



Sesión 2 - Secuenciación Masiva Aplicaciones

Isabel Cuesta

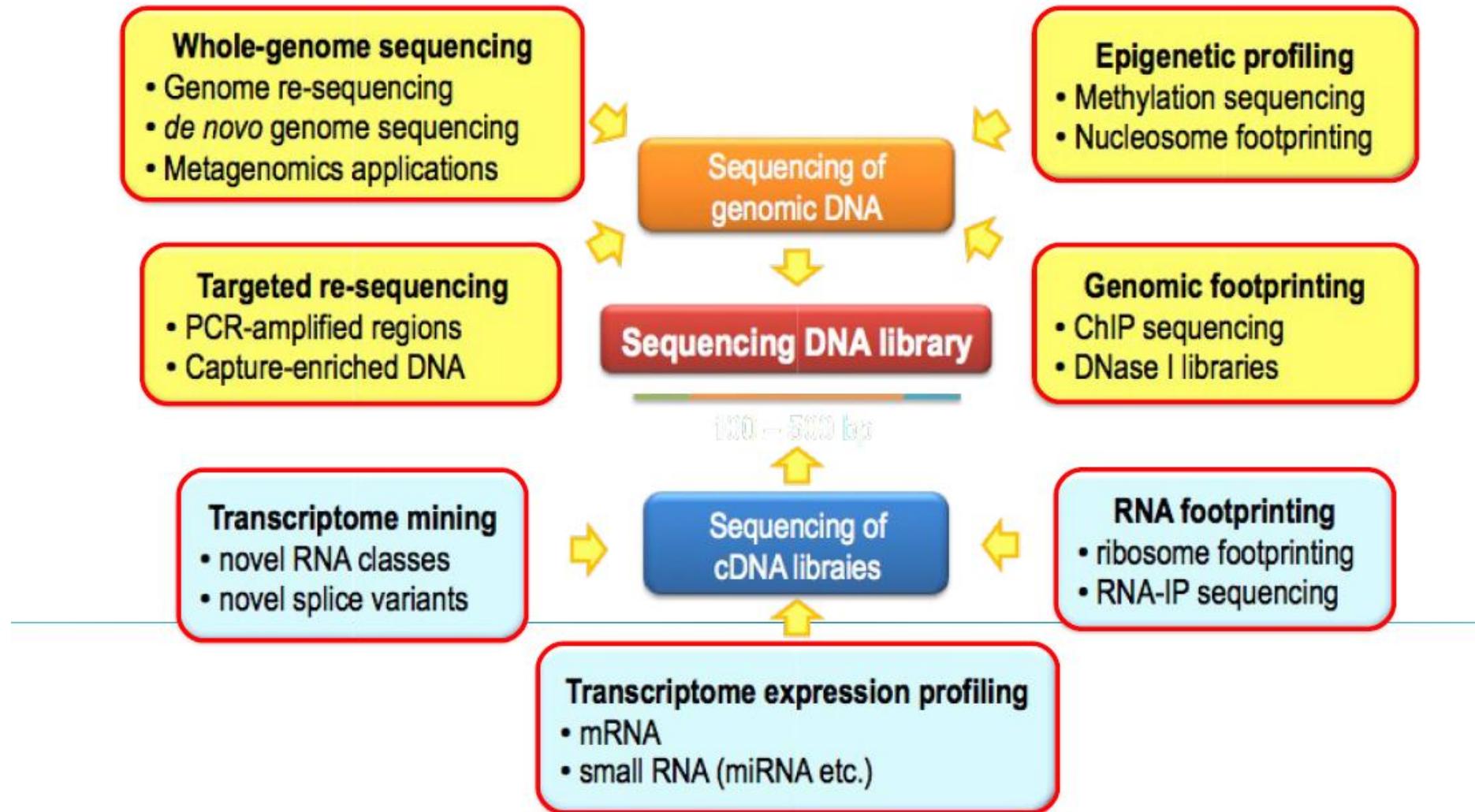
Unidad de Bioinformática (BU-ISCIII)
Unidades Centrales Científico Técnicas – SGSAFI-ISCIII

20-24 Mayo 2024, 11^a Edición
Programa Formación Continua, ISCIII

What has NGS changed?

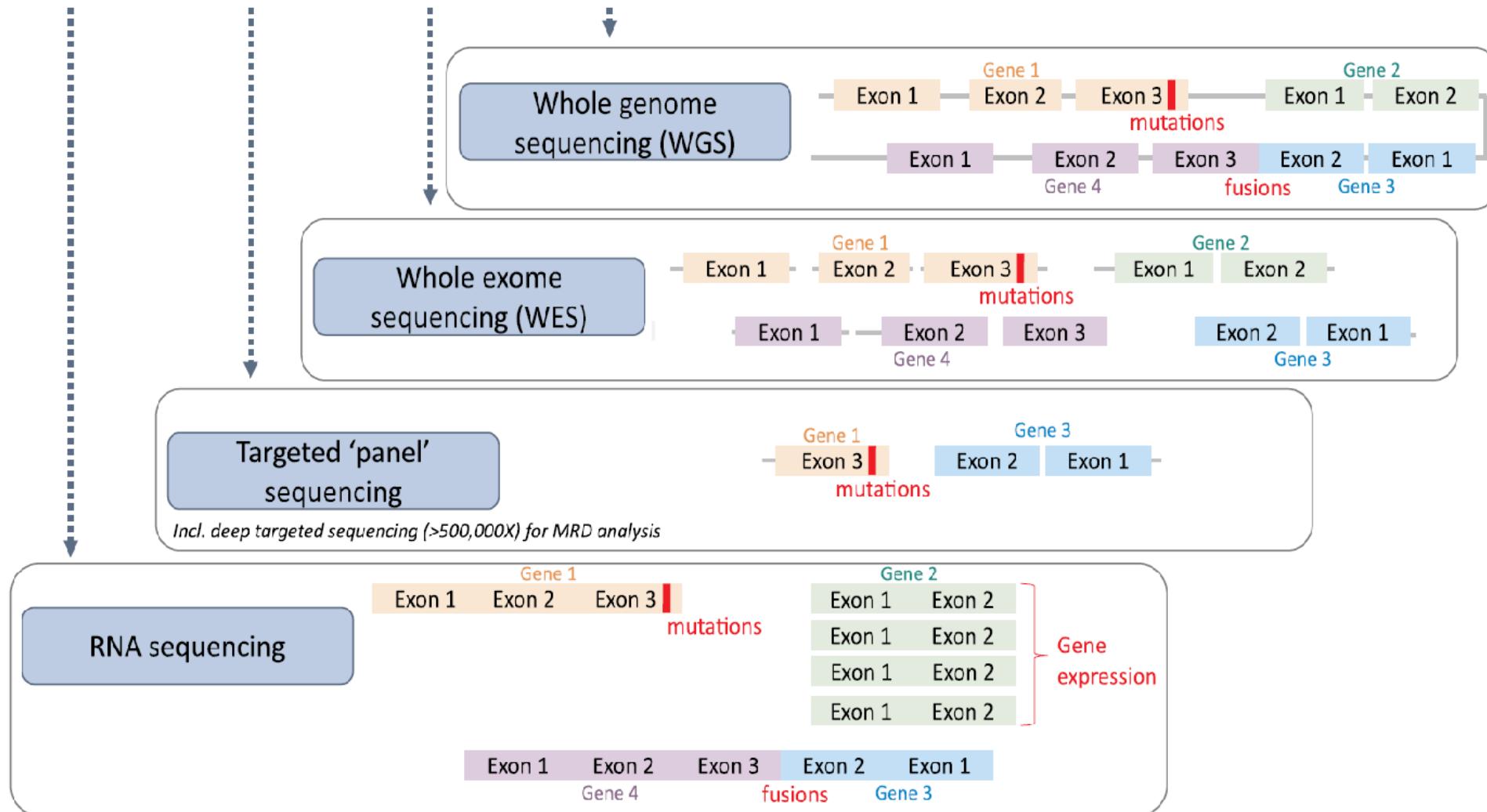
- ✓ **Functional genomics.** Genome-Seq. Epigenetics
- ✓ **Molecular diagnostics.** Complex diseases
- ✓ **Microbial Ecology.** Metagenomics
- ✓ **Molecular Ecology.** Population Genetics
- ✓ **Evolutionary Genomics**
- ✓ **DNA-Protein Interactions.** ChIPSeq
- ✓ **Pharmacogenomics**
- ✓ **Transcriptomics.** RNAseq
- ✓ **Systems Biology**

Aplicaciones de la secuenciación masiva

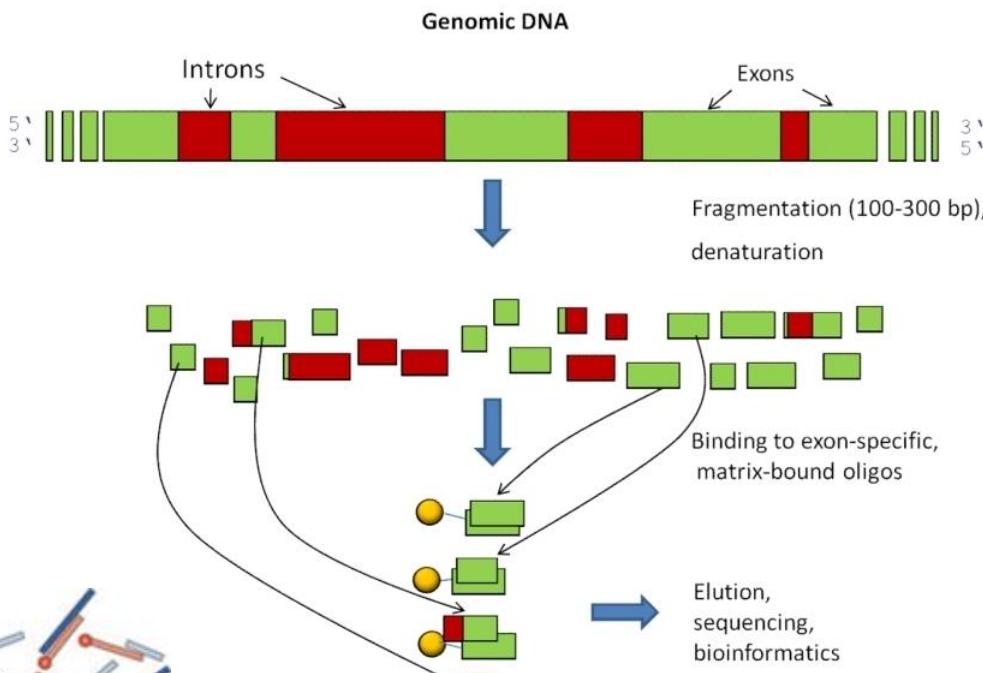
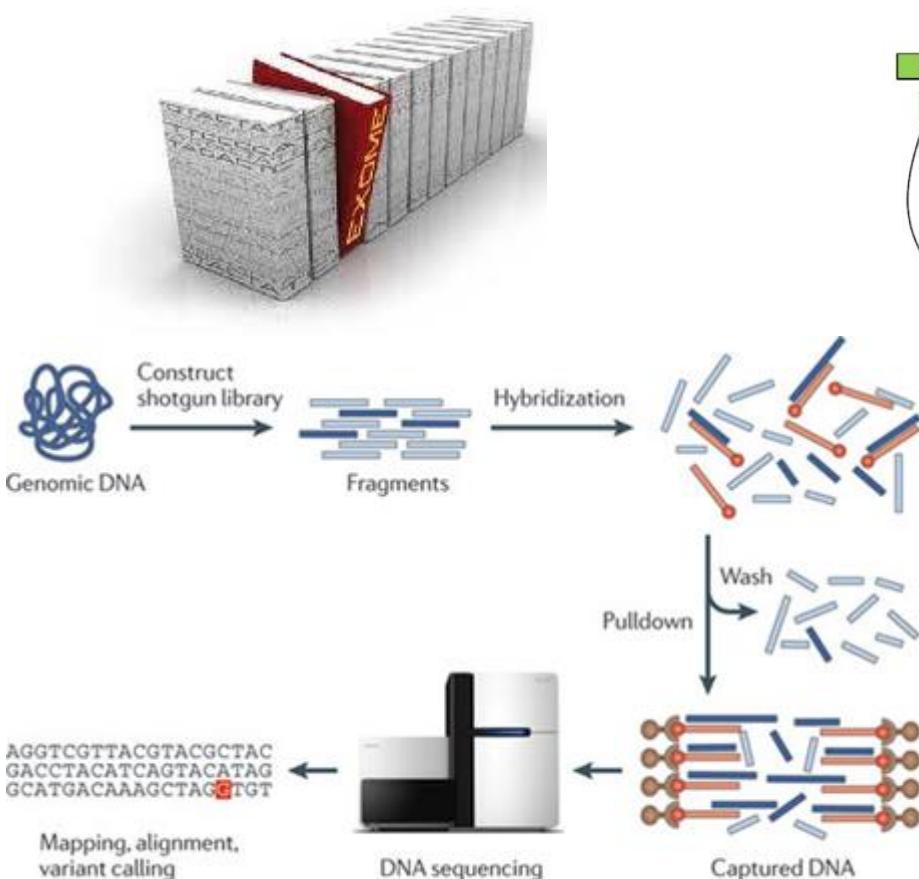


Human Genetics Applications

Next-generation sequencing (NGS) in human genetics



EXOME



30-50 Mb
Human Exome
\$500

Genoma, Exoma, Panel? desde un punto de vista clínico

PANEL

- Barato y rápido
- Util en enfermedades monogénicas
- Datos mas manejables, análisis y almacenamiento

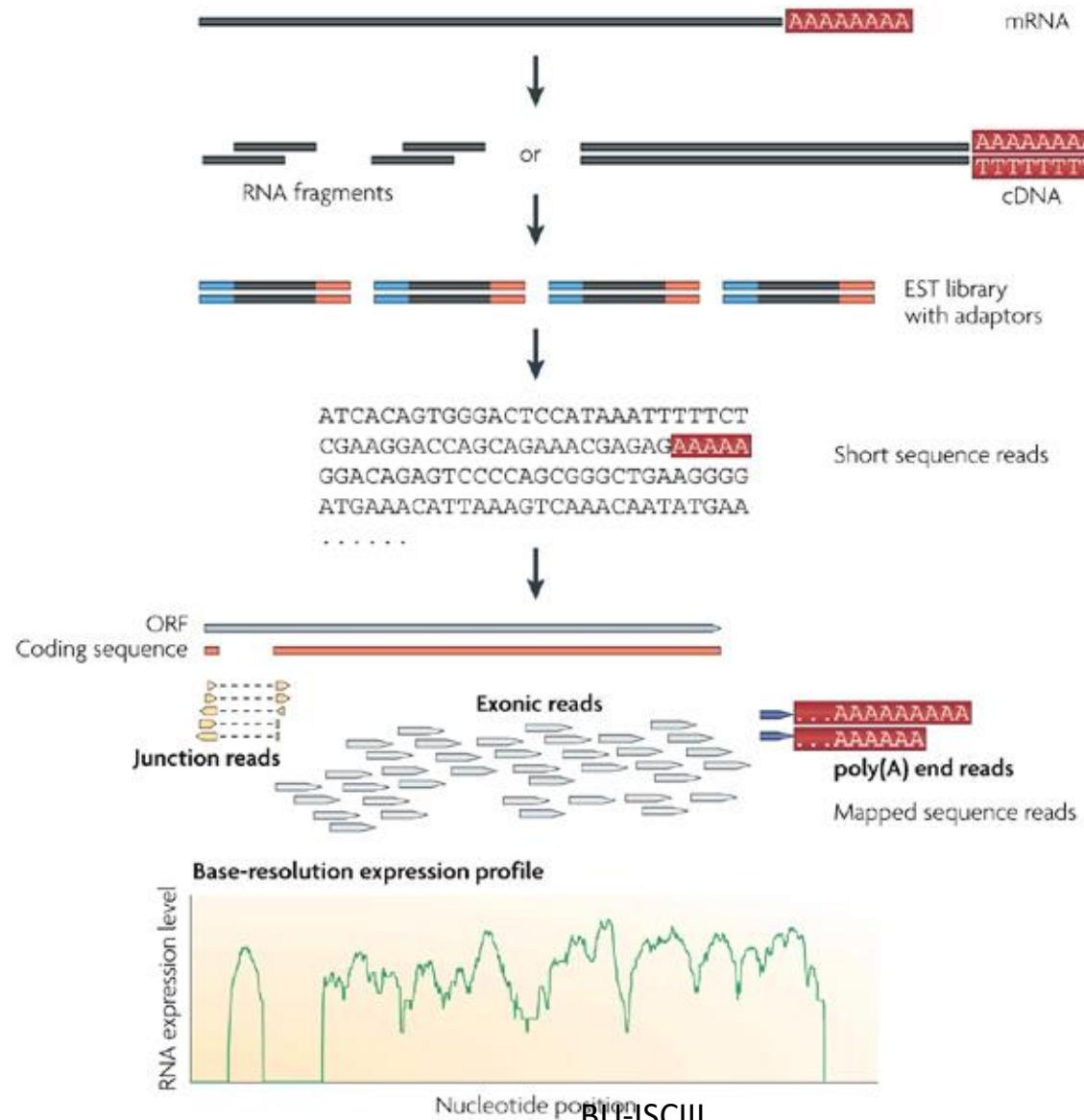
EXOMA

- Mas complejo y lento
- Necesario en enfermedades complejas
- Análisis mas complejo
- Mayor volumen de datos

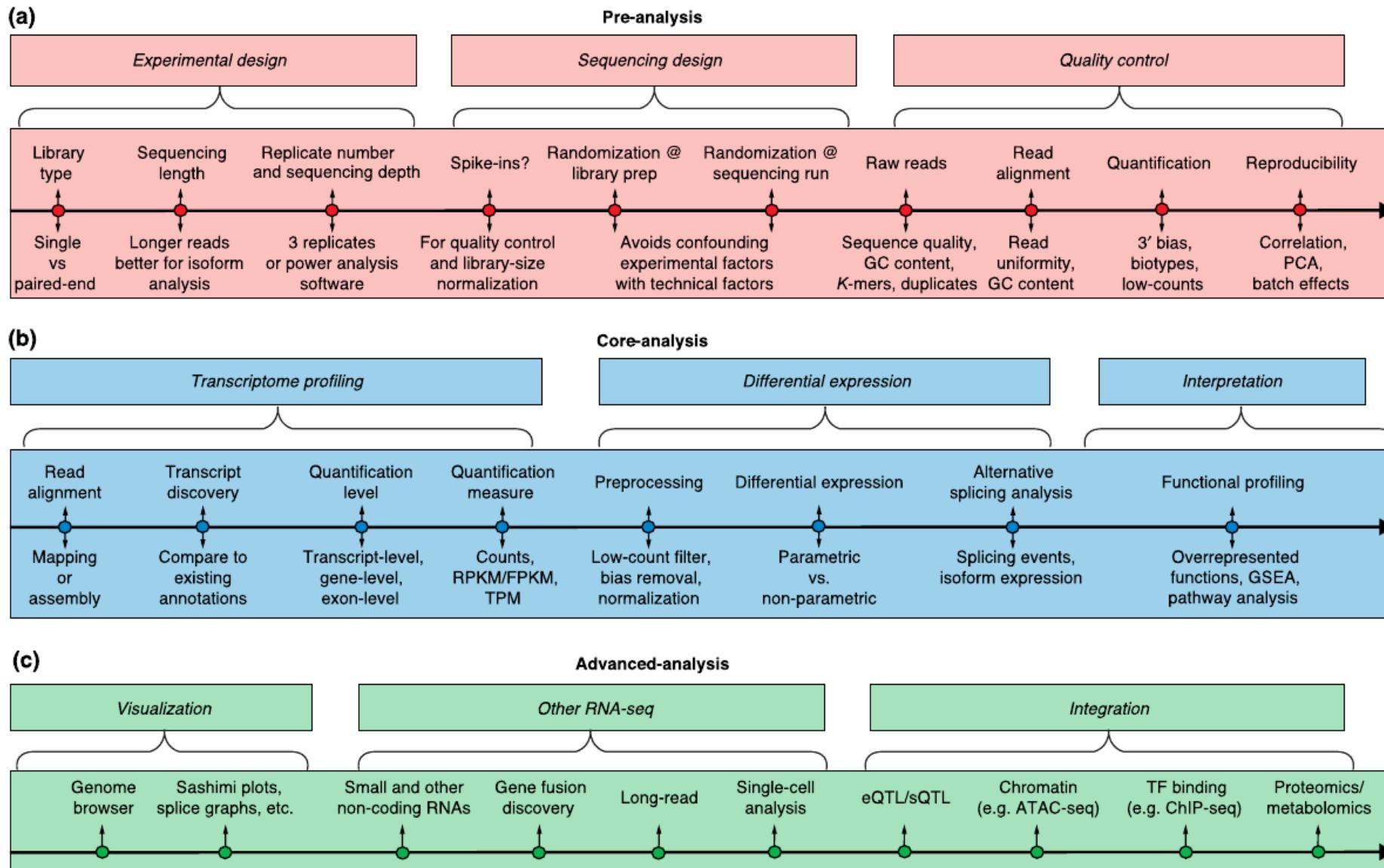
GENOMA

- Maxima complejidad en secuenciación y coste
- Información de regiones no codificantes
- Análisis de variaciones estructurales
- Elevado volumen de datos

