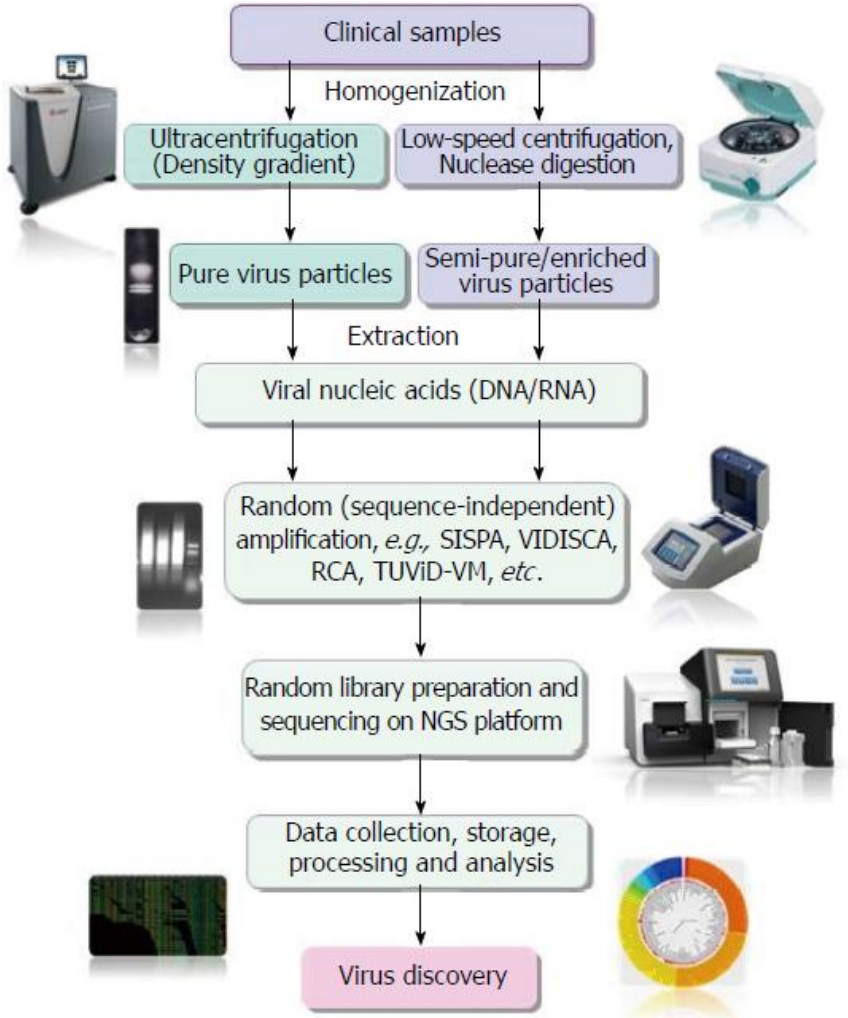
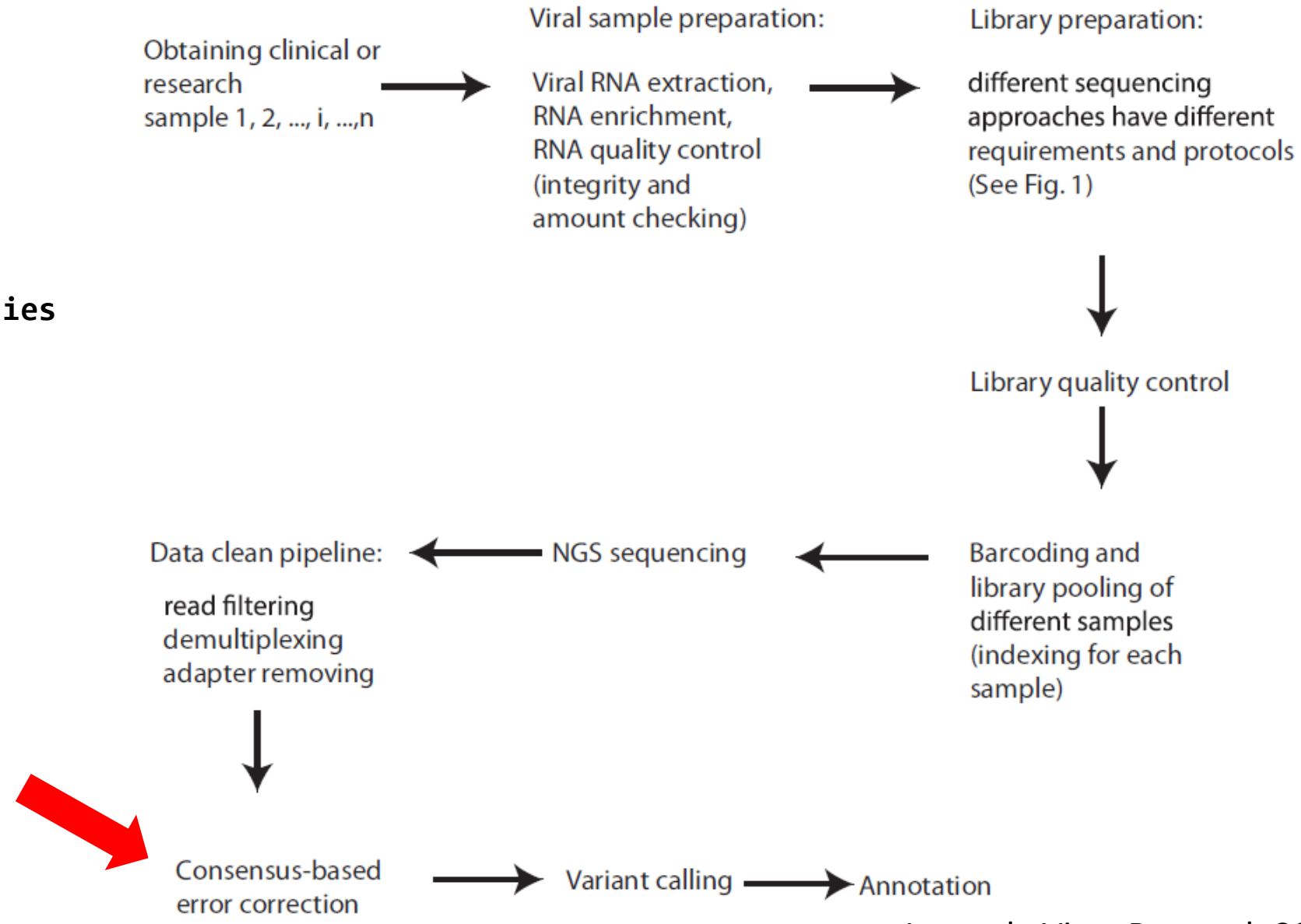