RNA seq

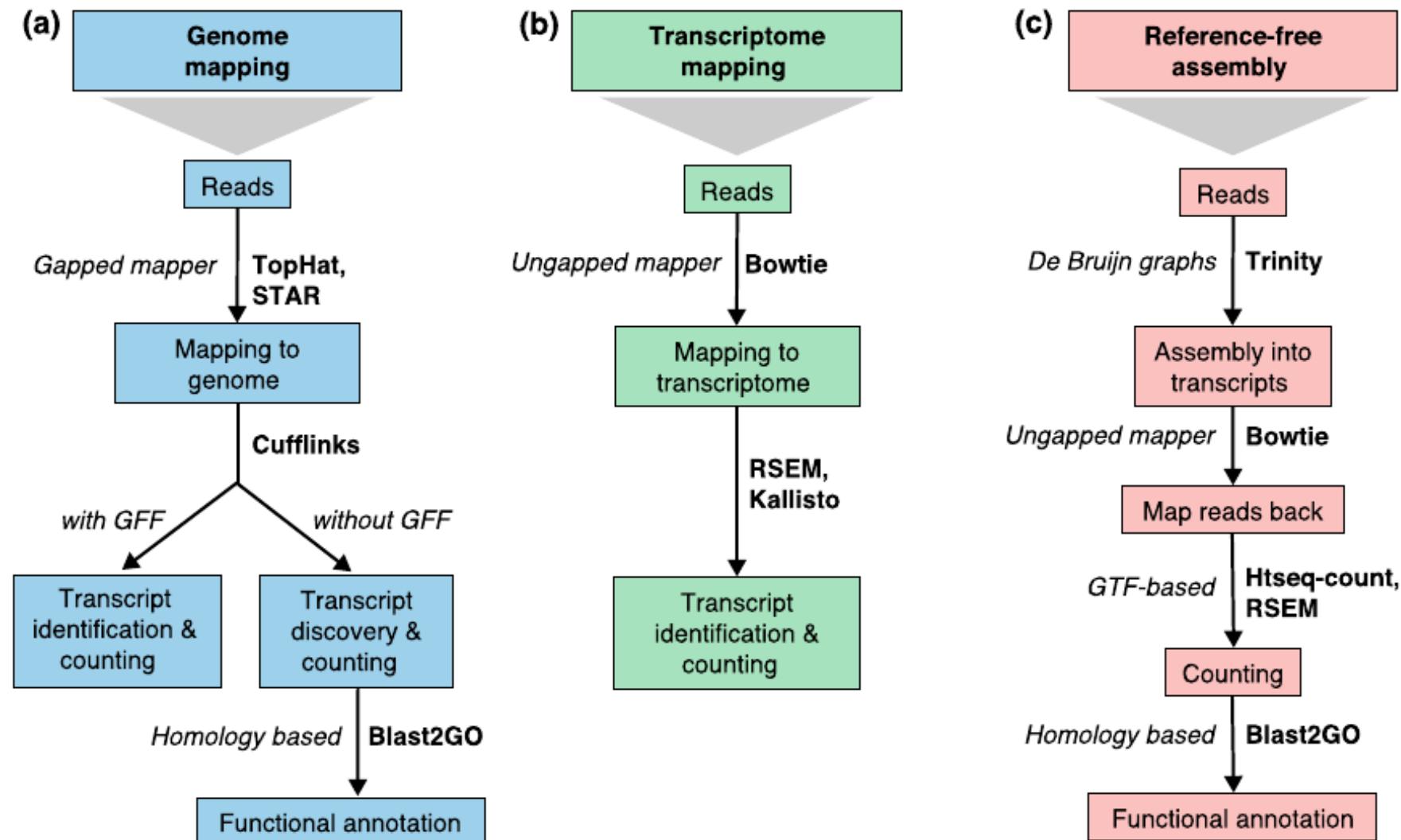


RNA seq



Conesa et al., Genome Biology (2016) 17:13
BU-ISCIII

RNA seq: transcript identification strategies

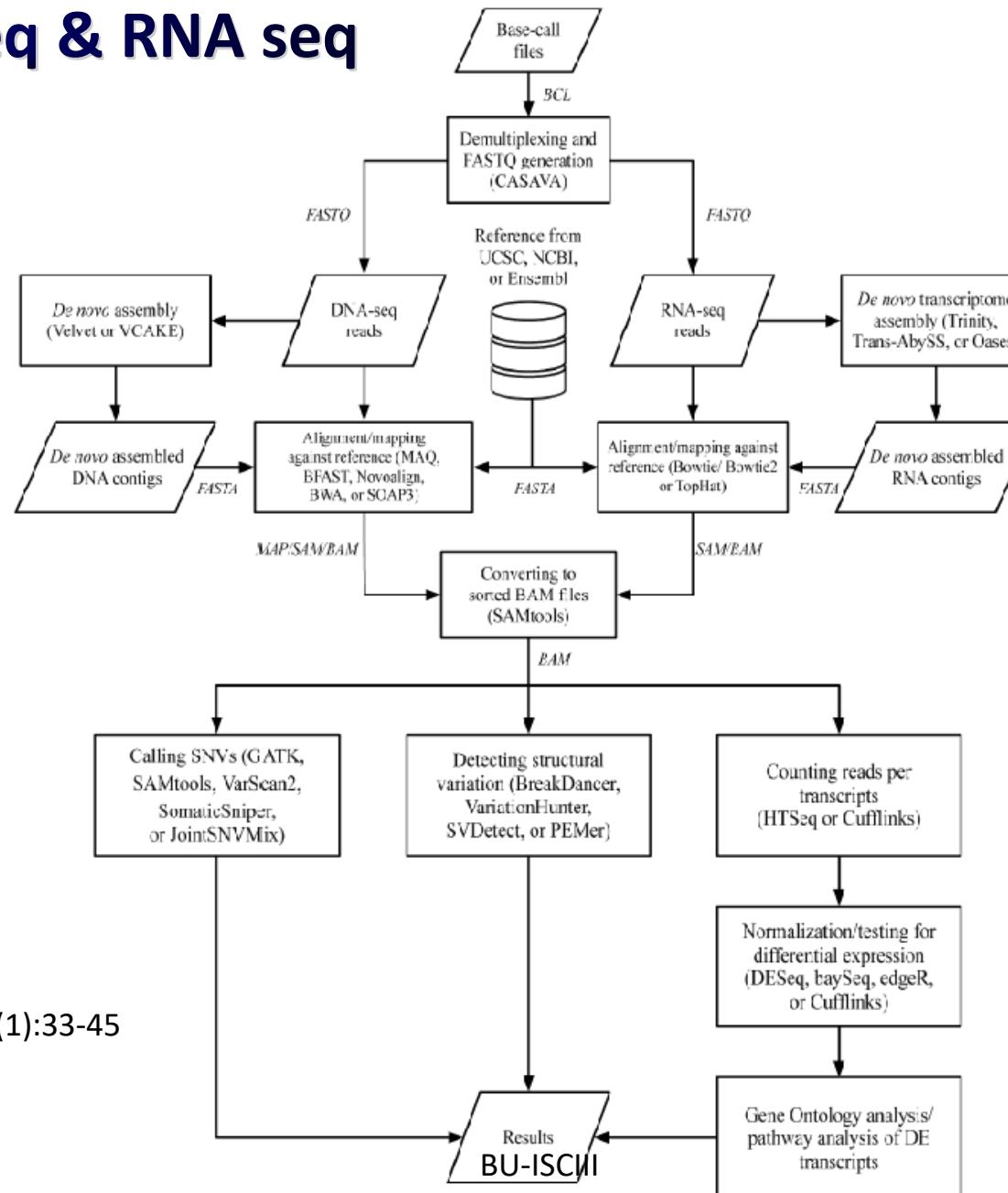


Conesa et al., Genome Biology (2016) 17:13
BU-ISCIII

RNA seq, ventajas

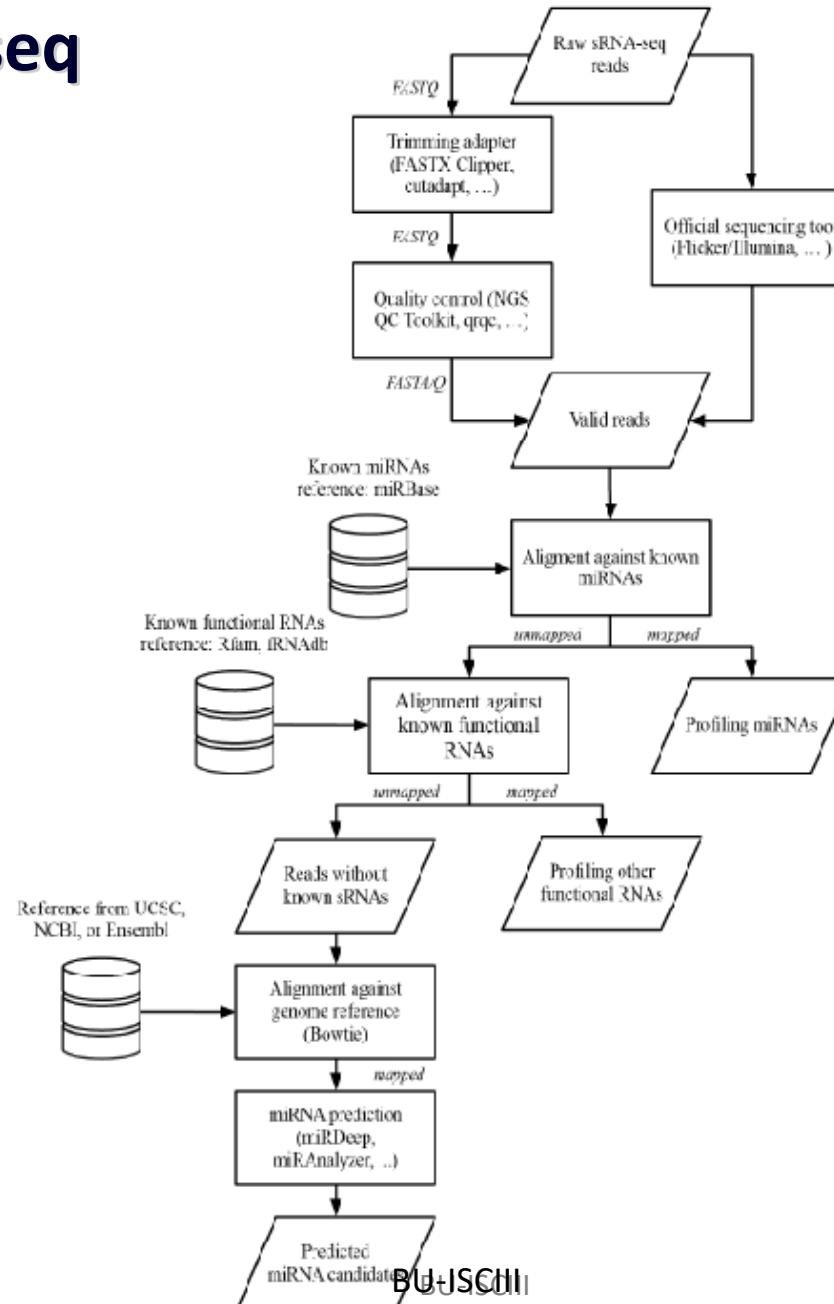
- A diferencia de microarrays no requiere conocimiento de la secuencia del genoma
- Bajo nivel de ruido (las lecturas se mapan correctamente)
- Cuantificación de los transcritos
- Identificación de nuevos transcritos
- Identificación de variaciones (SNPs)
- Disminución progresiva del coste
- Pipelines de análisis disponibles para organismos eucariotas superiores

Strategies of DNA-seq & RNA seq



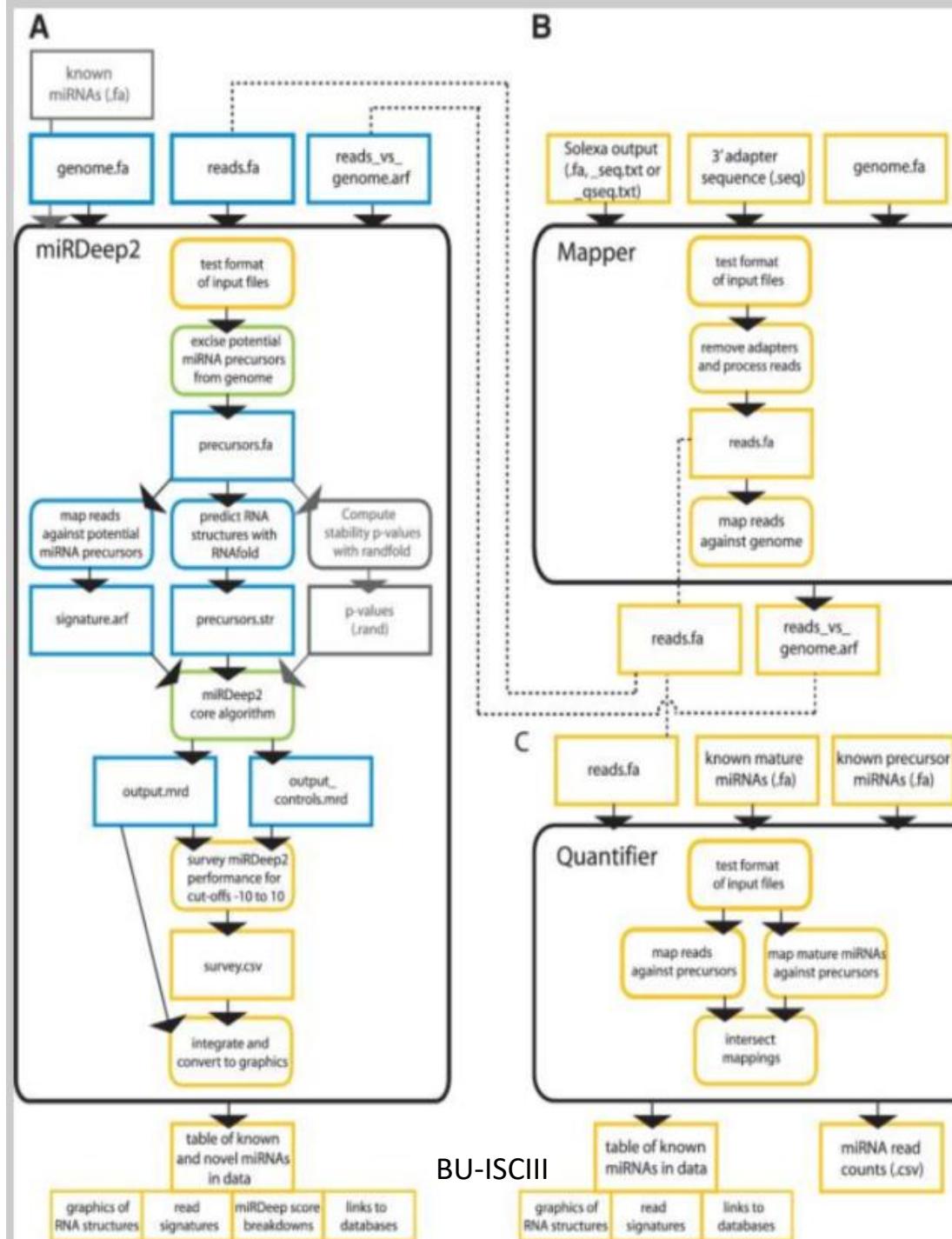
Lee et al., Transl Cancer Res 2013;2(1):33-45

Strategies of small RNA seq



Lee et al., Transl Cancer Res 2013;2(1):33-45

miRNAsq analysis



Applications in Human Genetics

- Identification of polymorphisms –SNVs (SNPs, indels)
- Identification of structural variants –SVs (1kb-3Mb) (indels, CNVs, inversions, translocations)
- Gene expression profiling analysis, differential expression – mRNA, SNVs
- Identification and quantification of : smallRNA, miRNA, lncRNA
- De novo transcriptome assembly
- Chip-Seq
- Metil-Seq
- Variant effect annotation
-

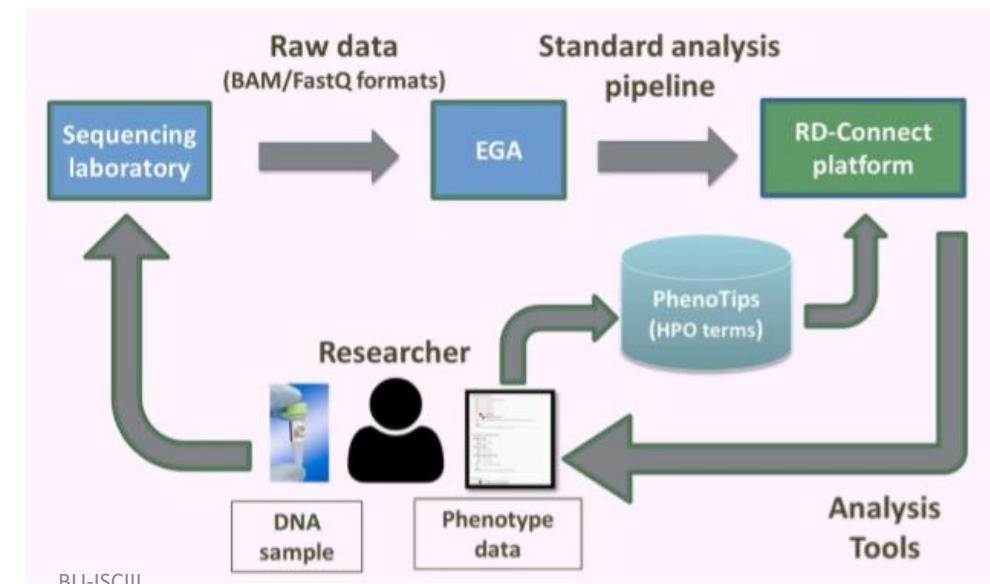
Clinical Bioinformatics - Precision Medicine

SpainUDP: “Spanish Undiagnosed Rare Diseases Program”



SpainUDP, Manuel Posada

- El programa SpainUDP - IIER se creó con el objetivo de intentar obtener un diagnóstico clínico en pacientes con enfermedades raras sin diagnosticar. Se basa en tres pilares: Estudio individualizado y exhaustivo de cada caso; Uso de técnicas de NGS; Compartir datos a nivel internacional (RD-Connect)
- Se han analizado 120 WES, llegando a un 50% de resolución de casos. Se incorporó en 2020 WGS y RNAseq.