Figure 1 Diagrammatic representation of main steps of clinical virus discovery by next-generation sequencer based technologies.

Datta et al., World J Virol 2015
DOI: 10.5501/wjv.v4.i3.265

Nucleic Acid Extraction
low frequency variant identification, quasispecies



Lu et al., Virus Research 2020

LIBRARY PREPARATION, strategies

SECUENCIACIÓN GENOMA, EXOMA, TRANSCRIPTOMA

1. Sin amplificación
2. Amplificación con PCR
3. Sondas captura

- Tamaño de fragmento
- Longitud de la lectura
- Single o Paired-end
- Número de bases por muestra
- Profundidad de cobertura x

SECUENCIACIÓN GENOMAS

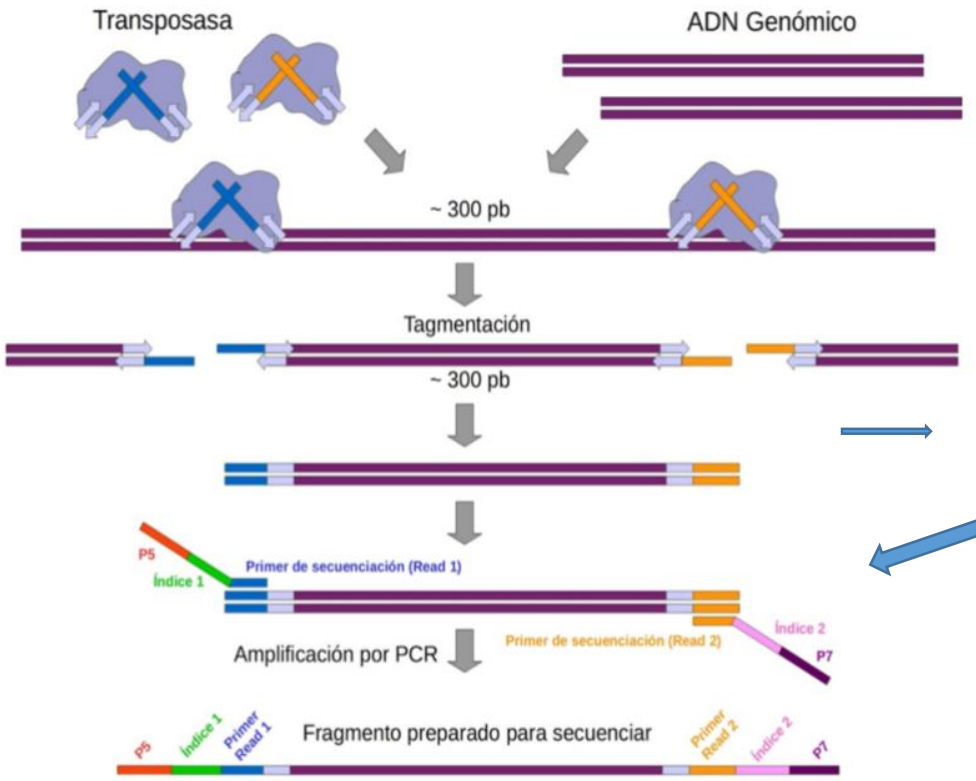
1. Metagenómica

IDENTIFICACIÓN MICROORGANISMOS

1. Metataxonomía o Targeted Metagenomics

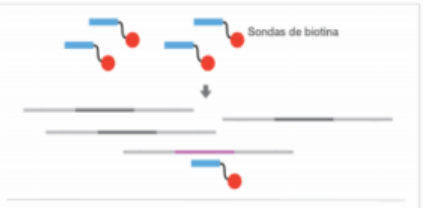
PREPARACIÓN LIBRERÍA

ENZIMÁTICA FÍSICA



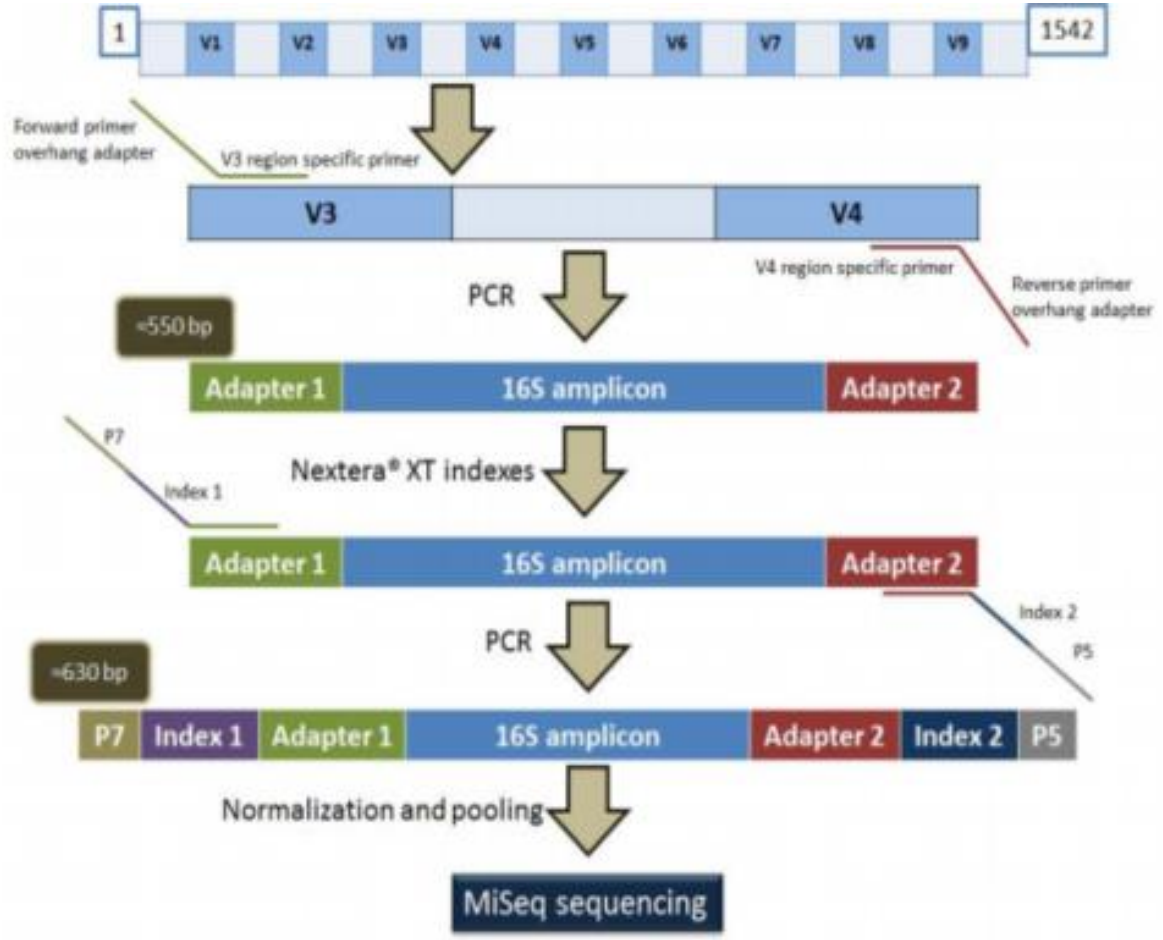
RNA -> cDNA

ENRIQUECIMIENTO:
PCR
CAPTURA SONDAS



Guia Práctica Genómica https://www.uv.es/varnau/GM_Cap%C3%ADtulo_2.pdf

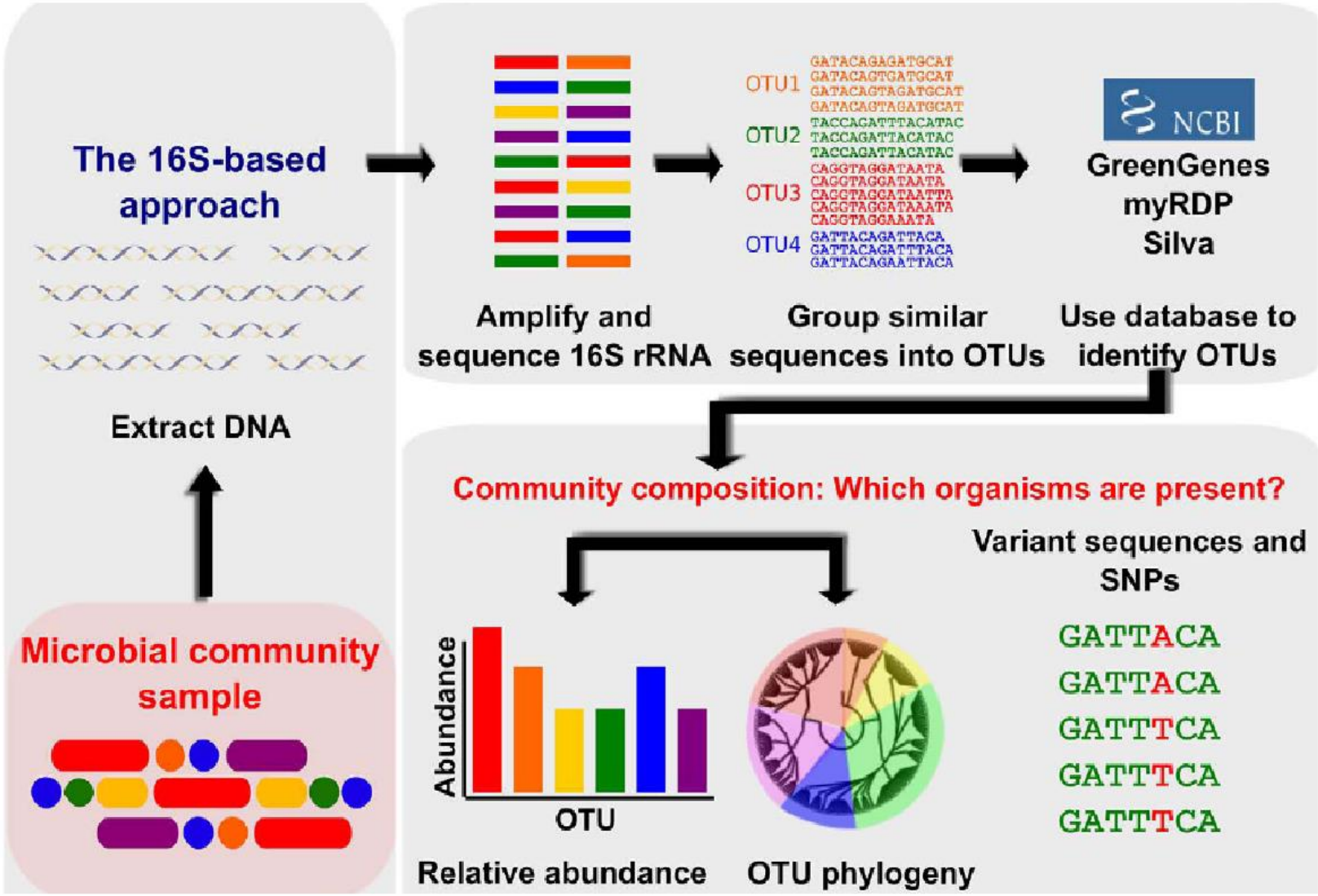
PREPARACIÓN LIBRERÍA, rRNA 16S, caracterización microbiota



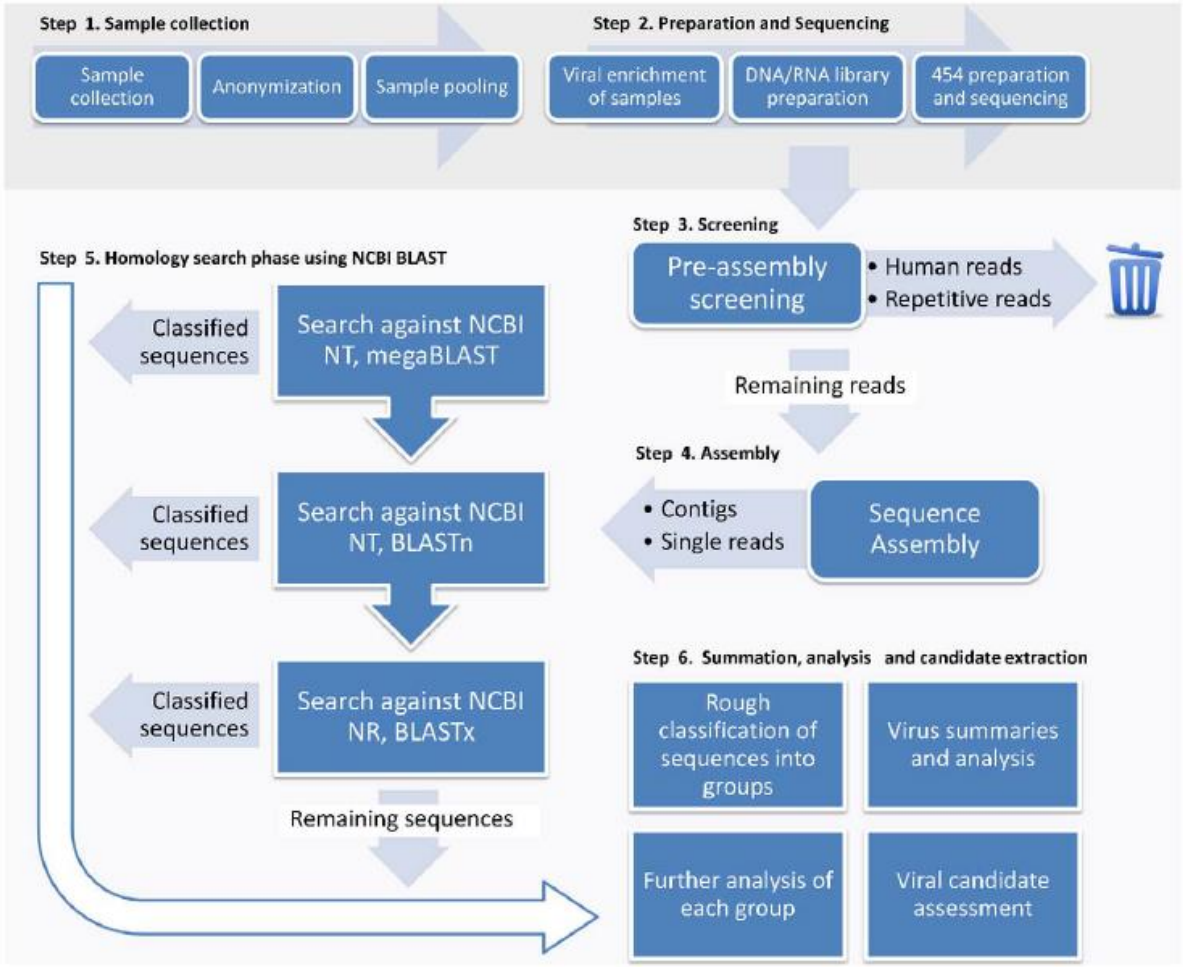
Targeted Metagenomics vs Metagenomics (16S vs Shotgun)

	Metagenetics	**Metagenomics**
Amplified sequence	Marker regions	Whole genome
Computing time	Usually short	Usually long
Taxonomic composition	Yes	Yes
New pathogen detection	No	Yes
Genome coverage information	No	Yes