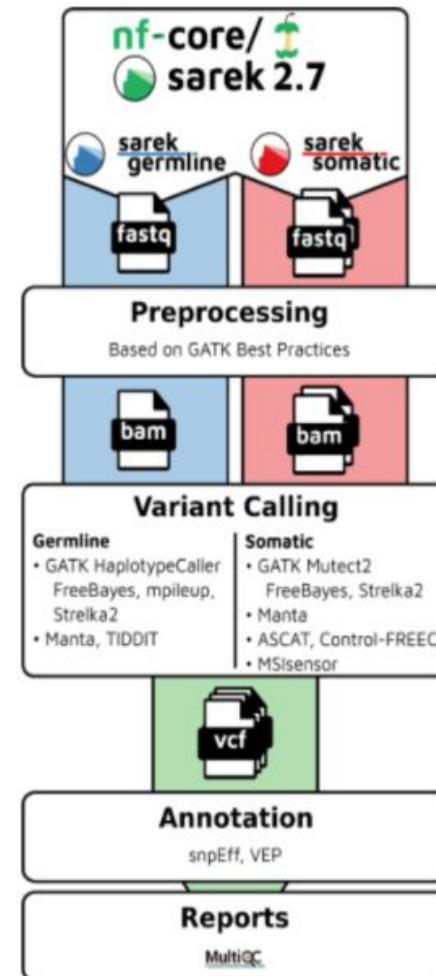
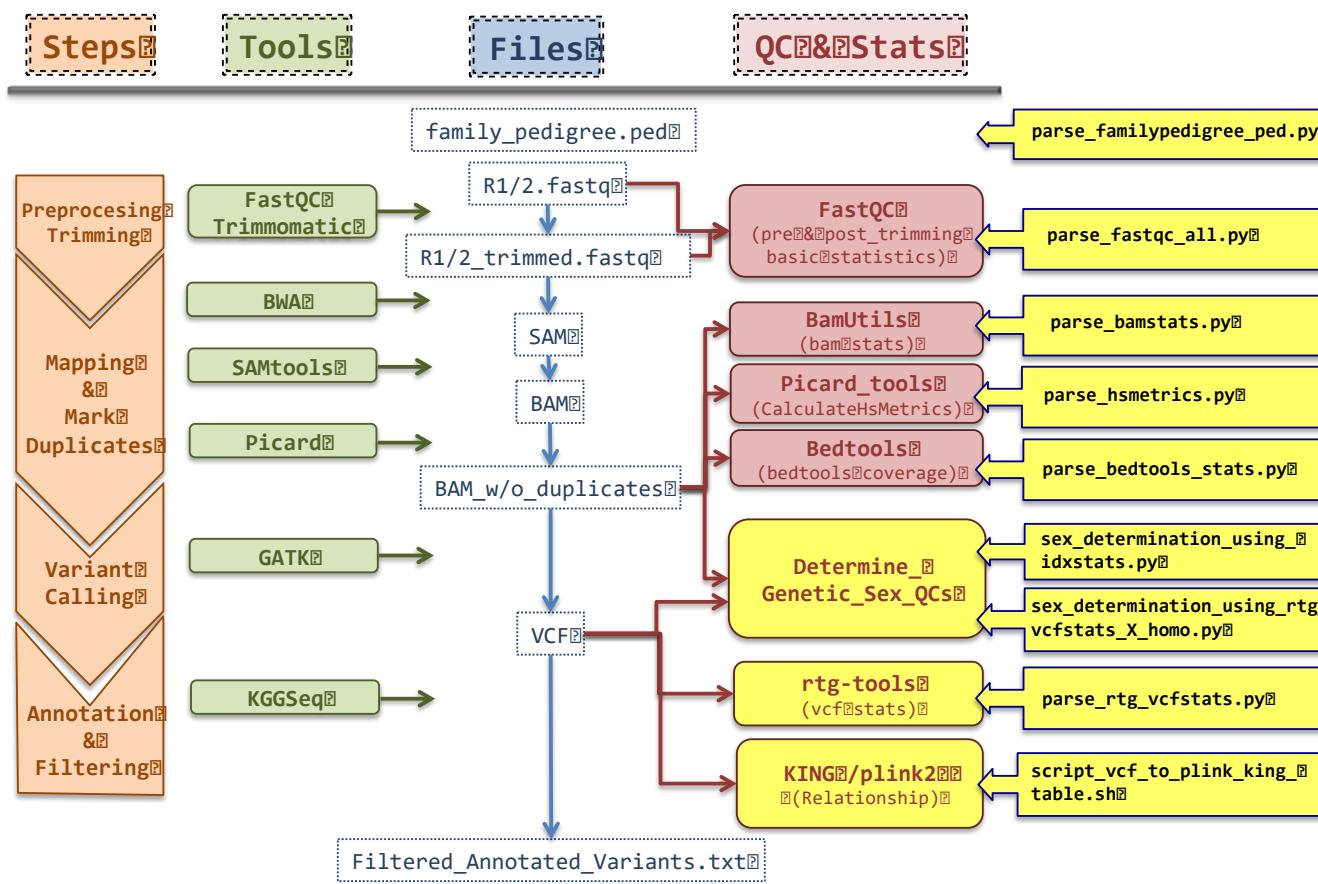
Spa*ii* UDP

RD Connect

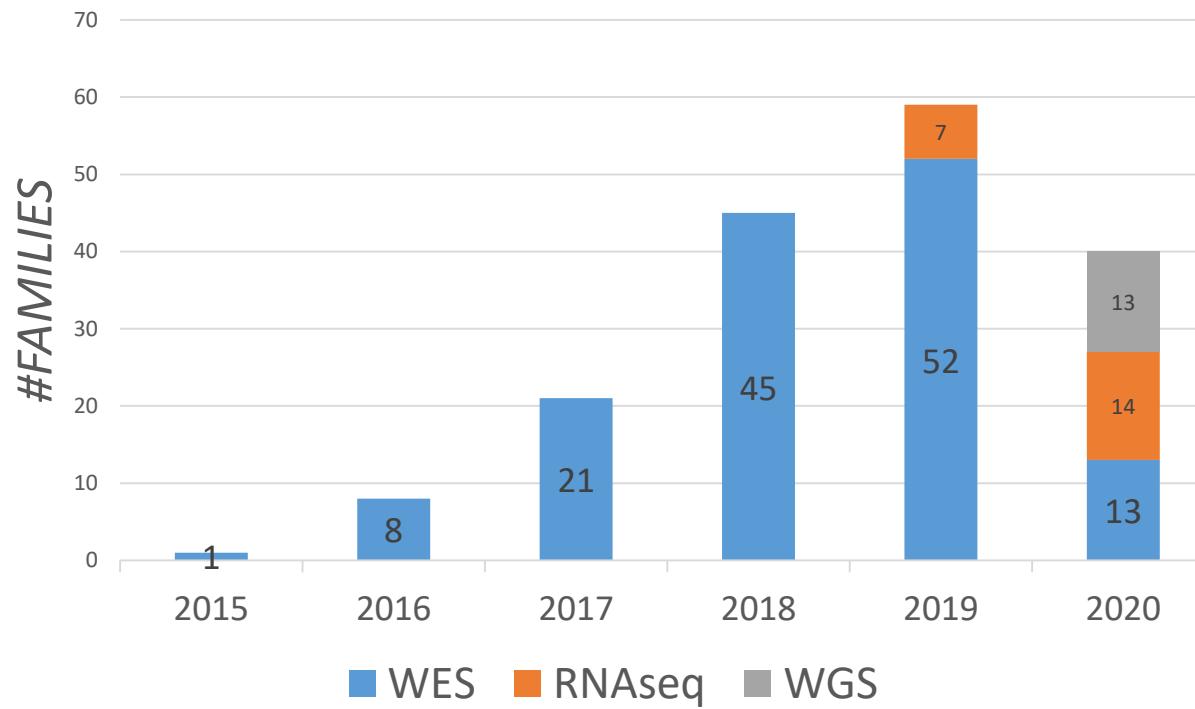
 **EUROPEAN JOINT PROGRAMME**
RARE DISEASES

Solve  **RD**

Data analysis: Pipeline



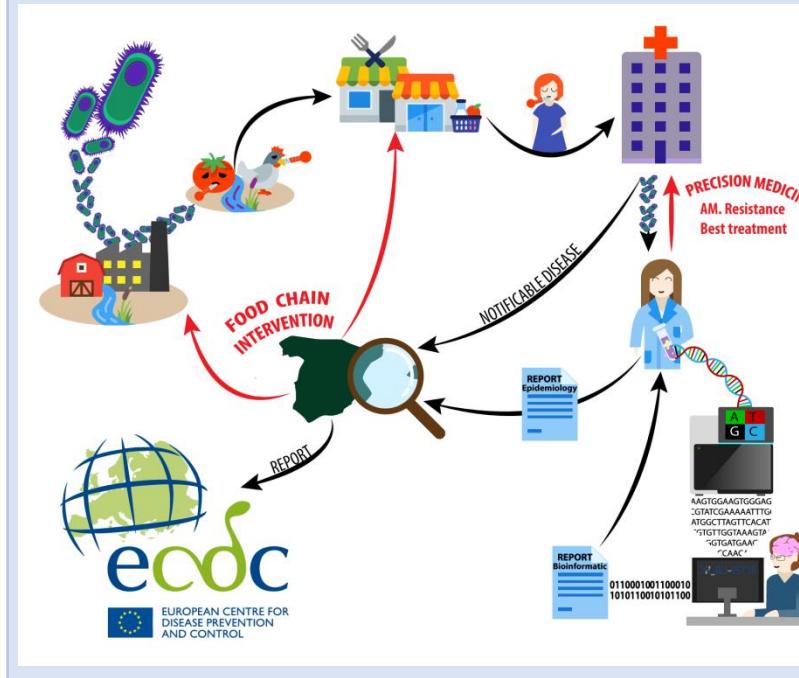
SpainUDP: number of families analyzed



Microbial Genomics Applications

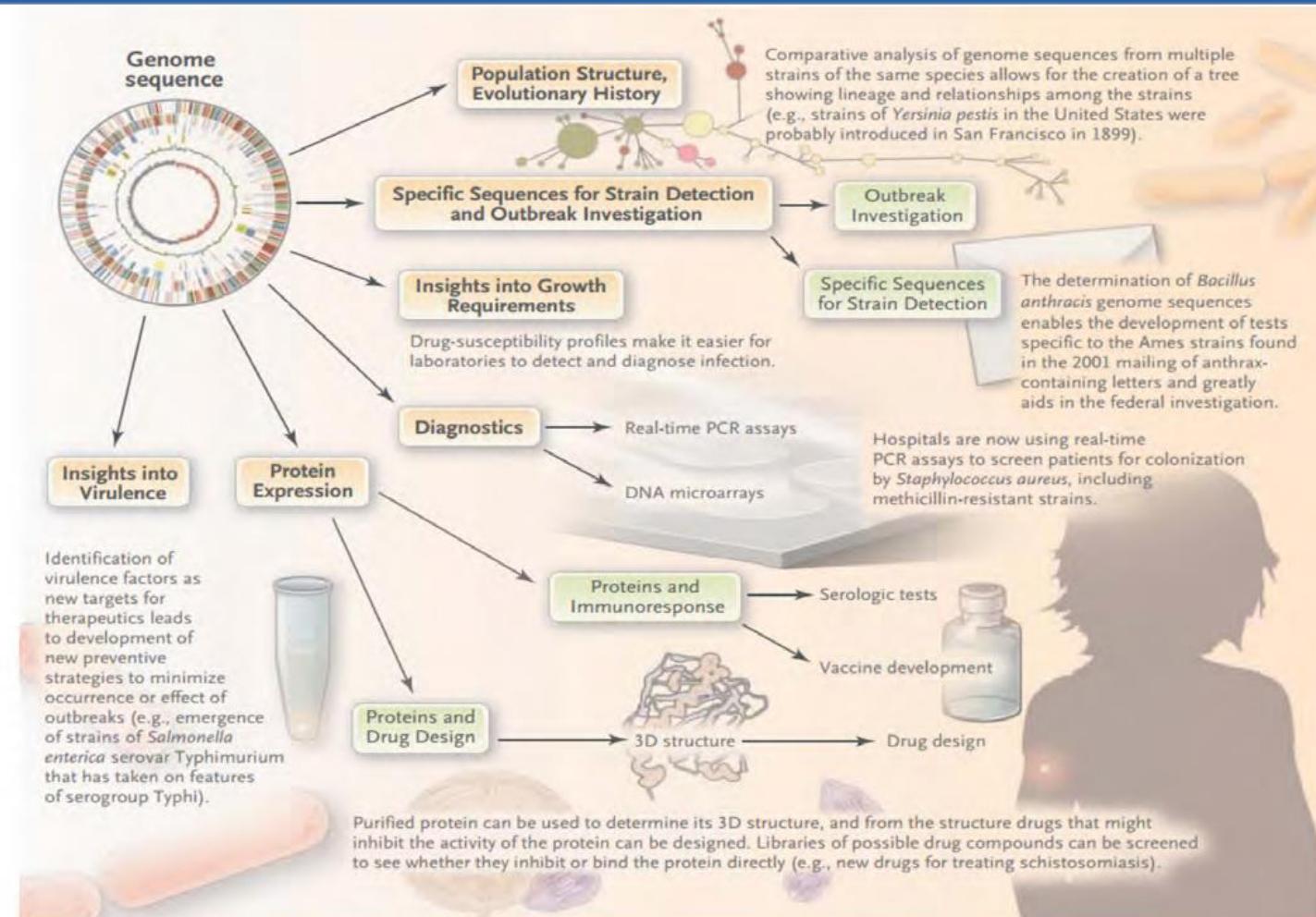
Research - Clinical Bioinformatics - Precision Medicine

CNM Pathogen associated



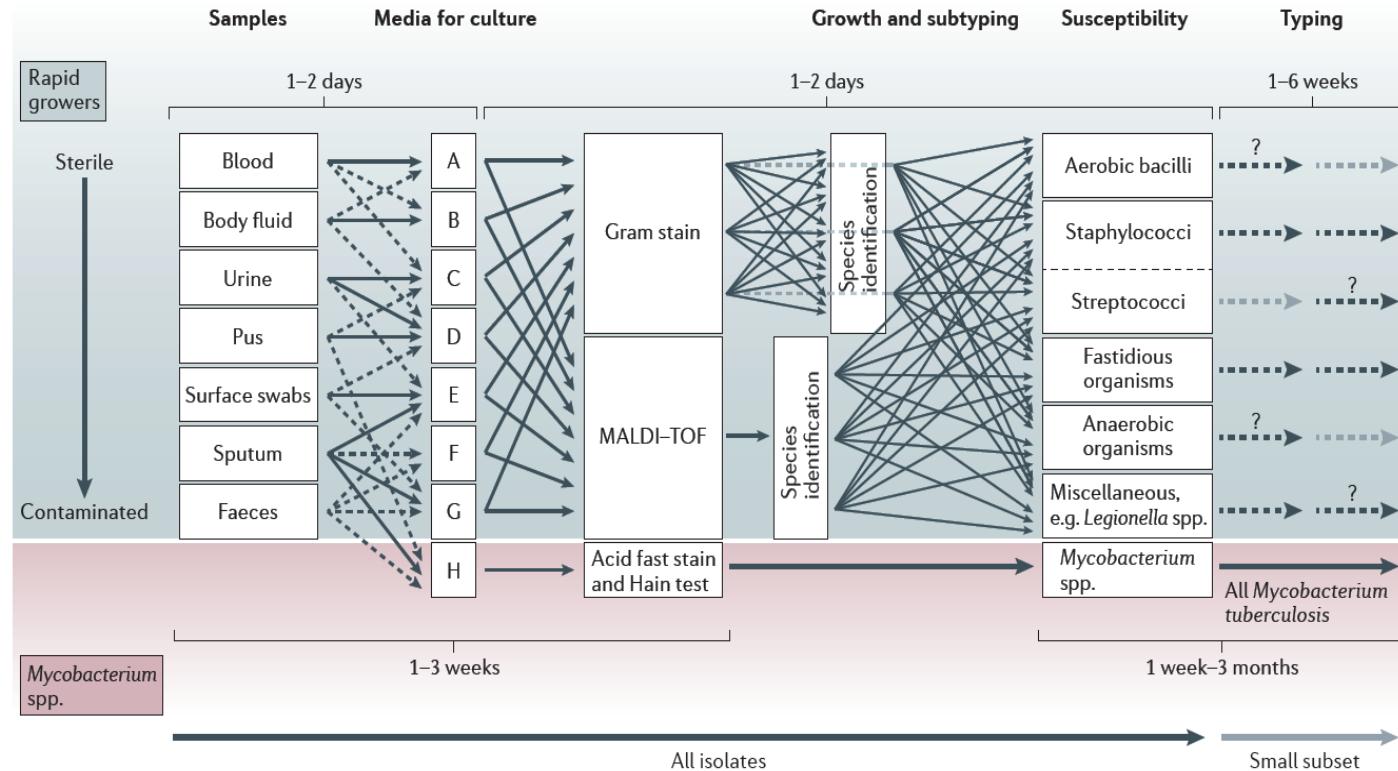
Use of microbial genomics for tool development

Report from The American Academy of Microbiology, 2015



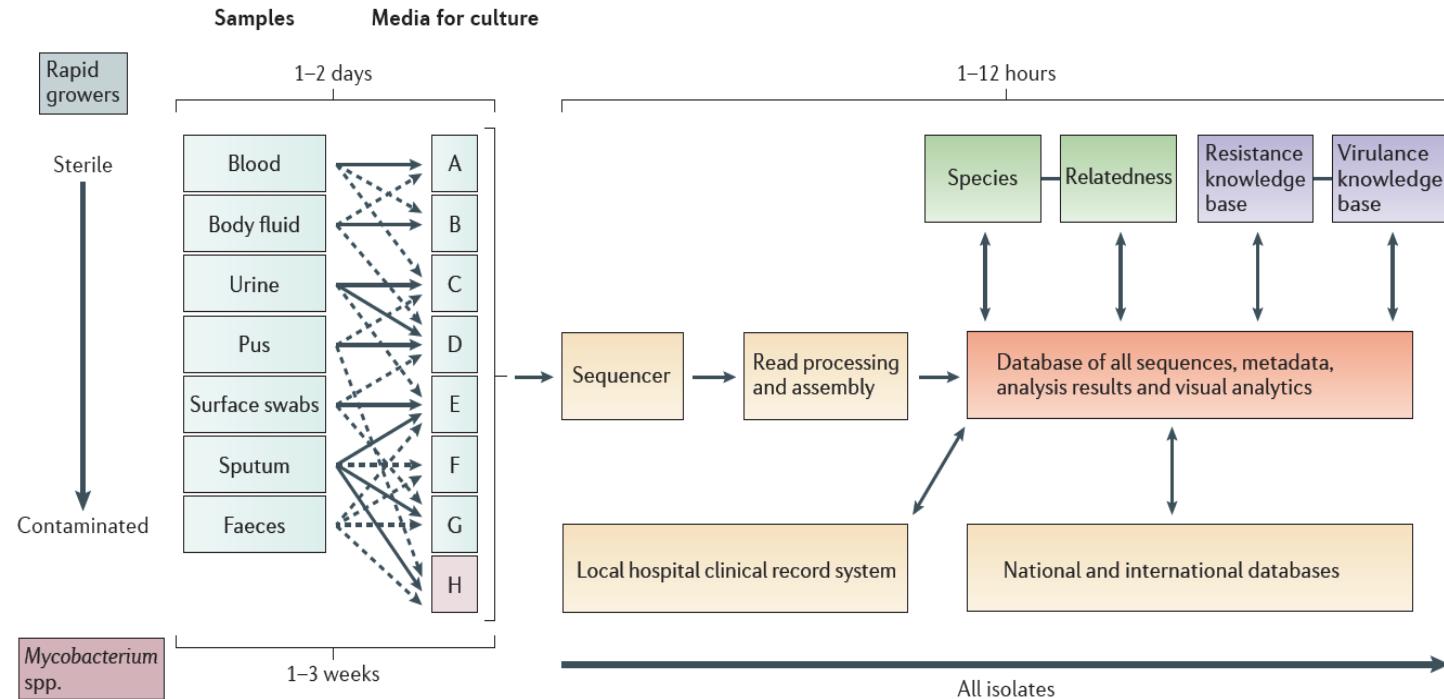
Workflow for processing samples for bacterial pathogens

Didelot et al., Nature Genet Review 2012, 13:601-612



Ongoing developments in DNA-sequencing technologies are likely to affect the diagnosis and monitoring of all pathogens, including viruses, bacteria, fungi and parasites.