Targeted Metagenomics

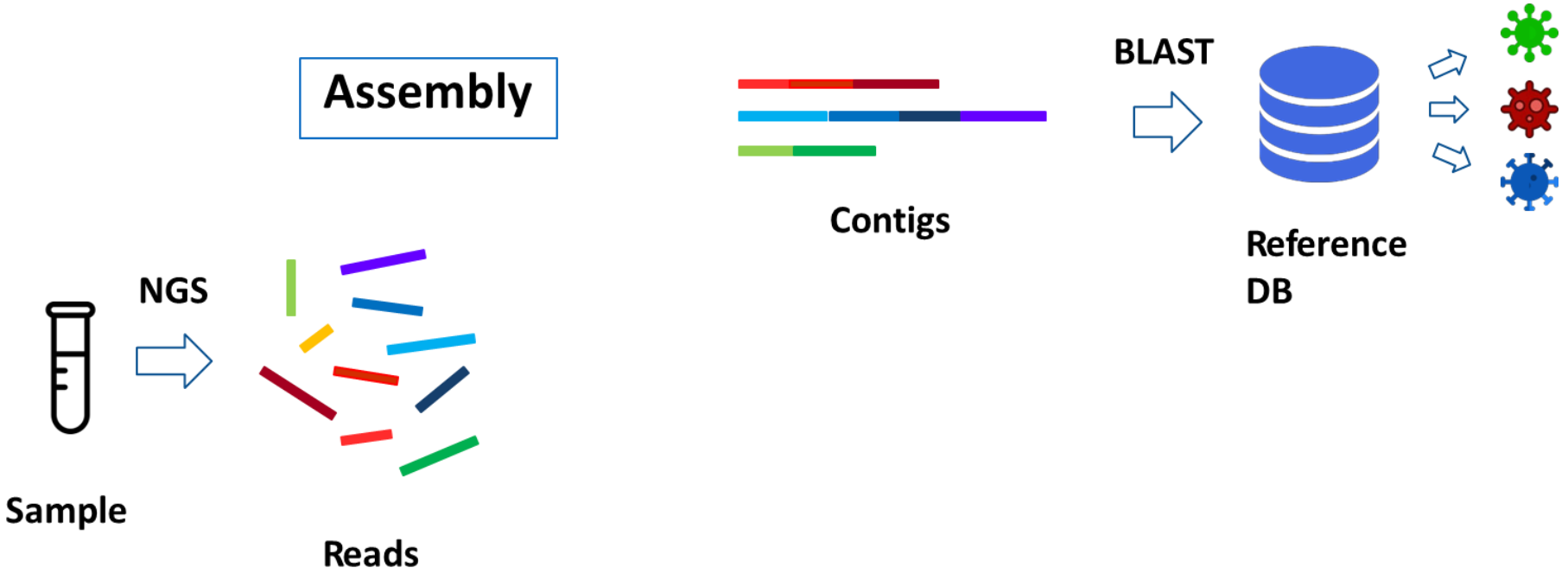


Metagenomics

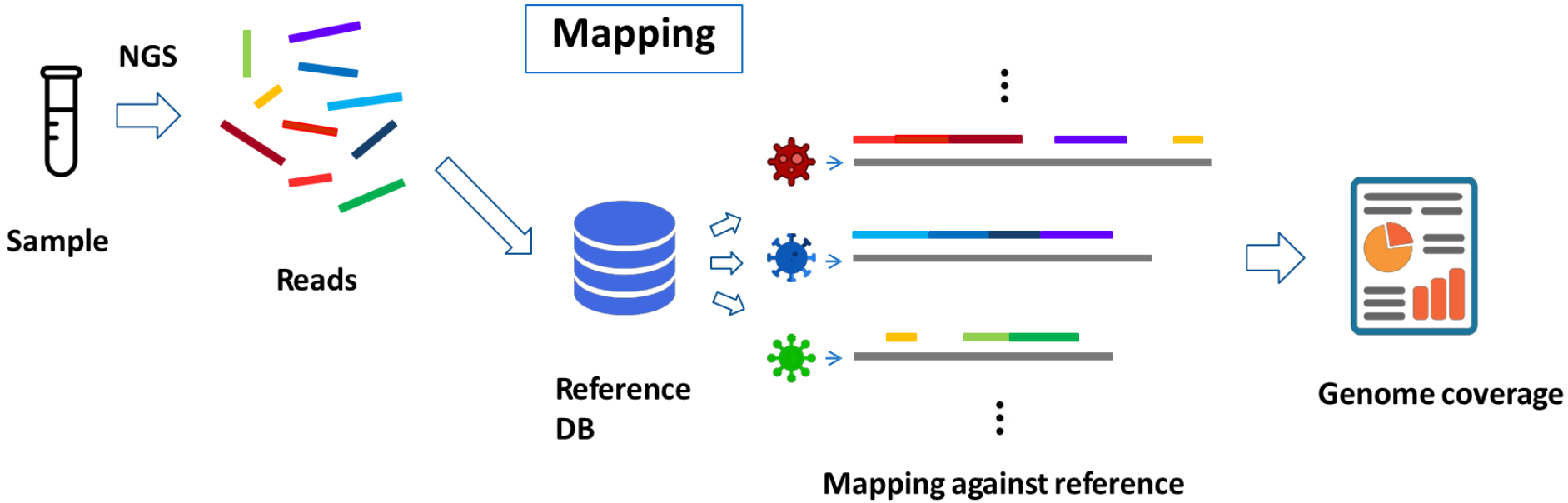