The diagnostic and clinical applications of bacterial WGS



Didelot et al., Nature Genet Review 2012, 13:601-612

Foodborne outbreak identification “Crisis del pepino”

2011

Mayo

- 24 Primera muerte en Alemania
- 26 Alemania acusa a los pepinos españoles
- 30 Prohibición de importaciones de verduras de España y Alemania
- 31 Laboratorios alemanes desmienten oficialmente que los pepinos españoles sean el foco de infección

Junio

- 10 Resolución de la crisis

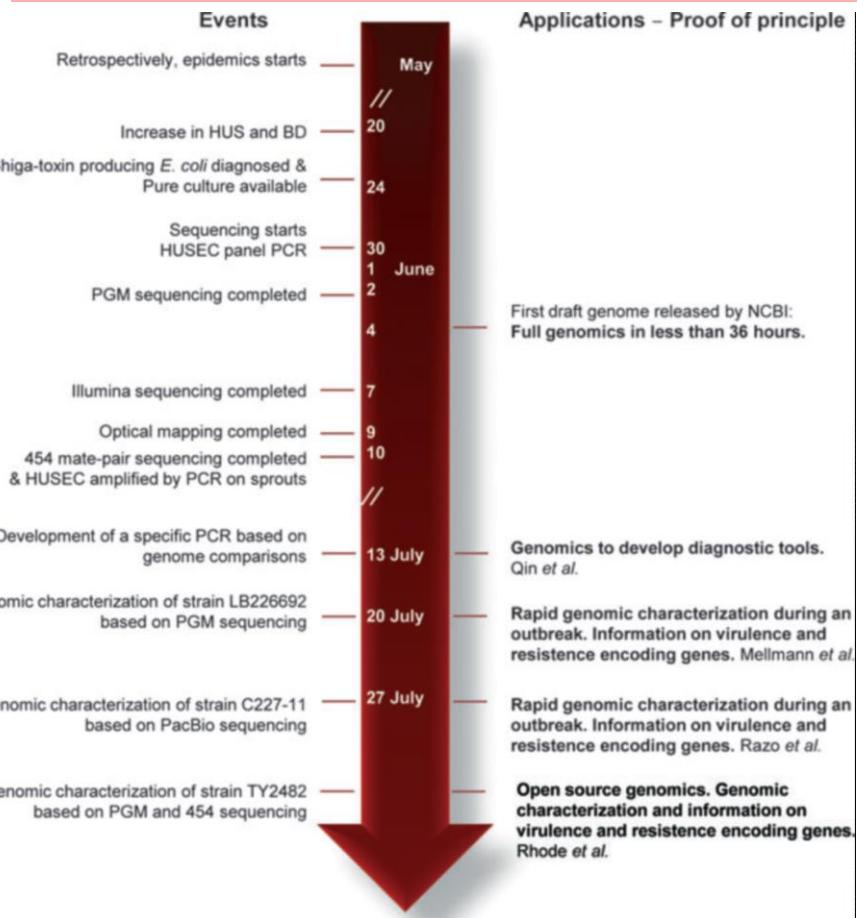
Secuenciación Genoma



Universitätsklinikum
Hamburg-Eppendorf

华大基因
BGI

The Escherichia coli O104:H4 epidemics: event timeline and major outputs



Foodborne outbreak identification “Crisis del pepino”

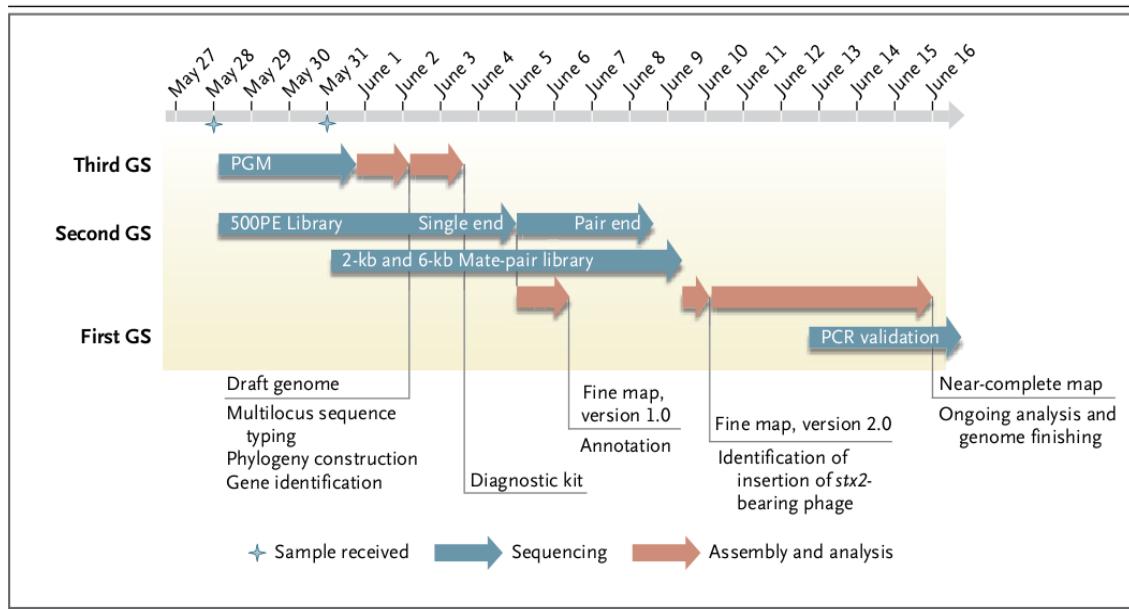


Figure 1. Timeline of the Open-Source Genomics Program.

After receiving the first batch of DNA samples on May 28, 2011, sequencing runs with the use of the Ion Torrent Personal Genome Machine (PGM) and Illumina (small-insert library) were initiated simultaneously. On May 31, the second batch of DNA was received and used for Illumina large-insert sequencing. An assembly of the Ion Torrent reads was released on June 2, which enabled subsequent analyses (multilocus sequence typing, phylogenetic analysis, and genome comparisons). Errors in the Ion Torrent data were corrected with the use of later Illumina data, and a high-quality draft genome sequence was created. GS denotes generation of sequencing technology. The symbols at May 28 and May 31 in the timeline indicate the arrival of DNA samples.

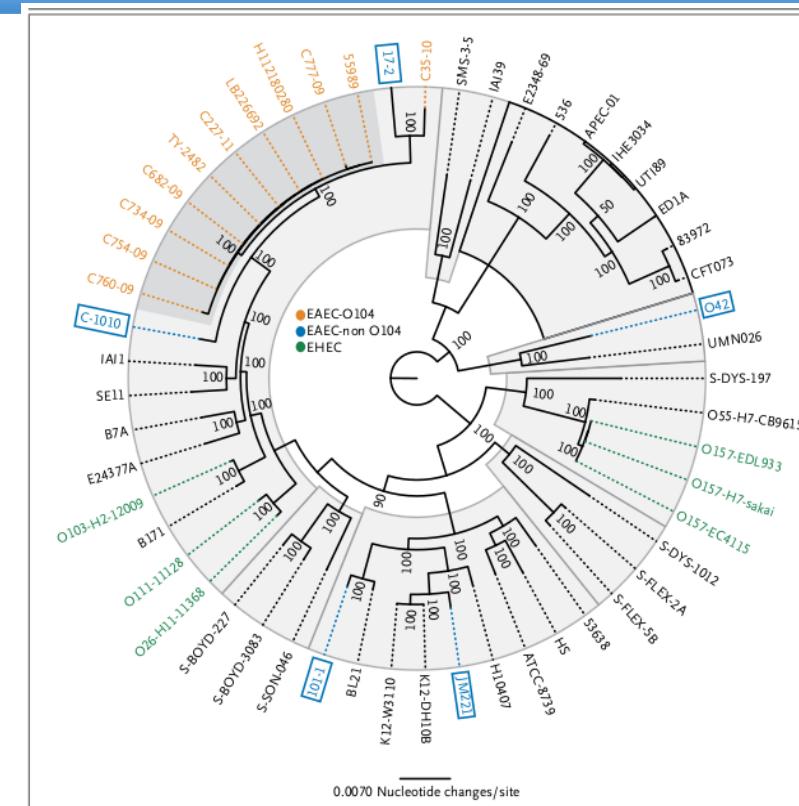


Figure 2. Phylogenetic Comparisons of 53 *Escherichia coli* and *Shigella* Isolates.

Genomic sequences were compared with the use of 100 bootstrap calculations, as described by Sahl et al.³⁵ The species-based phylogeny was inferred with the use of 2.56 Mbp of the conserved core genome. The O104:H4 isolates are shown in orange, the reference enteroaggregative *E. coli* (EAEC) isolates in blue, and the enterohemorrhagic *E. coli* isolates in green. (The classification of the other strains is shown in Fig. 4 and Table 4 in the Supplementary Appendix.) The O104:H4 isolates cluster into a single clade (dark gray); in contrast, the reference EAEC isolates are extremely divergent and are represented throughout the phylogeny.

Andalusian Listeria Outbreak

Actualización de información sobre el brote de intoxicación alimentaria causado por Listeria monocytogenes.

Publica: Agencia Española Seguridad alimentaria y Nutrición
Fecha: 29 agosto 2019
Sección: Seguridad Alimentaria

Jueves 29 de agosto de 2019, 12.00 horas

ACTUALIZACIÓN EN RELACIÓN CON LA DISTRIBUCIÓN DE PRODUCTOS RELACIONADOS CON LA ALERTA.

La Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) recomienda a las personas que tengan en su domicilio algún producto de la marca "La Mechá" se abstengan de consumirlo. Si se dispone del producto se debe devolver al punto de compra y, de no ser posible, desecharlo.

Brote de listeriosis: sube el número de afectados y se apunta a la falta de higiene en la carne como causa

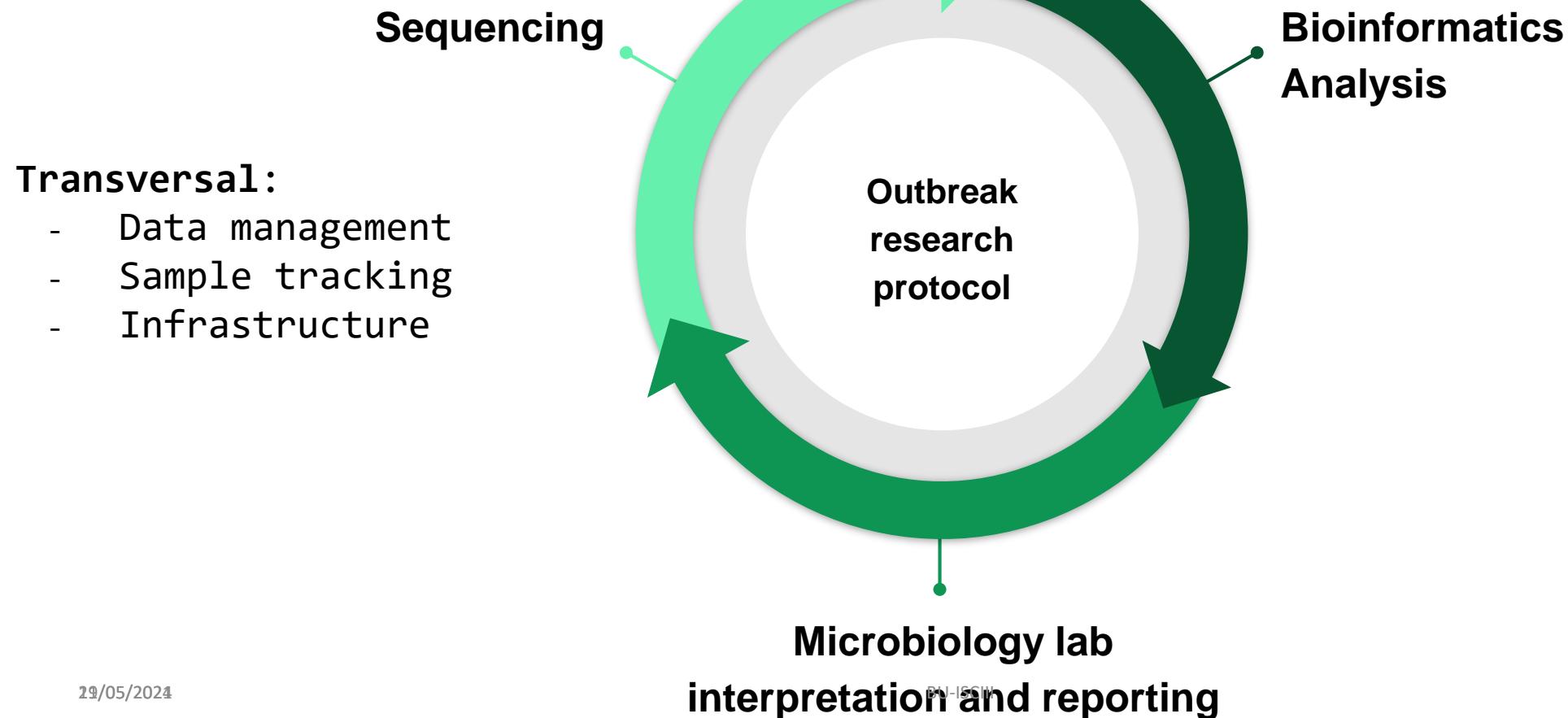
EFE 25.08.2019

- Tres nuevos casos, en Sevilla y Cádiz, dejan el número de personas afectadas en Andalucía en 192.
- [La carne con listeria de la marca blanca se vendió en los municipios de Sevilla.](#)
- La empresa que vendió la marca blanca de Magrudis dice que cumple los protocolos.



- Meat “La Mechá”. Margulis S.L.
- 250 cases related.
- Meat “"La Montanera del Sur". INCARYBE S.L”, suspicion. (Cádiz)
- Meat “Sabores de Paterna” (Málaga)

Andalusian Listeria Outbreak

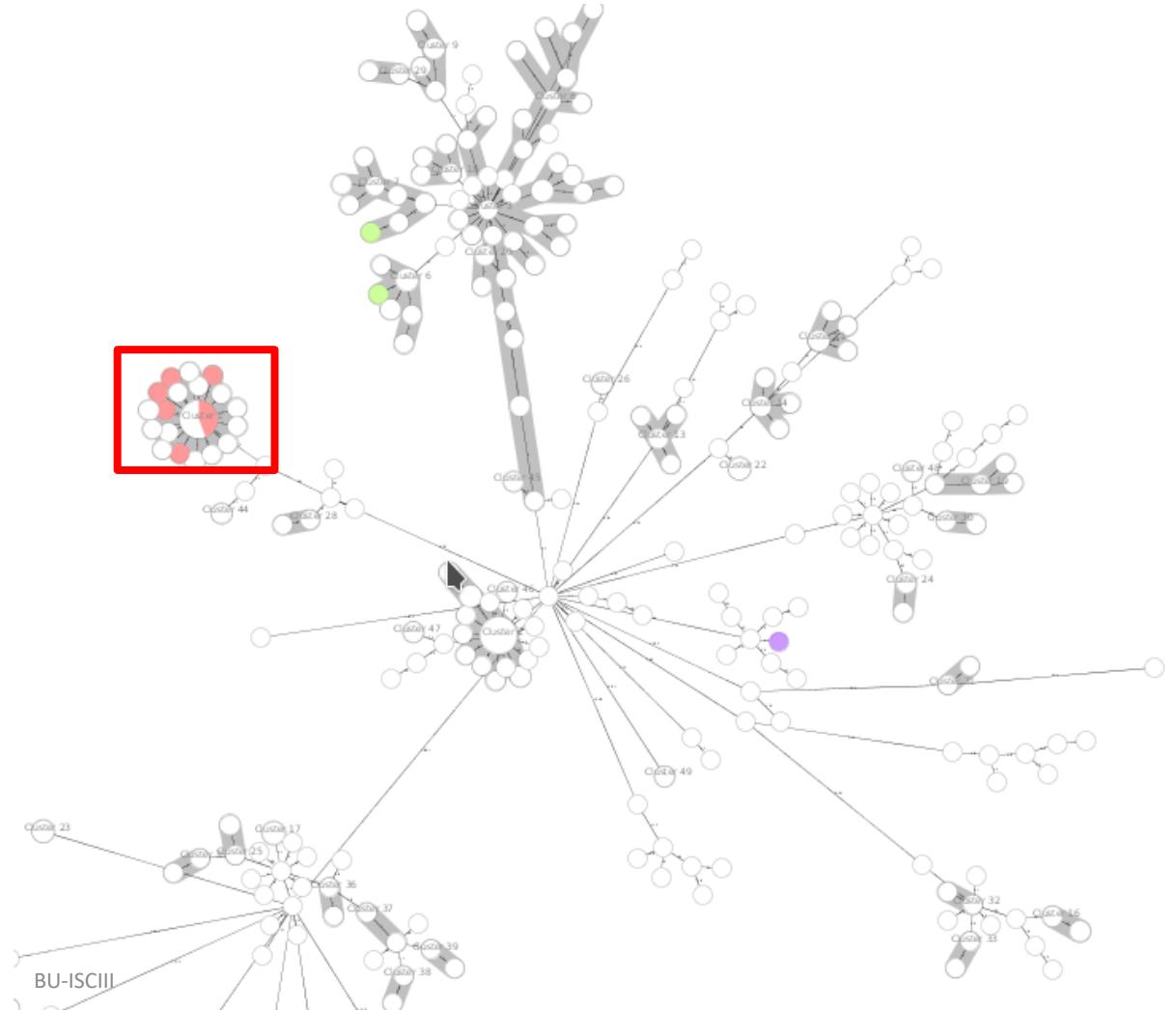


Andalusian Listeria Outbreak

- 625 listeria samples already sequenced
 - 258 suspected to be related to the outbreak (mid august to mid september)

Results:

- 233 related to the outbreak, confirmed to be caused by the meat “La Mechá”
 - 25 sporadic cases not related to the outbreak.