Lysholm et al., Plos One 2012:7,2, e30875

Metagenomic analysis approaches



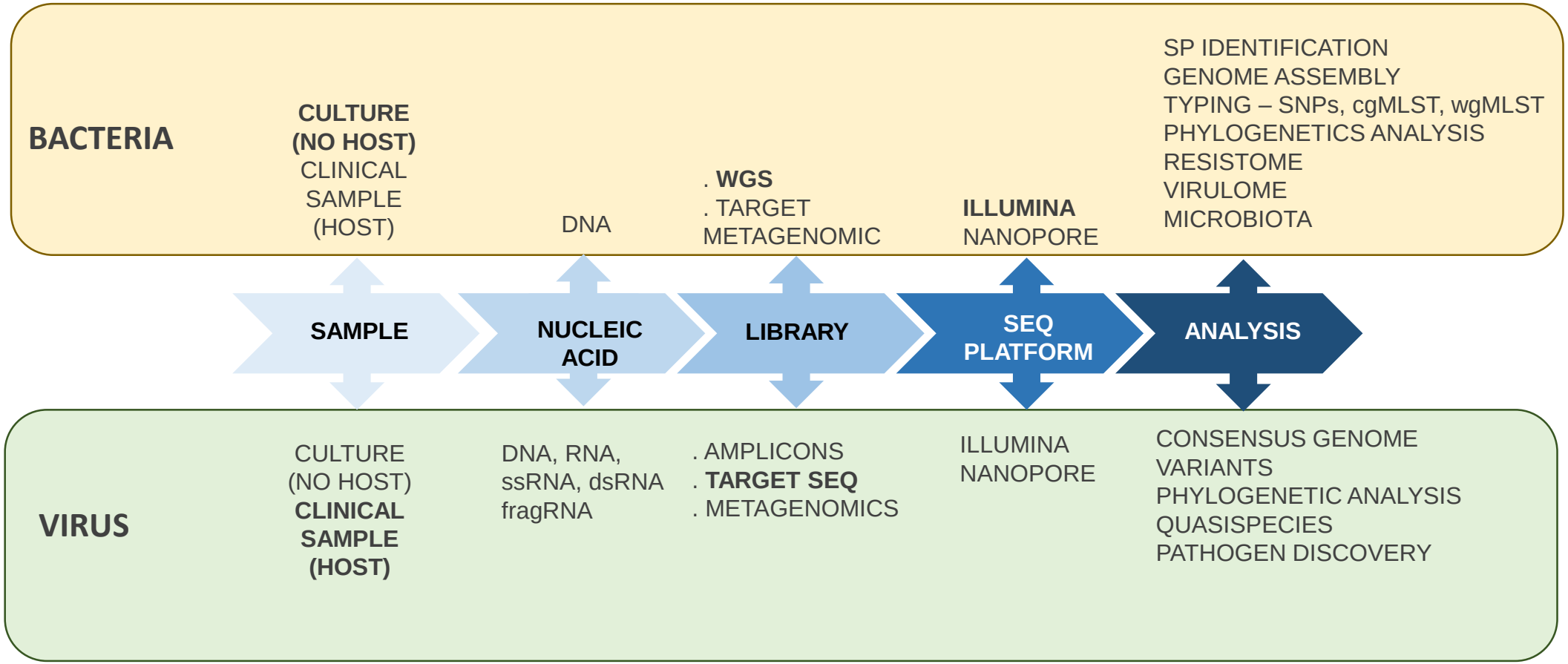