Pathogen discovery: new virus – SARS-CoV-2

Deep Meta-Transcriptomic Sequencing



bronchoalveolar lavage fluid (BALF)



Meta-transcriptomic library

2x150 MiniSeq

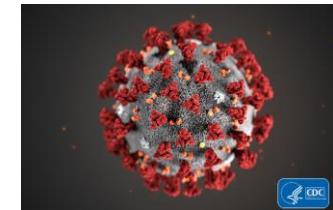
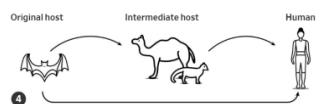
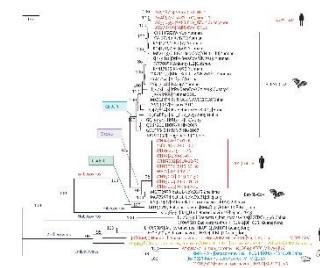
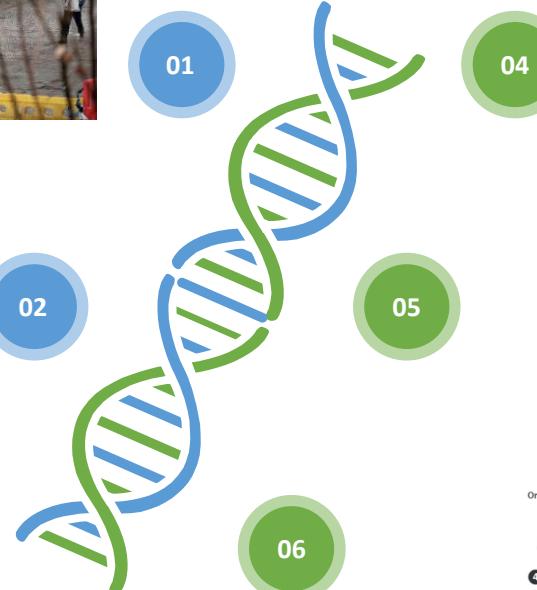
56,565,928 sequences reads



03

02

01

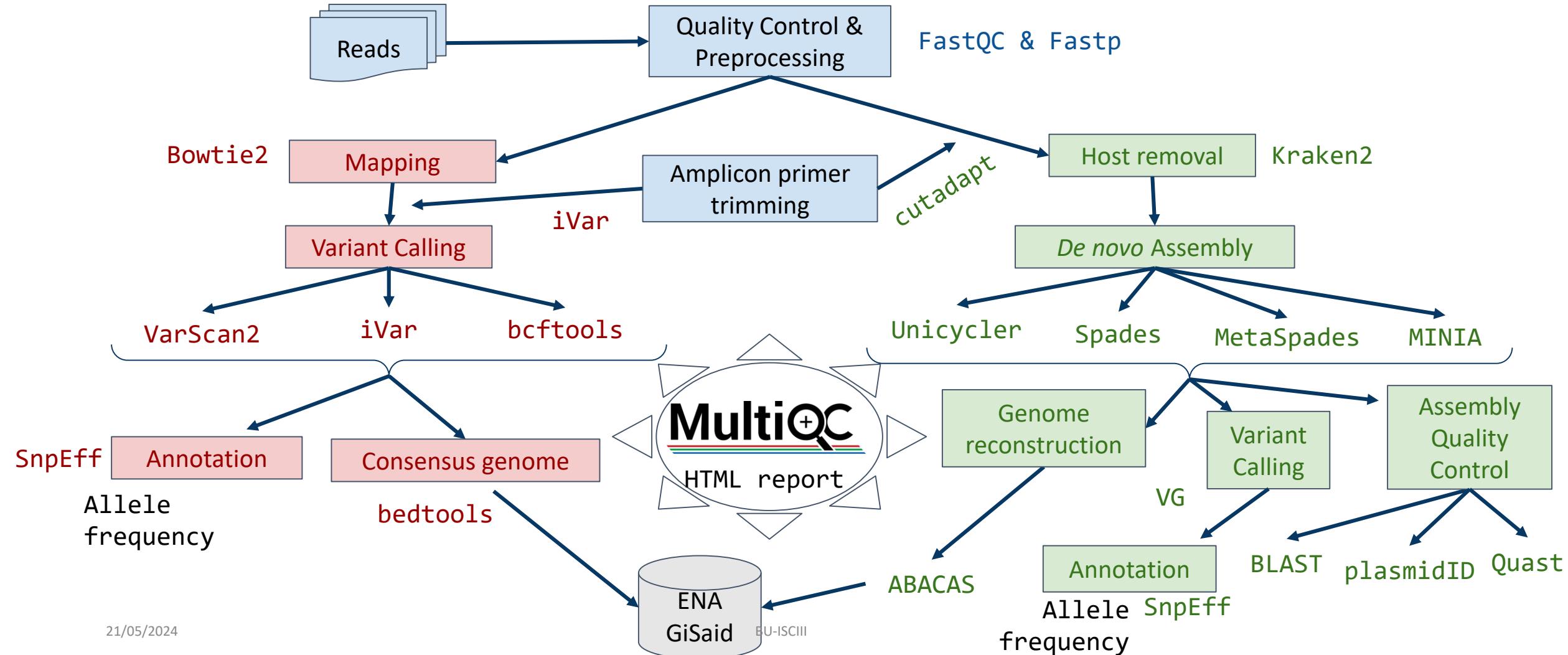


89.1% identity
Closely related to a bat SARS-like coronavirus

De novo-assembled - Megahit
384,096 Contigs
Screened for potential aetiological agents
The longest 30,474 nt

Wu et al., Nature 2020

Viralrecon



Surveillance-based reporting

Virussequence	sample	totalread	readshostR	readsho	%readsho	readsviru	%readsviru	unmappedread	%unmappedread	meanDPcov	coverageviru	Coverage>10x(%)	Variantsinconsensus	x1	MissenseVariants	%Ns1C	Lineages
NC_045512.2	211524	2040680	24760	49520	2.43	1980480	97.05	10680	0.52	4607.76	99.79		41		28	0,17	B.1.1.7
NC_045512.2	210710-2	2166652	12091	24182	1.12	2140652	98.80	1818	0.08	5331.12	99.77		40		25	0,18	B.1.1.7
NC_045512.2	211496	2092444	2299	4598	0.22	2081982	99.50	5864	0.28	4684.85	99.55		35		23	0,22	B.1.1.7
NC_045512.2	211429	1739402	1643	3286	0.19	1730009	99.46	6107	0.35	3891.88	98.84		46		27	0,24	B.1.1.7
NC_045512.2	211507	1394744	1500	3000	0.22	1386515	99.41	5229	0.37	3438.01	99.05		39		26	0,25	B.1.1.7
NC_045512.2	211517	2284388	6870	13740	0.60	2263829	99.10	6819	0.30	4882.96	99.66		40		26	0,26	B.1.1.7
NC_045512.2	211405	1946156	19779	39558	2.03	1900227	97.64	6371	0.33	4174.87	99.57		37		25	0,32	B.1.1.7
NC_045512.2	211497	1823806	104	208	0.01	1821435	99.87	2163	0.12	4889.73	99.65		39		25	0,33	B.1.1.7
NC_045512.2	211430	1859150	378	756	0.04	1853758	99.71	4636	0.25	4475.72	99.66		20		7	0,34	B.1.1.77
NC_045512.2	211498	1974394	713	1426	0.07	1963930	99.47	9038	0.46	4331.72	99.63		38		25	0,34	B.1.1.7
NC_045512.2	211506	2054730	28336	56672	2.76	1987951	96.75	10107	0.49	3822.90	99.15		41		27	0,49	B.1.1.7
NC_045512.2	211516	2240664	446	892	0.04	2232598	99.64	7174	0.32	4427.42	99.16		19		8	0,61	B.1.1.77
NC_045512.2	211495	2245802	4263	8526	0.38	2218628	98.79	18648	0.83	3620.33	98.60		35		23	0,62	B.1.1.7
NC_045512.2	211513	2191920	1071	2142	0.10	2183372	99.61	6406	0.29	4394.22	99.17		18		8	0,66	B.1.1.77
NC_045512.2	211489	1995358	619	1238	0.06	1990170	99.74	3950	0.20	4938.41	99.14		39		27	0,7	B.1.1.7
NC_045512.2	211512	2032422	11430	22860	1.12	1985067	97.67	24495	1.21	3070.94	98.21		39		26	0,7	B.1.1.7
NC_045512.2	211419	2143186	2827	5654	0.26	2125398	99.17	12134	0.57	4100.12	99.08		41		24	0,77	B.1.1.7
NC_045512.2	211509	1905366	1412	2824	0.15	1895839	99.50	6703	0.35	4146.72	98.79		40		26	0,78	B.1.1.7
NC_045512.2	211411	2192376	1420	2840	0.13	2180537	99.46	8999	0.41	3849.04	98.07		29		18	0,96	B.1.351
NC_045512.2	211519	2406852	382	764	0.03	2390245	99.31	15843	0.66	3833.59	98.42		27		12	0,99	B.1.1.77
NC_045512.2	210790-2	2042452	32448	64896	3.18	1928075	94.40	49481	2.42	3288.34	98.38		32		18	1,08	B.1.160
NC_045512.2	211428	2055490	33405	66810	3.25	1975531	96.11	13149	0.64	3604.64	97.95		37		23	1,22	B.1.1.7
NC_045512.2	211511	1989742	549	1098	0.06	1980788	99.55	7856	0.39	4077.32	98.31		38		26	1,35	B.1.1.7
NC_045512.2	211425	1761330	11924	23848	1.35	1714479	97.34	23003	1.31	2725.52	97.38		39		26	1,55	B.1.1.7
NC_045512.2	211508	1711536	547	1094	0.06	1701951	99.44	8491	0.50	3551.33	98.30		20		13	1,57	C.4
NC_045512.2	211518	2215498	22494	44988	2.03	2158117	97.41	12393	0.56	3630.94	98.03		39		26	1,58	B.1.1.7

One Health approach, infectious diseases could be better controlled and prevented



Spanish National Microbiology Center (CNM)

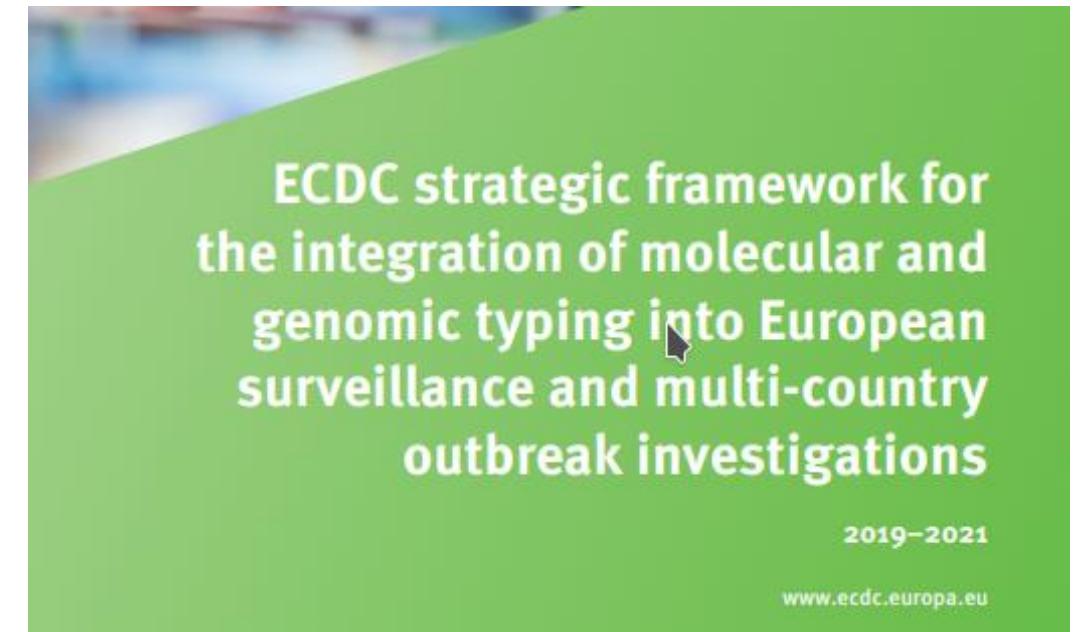


Mission: Provide support to the National Health System and the different Spanish Regions in the diagnosis and control of infectious diseases. In order to fulfill this mission it acts as Reference center offering a series of scientific activities:

- Diagnosis
- **Surveillance** →
- Infectious diseases research
- Training

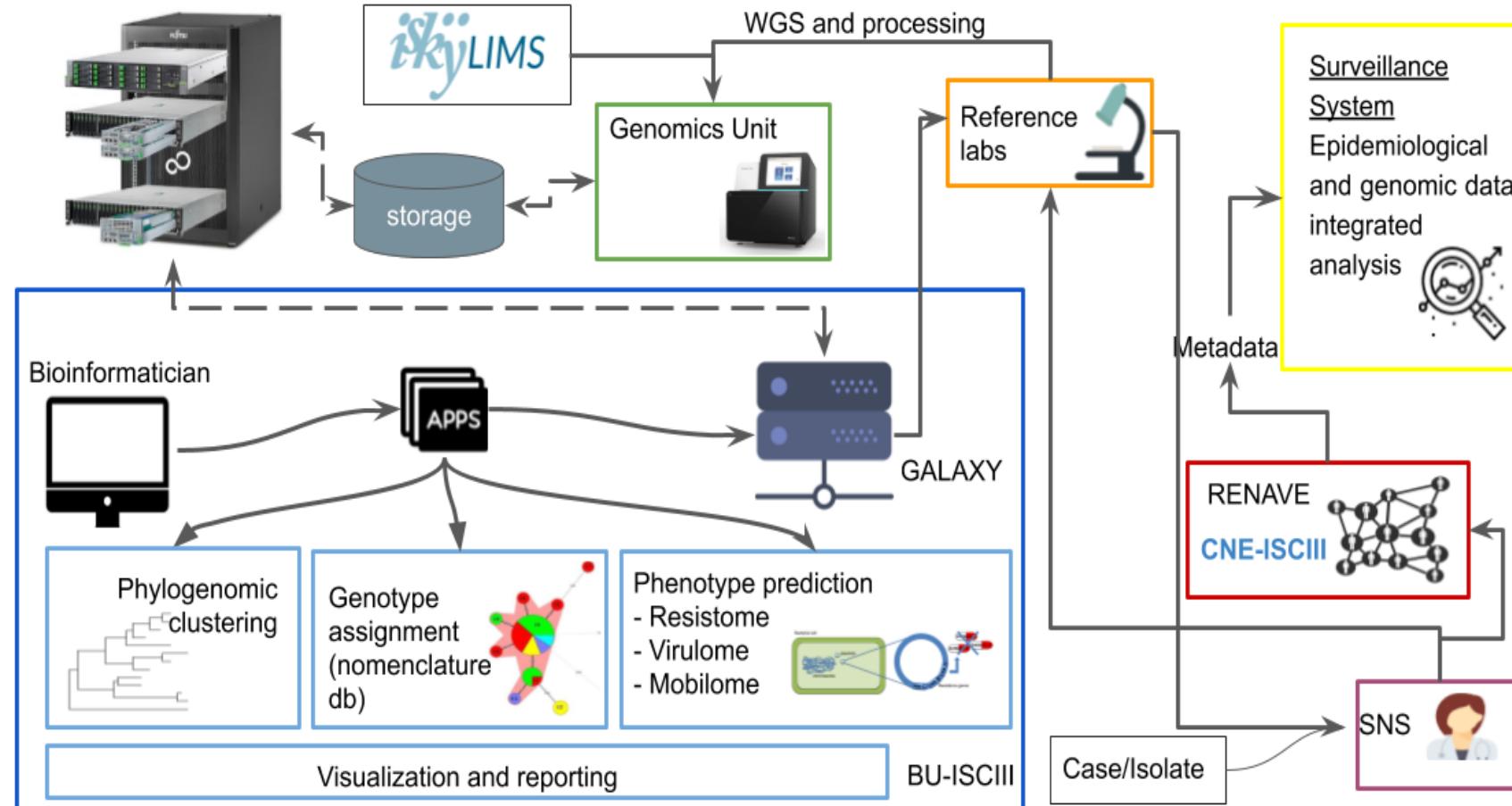
Outbreak research:
Molecular source
detection

ECDC roadmap and international commitment



- **Operationalisation of EU-wide WGS-based surveillance systems in the near term:** start implementation of WGS-based surveillance for *Listeria monocytogenes*, *Neisseria meningitidis*, Carbapenemase-producing *Enterobacteriaceae* and antibiotic-resistant *Neisseria gonorrhoeae*; 2018

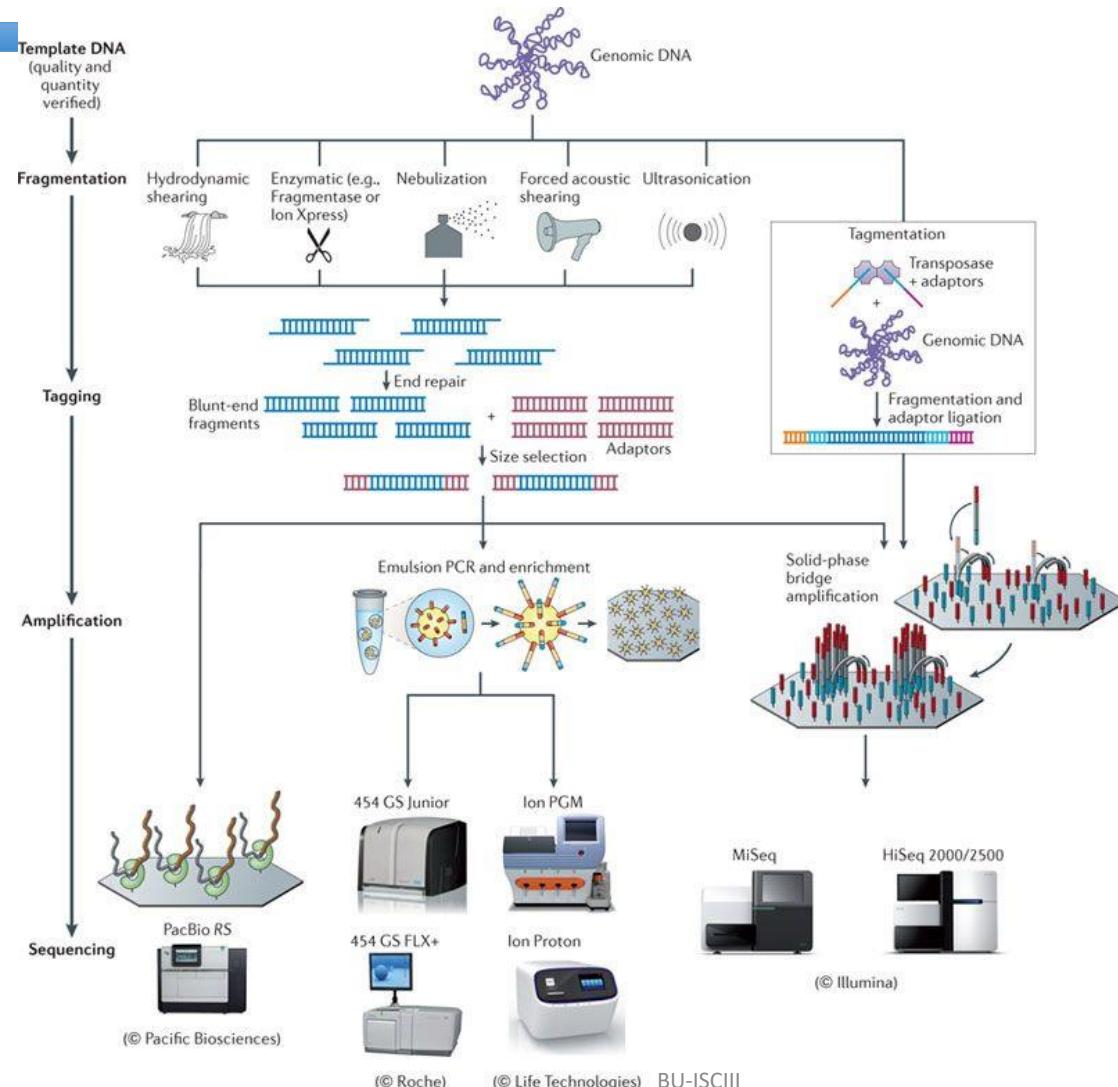
Bioinformatics Unit preparedness



- Infrastructure
- Data
- Sample tracking
- Sharing and intercommunication
- Bioinformatics analysis

Adapted and extended from Expert opinion on whole genome sequencing for public health surveillance. ECDC

High-throughput sequencing platforms



Loman et al, 2012

PREPARACIÓN LIBRERÍA, estrategias

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

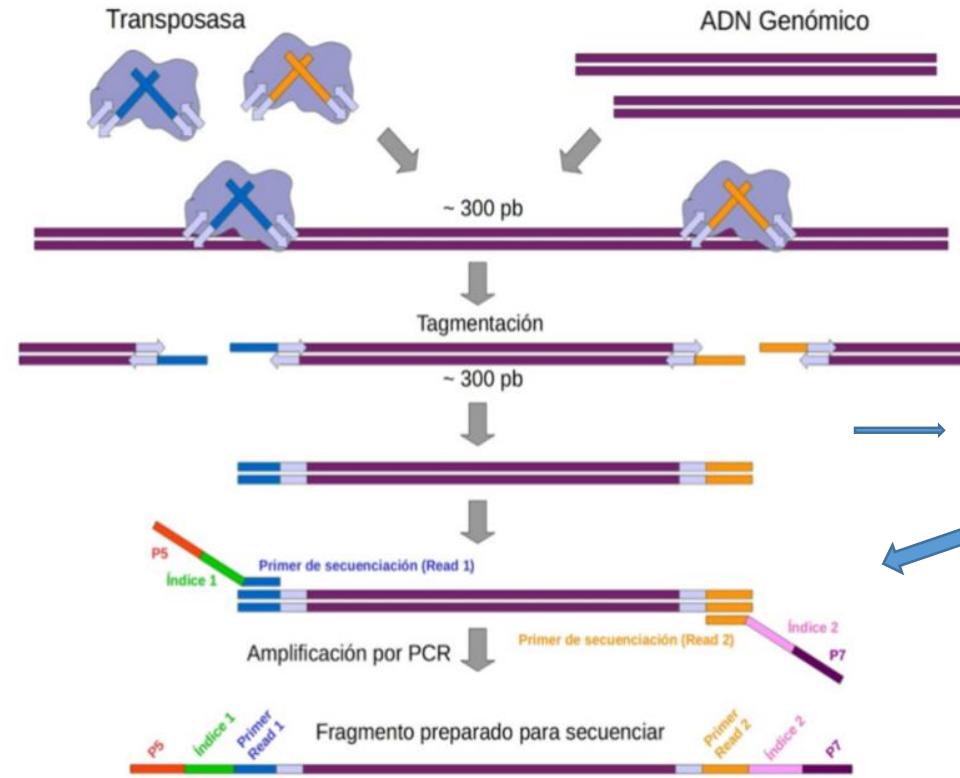
1. Metagenómica

IDENTIFICACIÓN MICROORGANISMOS

1. Metataxonomía

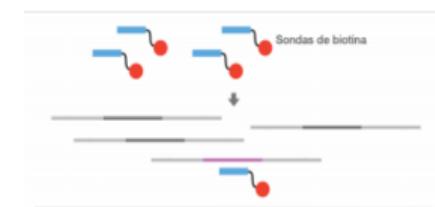
PREPARACIÓN LIBRERÍA

ENZIMÁTICA FÍSICA



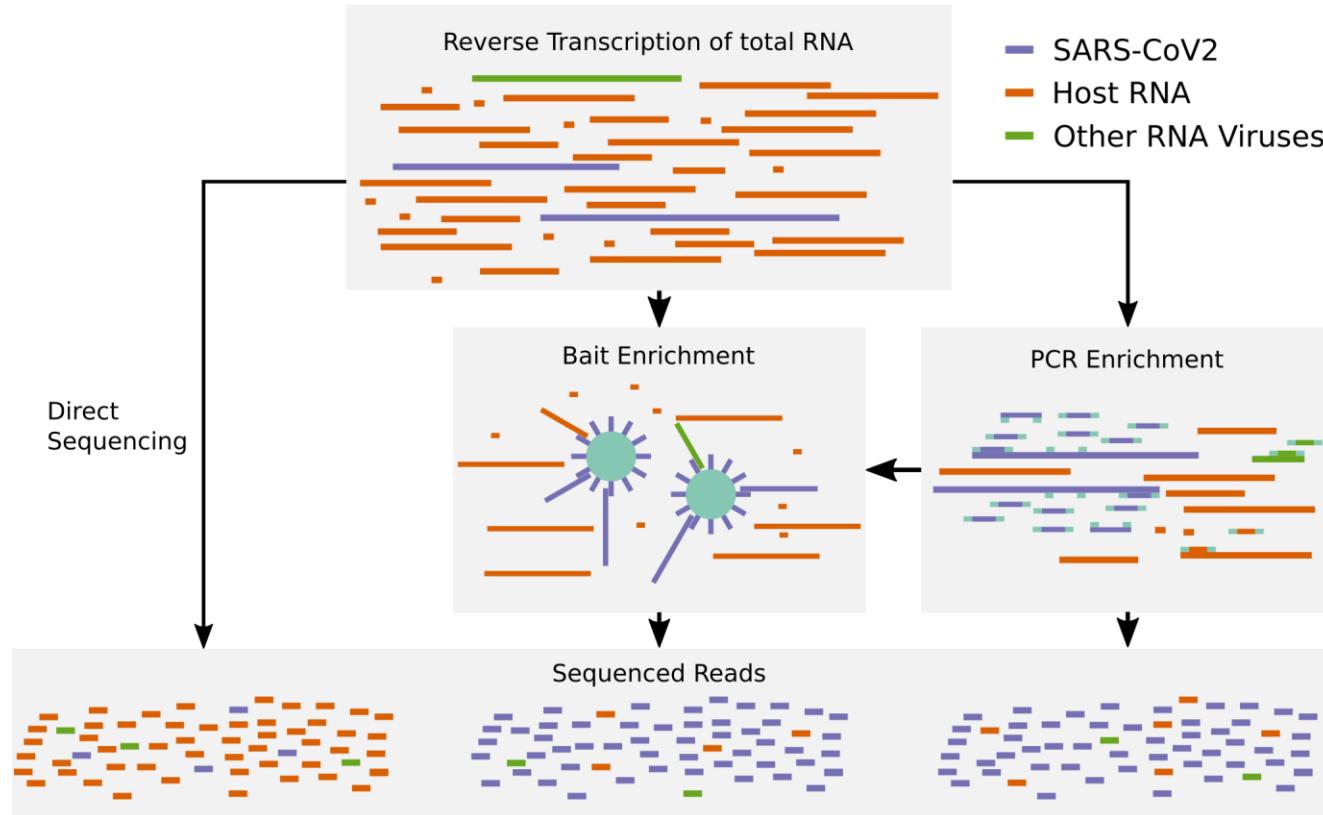
RNA → cDNA

ENRIQUECIMIENTO:
PCR
CAPTURA SONDAS

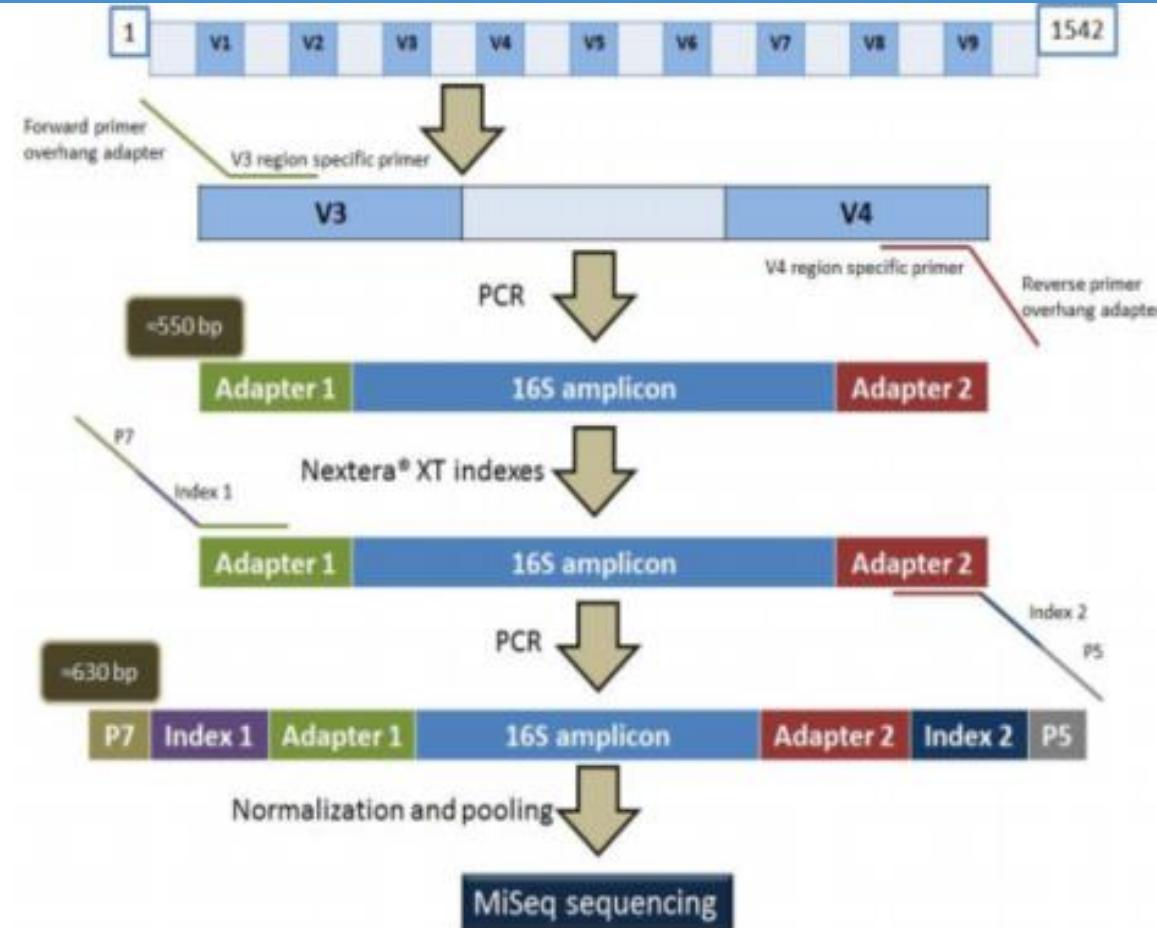


Adaptado de Guía Práctica Genómica https://www.uv.es/varnau/GM_Cap%C3%ADtulo_2.pdf

PREPARACIÓN LIBRERÍA



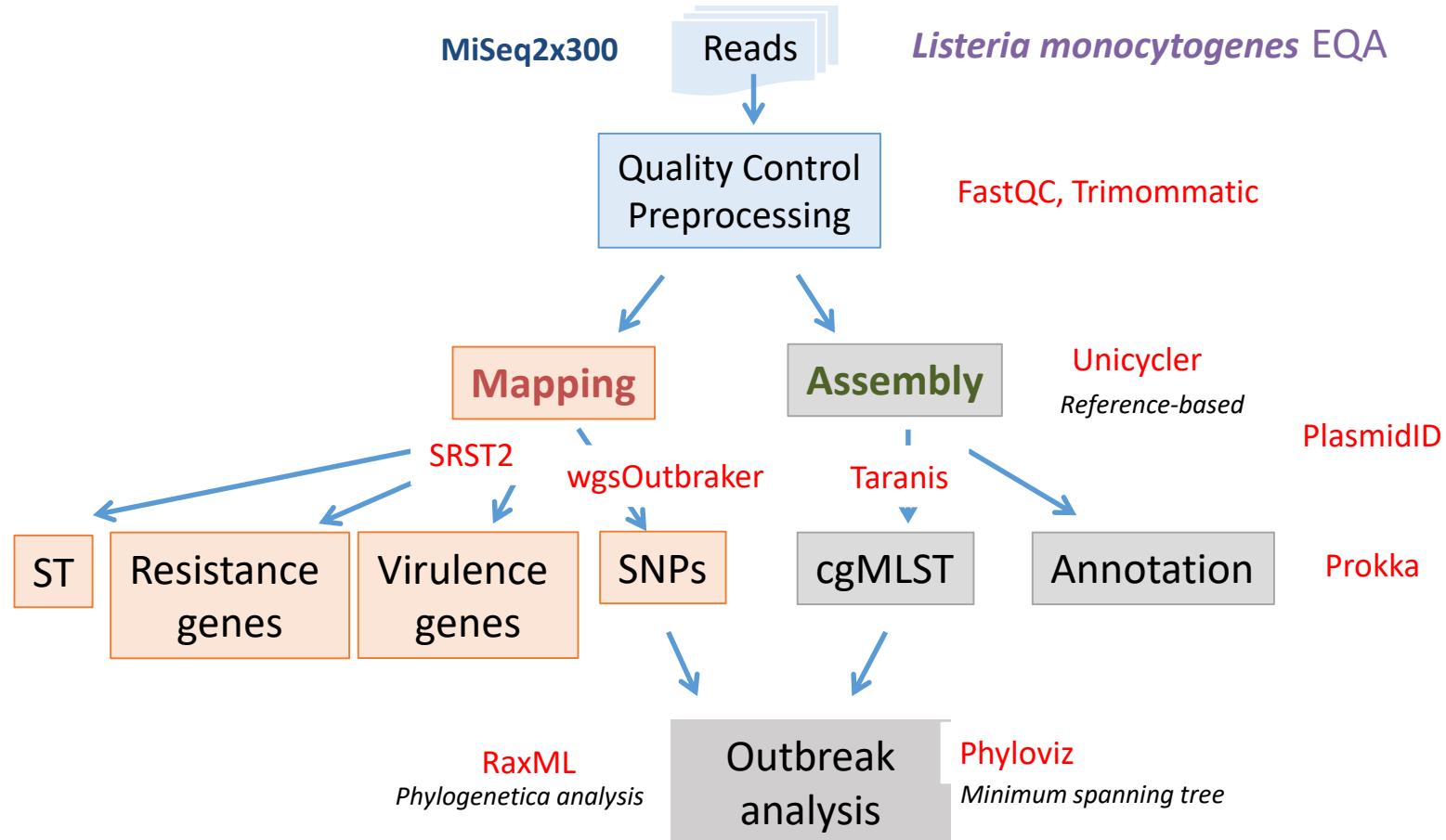
PREPARACIÓN LIBRERÍA, rRNA 16S, caracterización microbiota



Bioinformatics analysis in microbial genomics

- SPECIE IDENTIFICATION
 - WGS - Kmers analysis
 - TARGETED METAGENOMIC, rRNA - MICROBIOTA
- ASSEMBLY GENOME
 - de NOVO or REFERENCE -BASED
 - cgMLST, wgMLST - MINIMUM SPANING TREE
 - METAGENOMIC - HOMOLOGY -BASED
- VARIANT CALLING
 - REFERENCE GENOME SELECTION
 - HAPLOID GENOME
 - LOW FREQUENCY VARIANT - QUASISPECIES
 - SNPs MATRIX - PHYLOGENETIC ANALYSIS
- STRUCTURAL AND FUNCTIONAL ANNOTATION
 - RESISTOME, VIRULOME, SEQUENCE-TYPE

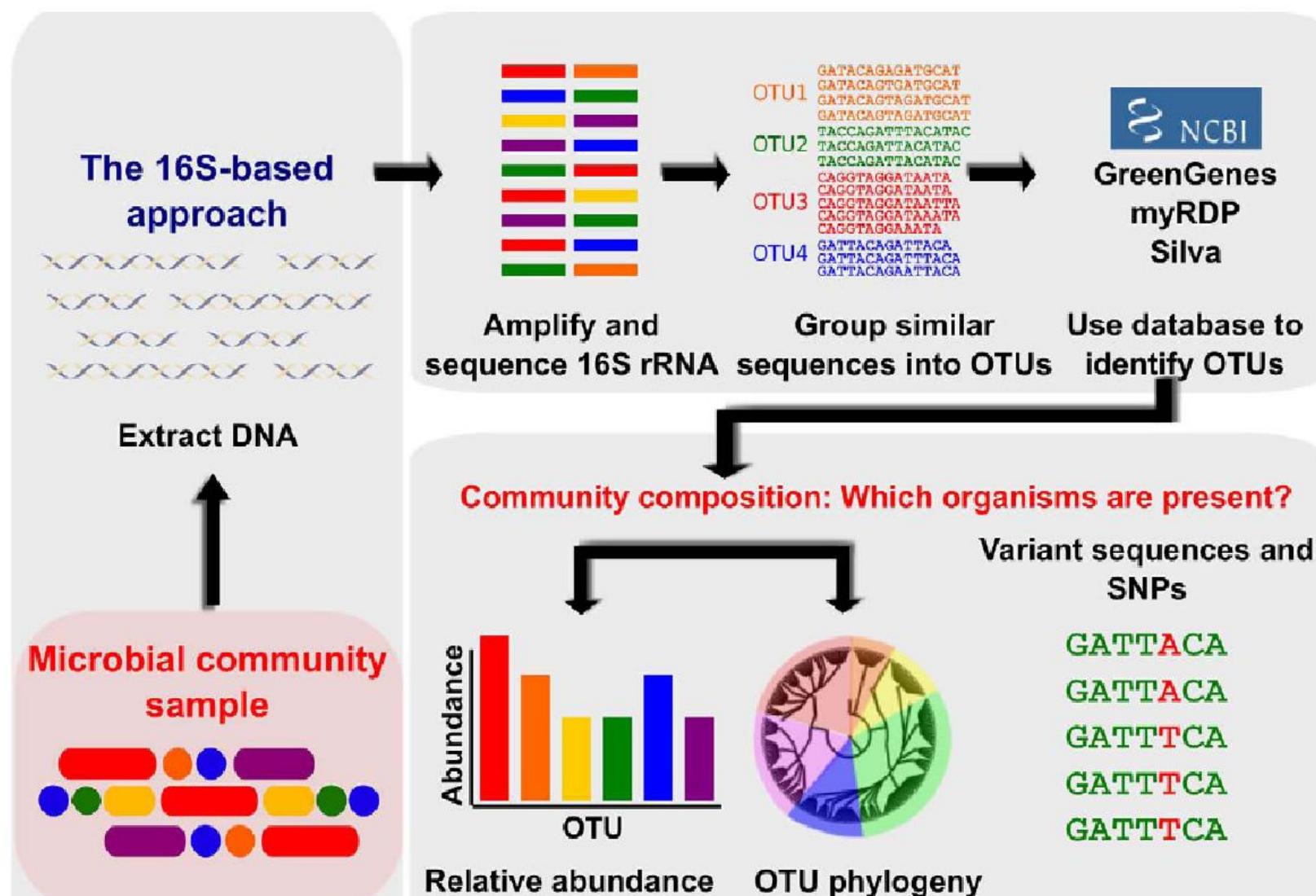
Workflow example



Metataxonomics or targeted metagenomics vs Metagenomics (16S - targeted 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