Metagenomic analysis approaches



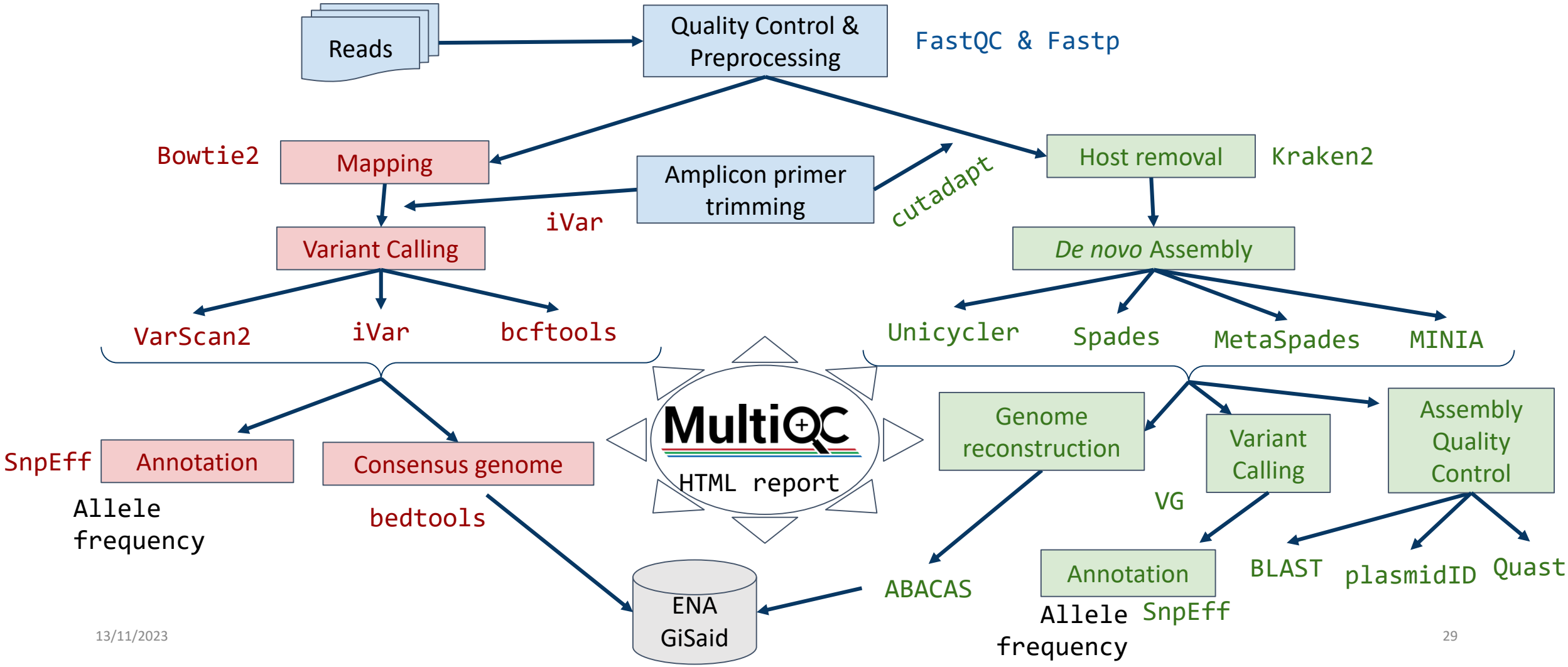
Metataxonomics vs Metagenomics (16S vs Shotgun)

Software	Organism	Genetic portion used		Binning algorithm used			Genome coverage	Novel pathogen discovery
		Genetic markers	Whole Genome	Clustering	Mapping	Assembly		
Mothur	Bacteria	X		X			No	No
QIIME	Bacteria	X		X		X	No	No
MEGAN	Bacteria		X			X	No	No
Platypus	Bacteria		X		X		No	No
SURPI	Virus		X			X	No	Yes
Virus-TAP	Virus		X			X	No	Yes
VIP	Virus		X		X		No	Yes
Pathosphere	Virus, Bacteria, Eukarya		X			X	No	Yes

Bacterial and Viral Genome Sequencing



Viralrecon


Reads
Quality Control & Preprocessing
FastQC & Fastp
Bowtie2
Mapping
Amplicon primer trimming
cutadapt
Host removal
Kraken2
iVar
Variant Calling
De novo Assembly
VarScan2
iVar
bcftools
Unicycler
Spades
MetaSpades
MINIA
MultiQC
HTML report
SnpEff
Annotation
Consensus genome
Genome reconstruction
Variant Calling
Assembly Quality Control
Allele frequency
bedtools
VG
ENA GiSaid
ABACAS
Annotation
BLAST
plasmidID
Quast
Allele frequency
SnpEff

Sequencing terms

Breadth of coverage

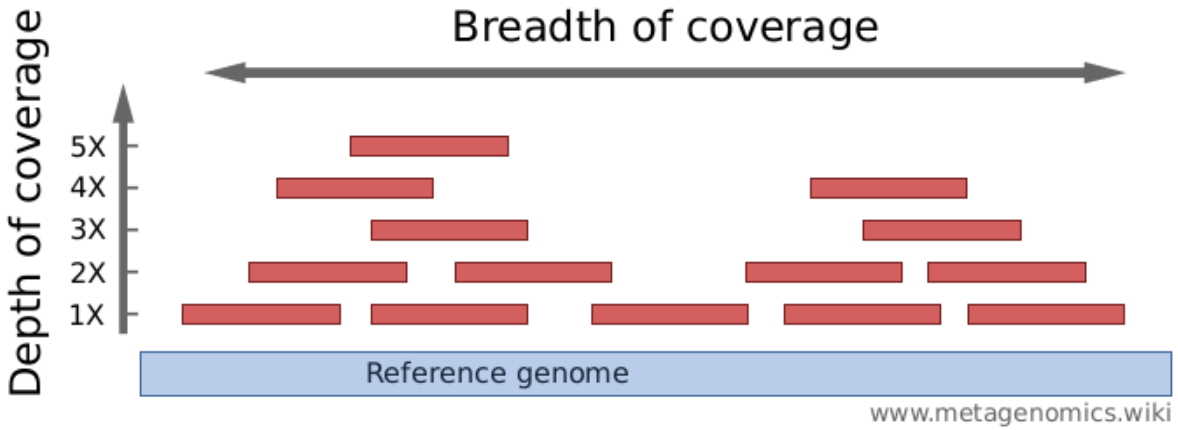
How much of a genome is "covered" by short reads? Are there regions that are not covered, even not by a single read?

Breadth of coverage is the percentage of bases of a reference genome that are covered with a certain depth. For example: 90% of a genome is covered at 1X depth; and still 70% is covered at 5X depth.

Depth of coverage

How strong is a genome "covered" by sequenced fragments (short reads)?

Per-base coverage is the average number of times a base of a genome is sequenced. The coverage depth of a genome is calculated as the number of bases of all short reads that match a genome divided by the length of this genome. It is often expressed as 1X, 2X, 3X,... (1, 2, or, 3 times coverage).



Depth of coverage and genome coverage

Depth of coverage

$$\text{the sequencing coverage} = \frac{\text{the number of total reads} \times \text{the read length}}{\text{the length of target sequence or genome}}$$

Genome coverage

% length sequence genome

Increase number of raw reads

- For the low-frequency variants
- For assembly (also read lenght)

Thanks for your attention!

Questions???