Metataxonomics



Metataxonomics

Etapas:

- ① Filtrado
- ② Eliminación de quimeras y otras anomalías
- ③ Formación de OTU
- ④ Identificación de los OTU con organismos en bases de datos

Algunos paquetes permiten llevar a cabo todo el proceso:

- mothur: <http://www.mothur.org>
- QIIME: <http://qiime.org>

Metataxonomics

Problemas:

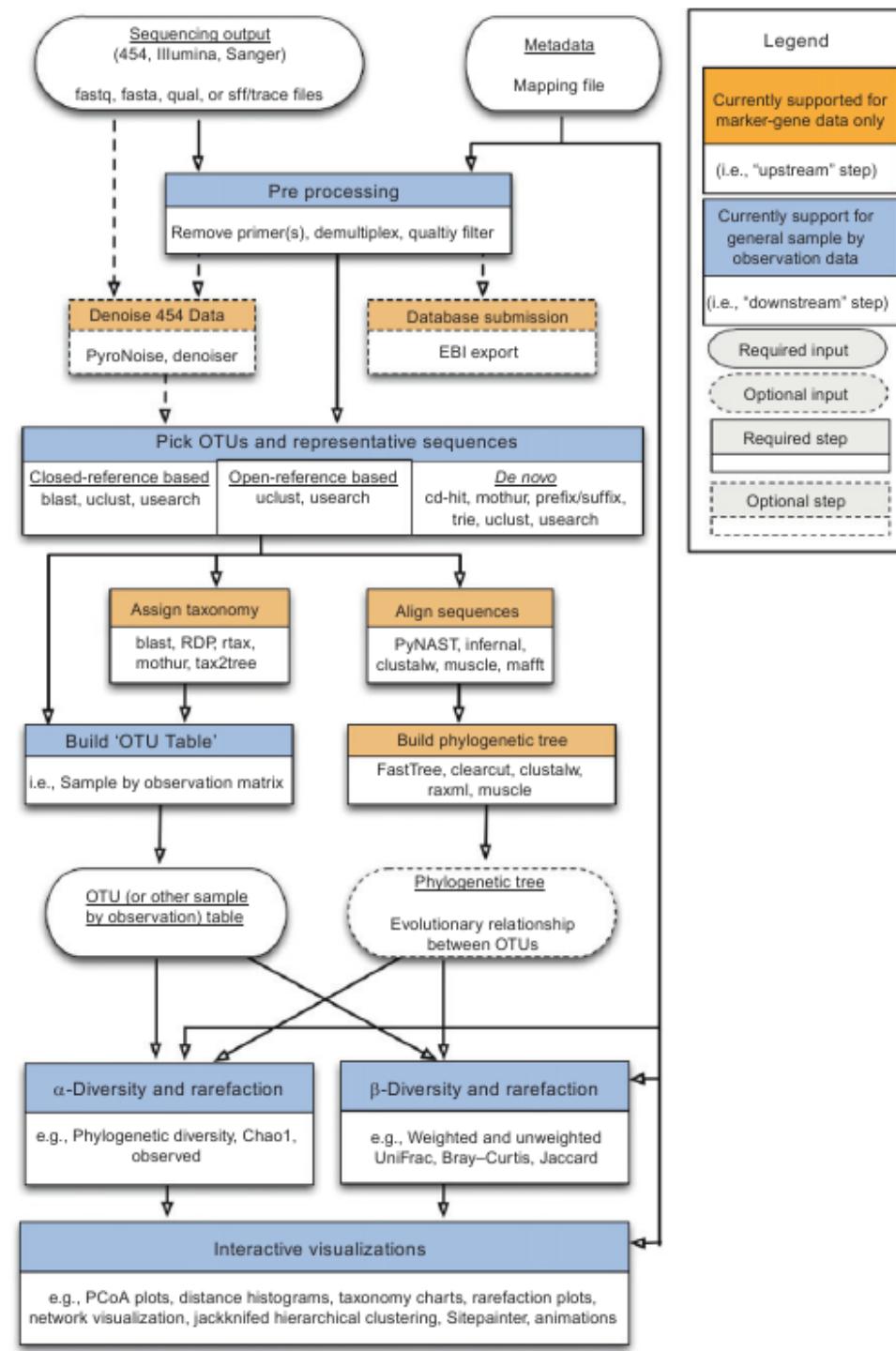
- Raros en el genoma (< 0.1%)
- Los trozos similares dificultan el ensamblado correcto de lecturas pequeñas
- No todos los rRNA se amplifican en la misma medida con los *primers* universales
- Especies con diversas copias de sus genes rRNA
- **No se conoce un umbral fijo de similitud que separe especies**
- Tendencia a producirse quimeras en la PCR

Targeted metagenomics

QIIME WORKFLOW

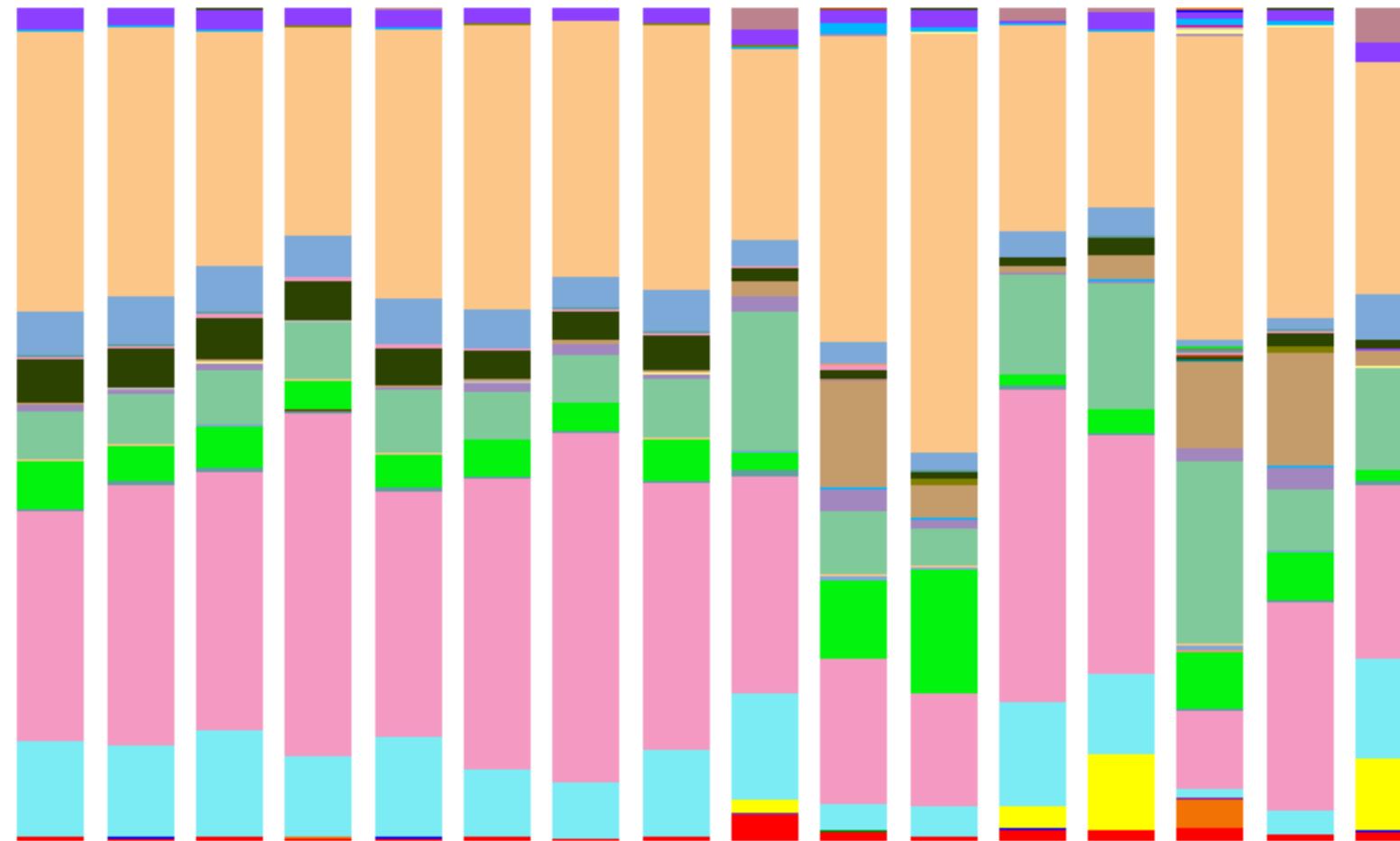
Integrated pipeline of
third-party tools

Navas-Molina et al., Methods in
enzymology 2013, 531: 371-439

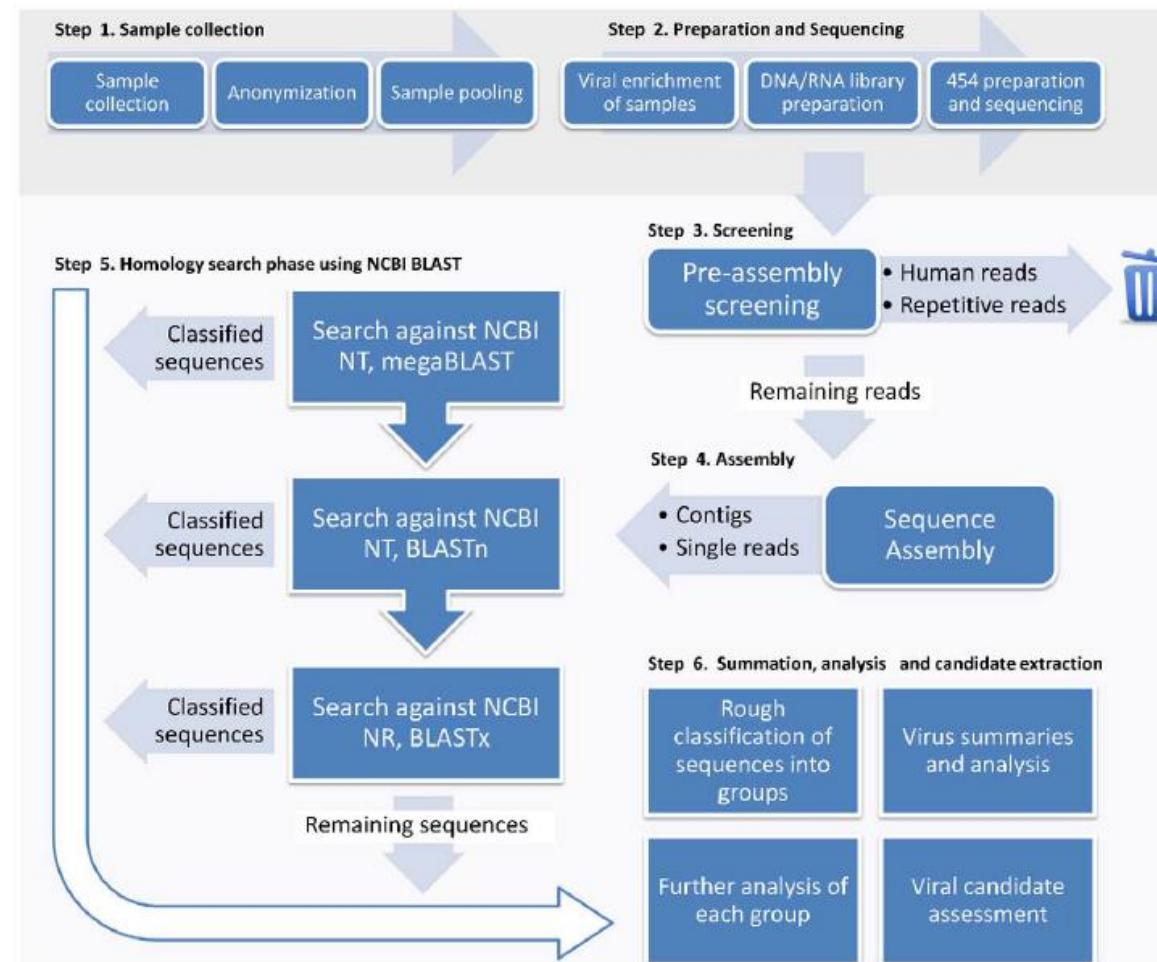


Currently supported for marker-gene data only (i.e., "upstream" step)
Currently support for general sample by observation data (i.e., "downstream" step)
Required input
Optional input
Required step
Optional step

Taxonomy summary (i.e. phylum level)

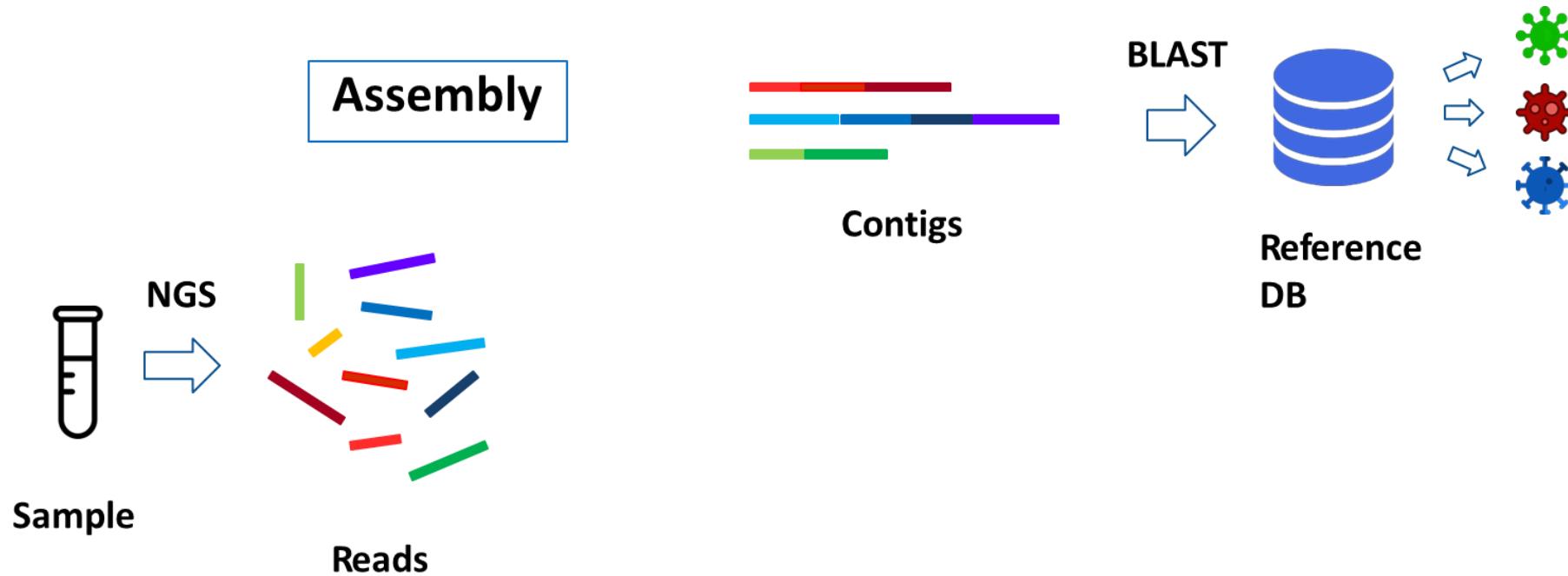


Metagenómica, pipeline de análisis

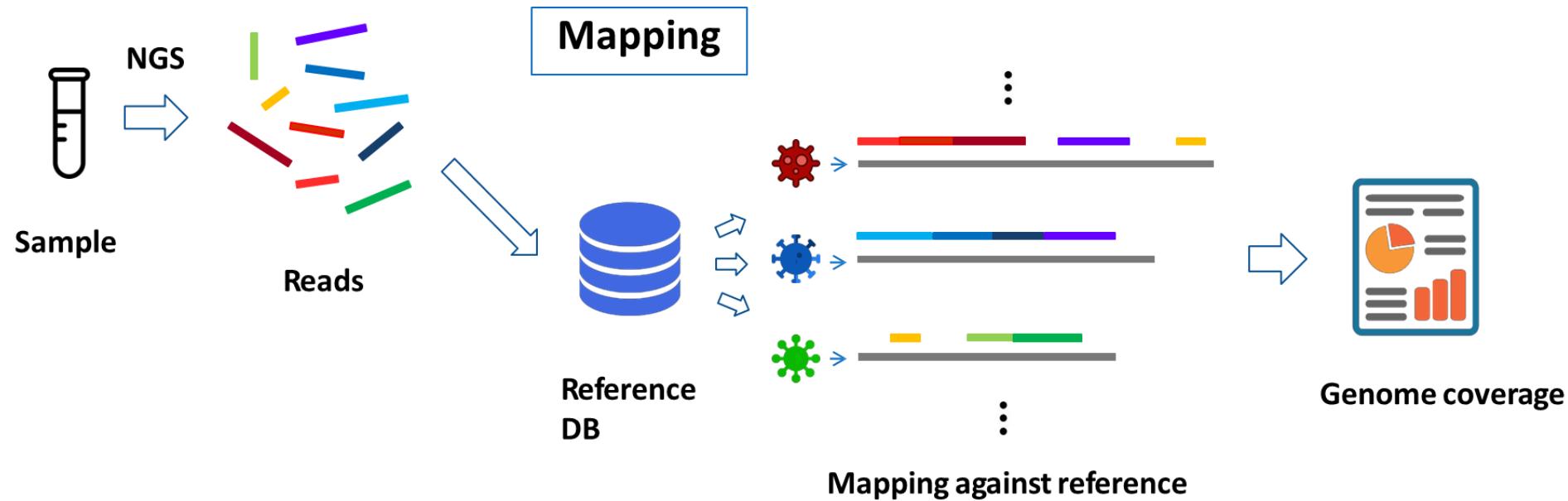


Lysholm et al., Plos One 2012:7,2, e30875

Metagenomic analysis approaches



Metagenomic analysis approaches

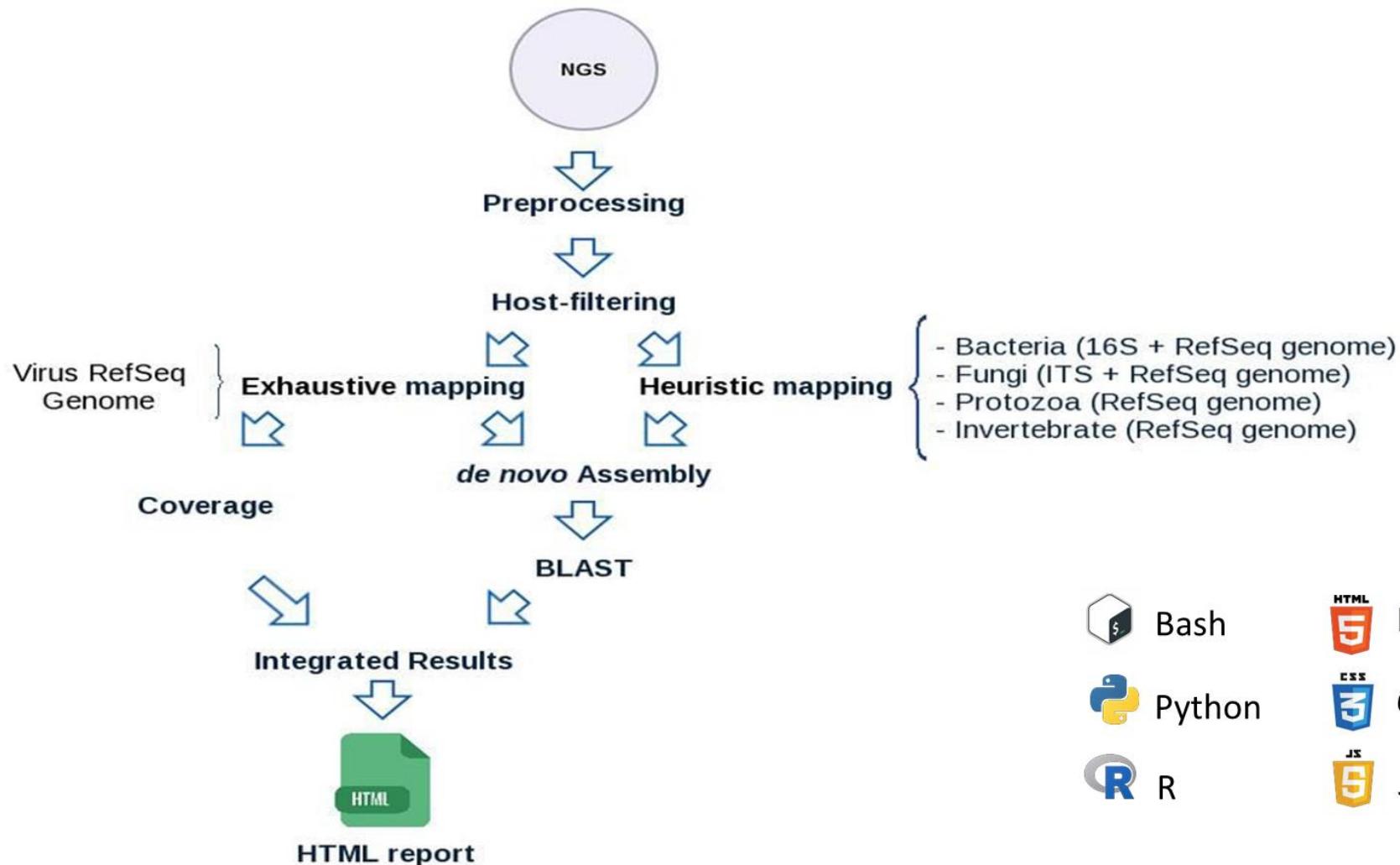


Metataxonomics vs Metagenomics (16S vs Shotgun)

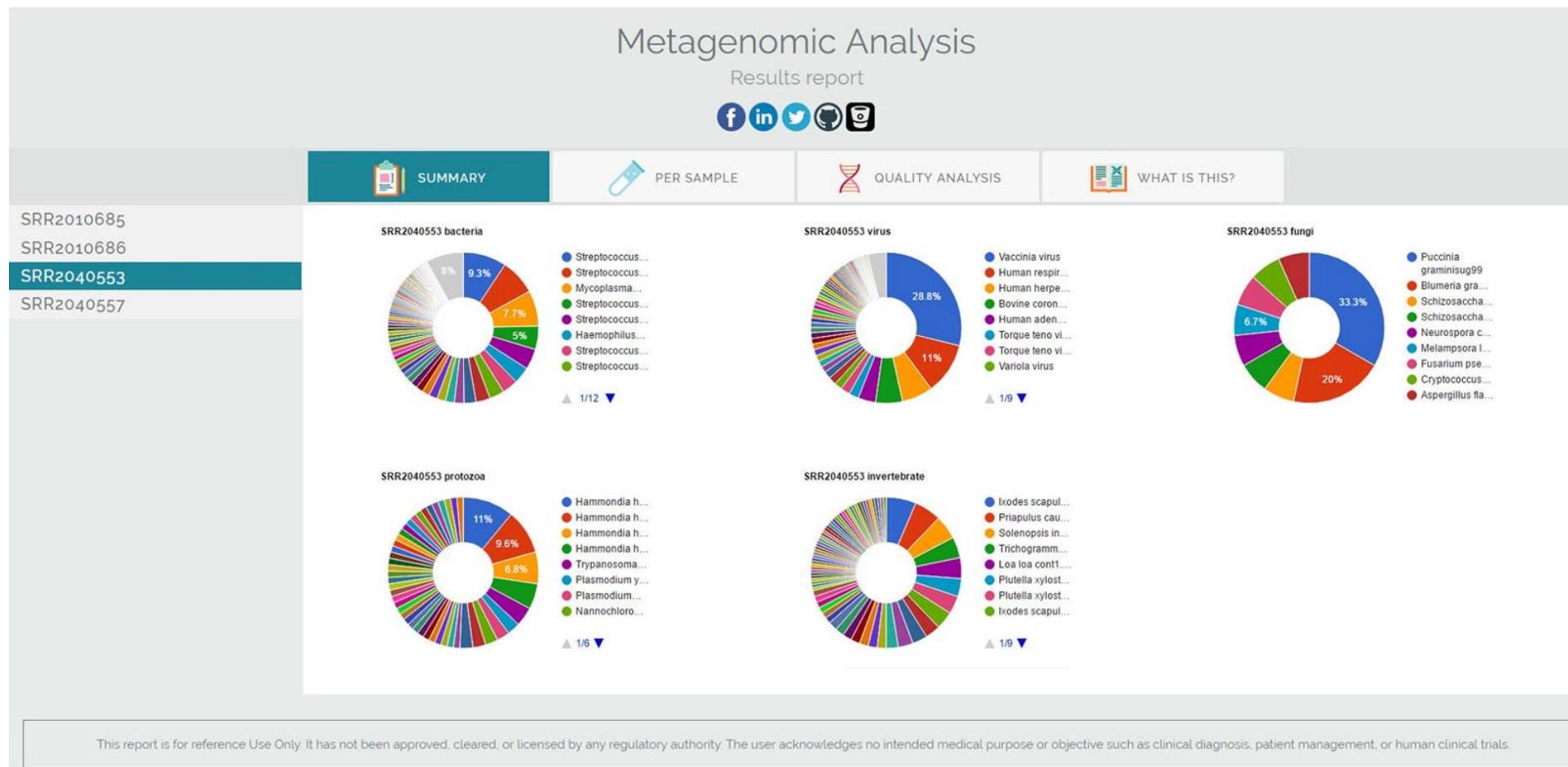
Software	Organism	Genetic portion used		Binning algorithm used			Genome coverage	Novel pathogen discovery
		Genetic markers	Whole Genome	Clustering	Mapping	Assembly		
Mothur	Bacteria	X		X			No	No
QIIME	Bacteria	X		X		X	No	No
MEGAN	Bacteria		X			X	No	No
Platypus	Bacteria		X		X		No	No
SURPI	Virus		X		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

Metagenomic analysis: PikaVirus

> BU-ISCIII



Metagenomic analysis: PikaVirus



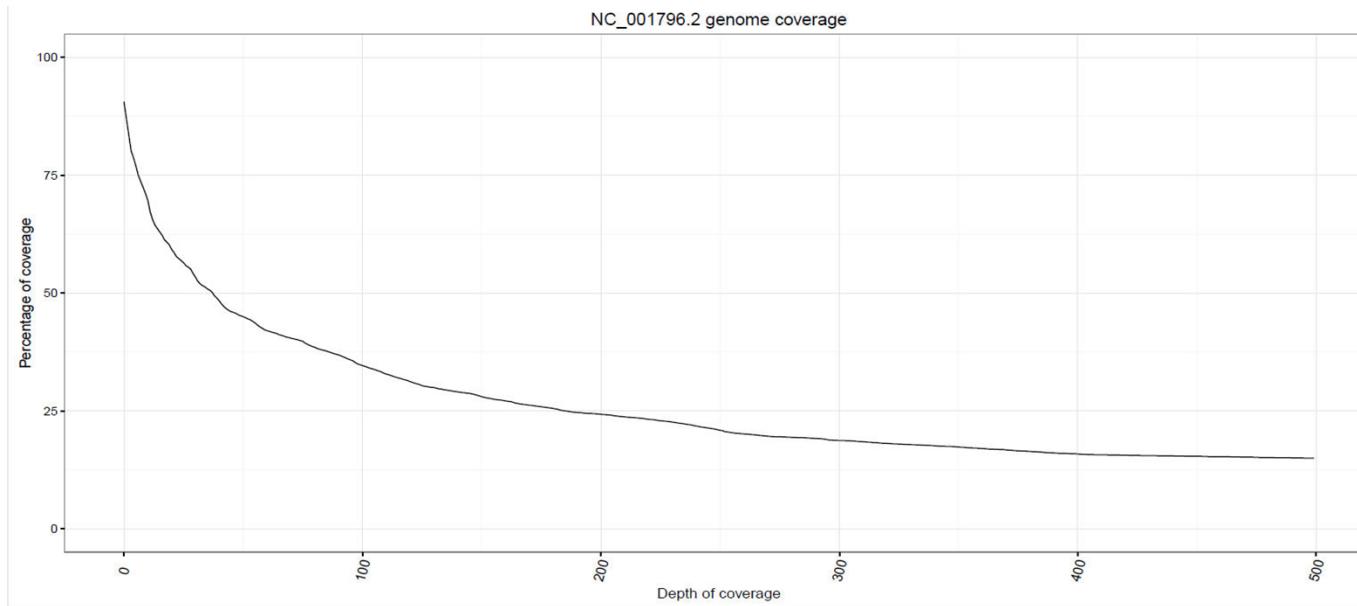
Metagenomic analysis: PikaVirus

Metagenomic Analysis
Results report

	SUMMARY	PER SAMPLE	QUALITY ANALYSIS	WHAT IS THIS?						
SRR2010685	SRR2010686 virus result	Reference Id	Reference name	Contig Id	% of identical matches	Alignment length	Number of mismatches	Number of gap openings	Start of alignment in query	End of alignment in query
SRR2010686		AC_000007.1	Human adenovirus 2, complete genome	NODE_206_length_317_cov_8.08779	99.12	227	1	1	66	291
	BACTERIA	AC_000008.1	Human adenovirus 5, complete genome	NODE_206_length_317_cov_8.08779	99.12	226	0	1	66	291
	VIRUS	AC_000010.1	Simian adenovirus 21, complete genome	NODE_245_length_289_cov_3.17949	91.02	256	23	0	1	256
	FUNGI	AC_000010.1	Simian adenovirus 21, complete genome	NODE_345_length_215_cov_2.625	93.85	179	7	2	40	214
	PROTOZOA	AC_000017.1	Human adenovirus type 1, complete genome	NODE_206_length_317_cov_8.08779	99.12	227	1	1	66	291
	INVERTEBRATE	AC_000018.1	Human adenovirus type 7, complete genome	NODE_228_length_302_cov_2.2996	100	302	0	0	1	302
SRR2040553		AC_000018.1	Human adenovirus type 7, complete genome	NODE_245_length_289_cov_3.17949	99.65	289	1	0	1	289
SRR2040557		AC_000018.1	Human adenovirus type 7, complete genome	NODE_250_length_285_cov_1.82609	96.68	241	8	0	45	285
		AC_000018.1	Human adenovirus type 7, complete genome	NODE_130_length_317_cov_31.7473	98.42	317	5	0	1	317
		AC_000018.1	Human adenovirus type 7, complete genome	NODE_308_length_241_cov_12.1237	98.46	130	2	0	112	241
		AC_000018.1	Human adenovirus type 7, complete genome	NODE_345_length_215_cov_2.625	92.61	230	2	2	1	215
		AC_000018.1	Human adenovirus type 7, complete genome	NODE_346_length_215_cov_2.1875	99.07	215	2	0	1	215

This report is for reference Use Only. It has not been approved, cleared, or licensed by any regulatory authority. The user acknowledges no intended medical purpose or objective such as clinical diagnosis, patient management, or human clinical trials.

Metagenomic analysis: PikaVirus

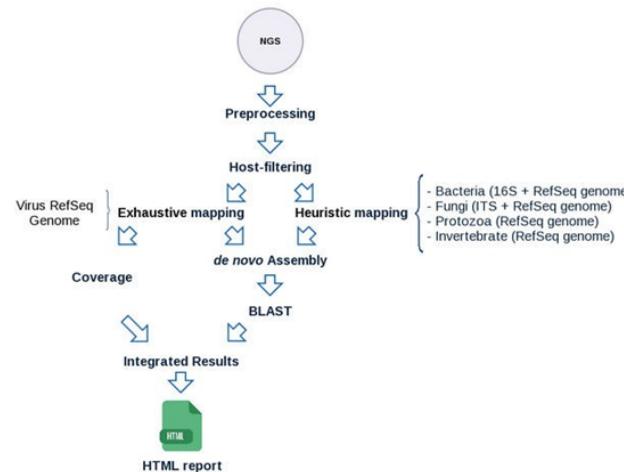


Wiki page and Project code

Welcome to pikAVIRUS

This project includes scripts to run metagenomic analysis on a single or several samples.

Workflow



Important!

First things first, for this to work there are a few dependencies you need to have installed. Also, it is necessary to have a refseq DB for every organism group you want to search for in your samples. You can find the dependency list in [Dependencies](#) and the procedure to generate the DB files in [References](#).

► Pages 31

- Home
- Dependencies
- References
 - Host
 - Bacteria
 - Virus
 - Fungi
 - Invertebrate
 - Protozoa
- Usage
 - i. Configuration file
 - ii. Quality control
 - iii. Mapping
 - Host filtering
 - Bacteria
 - Virus
 - Fungi
 - Parasite
 - iv. Assembly
 - v. BLAST
 - vi. Coverage
 - vii. Results
 - Summary
 - By Sample
 - Quality
 - Info
- Directory Structure

Clone this wiki locally

<https://github.com/AndreaRP/> 

 Clone in Desktop

<https://github.com/BU-ISCIII>

Table 3 Display of features of current bioinformatics pipelines for metagenomic data analysis

Tasks/Pipeline	Quality control	Assembly	Gene detection	Functional annotation	Taxonomic analysis	Comparative analysis	Data management
Clover metagenomics	No	No	Yes	Yes	Yes	Yes	Yes
Galaxy platform ^a	Yes	No	No	No	Yes	Yes	No
IMG/M	No	No	Yes	Yes	Yes	Yes	Yes
MetAMOS	Yes	Yes	Yes	Yes	Yes	Yes	Yes
MG-RAST	No	No	Yes	Yes	Yes	Yes	Yes
RAMMCAP	No	No	Yes	Yes	Yes	Yes	No
Smash Community	Yes	Yes	Yes	Yes	Yes	Yes	No

^a Refers to the metagenomics pipeline of galaxy

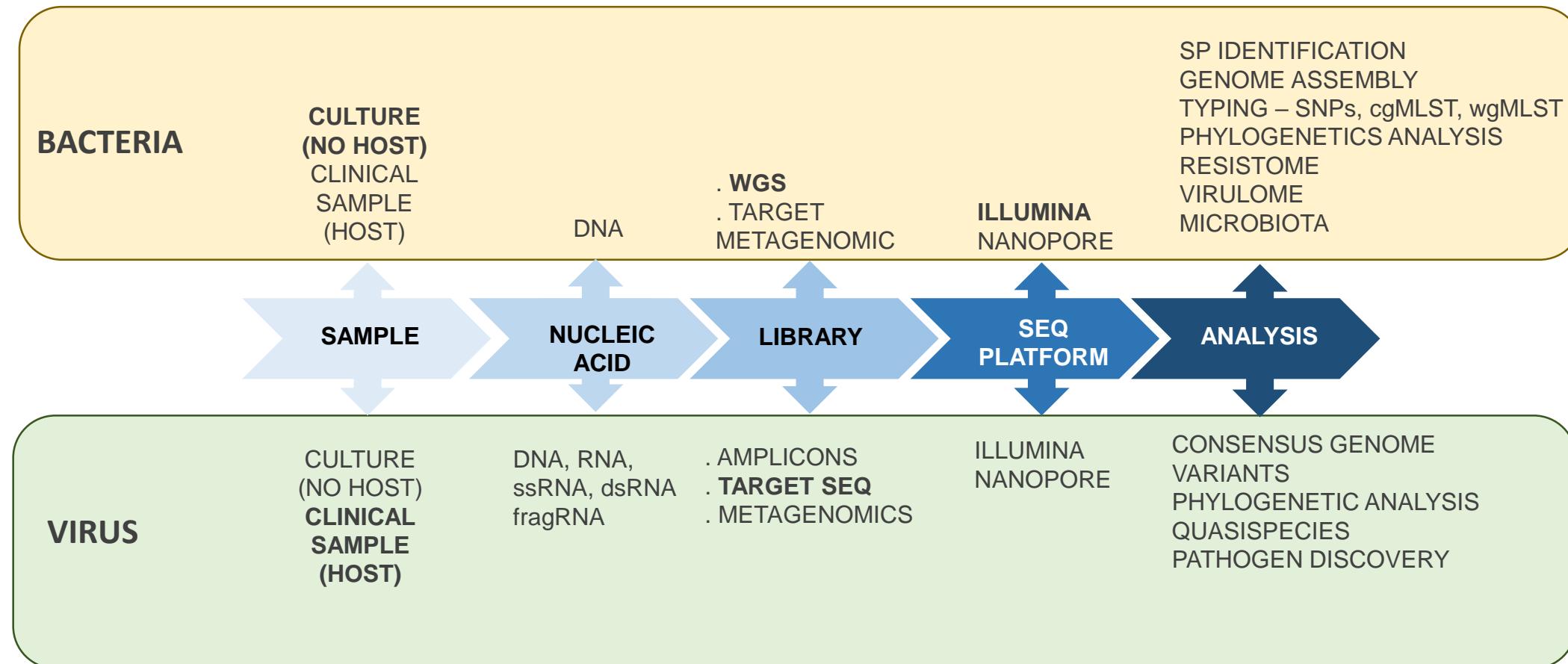
Navgire et al. Biological Procedures Online 2022

Table 1. Summary and comparison of some commercial kits and pros and cons of different sequencing techniques in COVID-19.

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	Shotgun Metagenomics	Capture-Based Enrichment Targeted Sequencing	Amplicon-Based Enrichment Targeted Sequencing
Examples of commercial kits	Illumina Stranded Total RNA Prep with Ribo-Zero Plus	Illumina Respiratory Virus Oligo Panel Illumina Respiratory Pathogen ID/AMR Enrichment Panel Kit Roche KAPA SARS-CoV-2 Target Enrichment Panel Biosystem TWIST. SARS-CoV-2 Research Panel	Illumina COVIDSeq Test ThermoFisher Ion AmpliSeq™ SARS-CoV-2 Research Panel Paragon Genomics CleanPlex® SARS-CoV-2 Panel Qiagen QIAseq SARS-CoV-2 Primer Panel
Characteristics			
Turnaround time	Long	Moderate	Short
Cost	High	Moderate to Low	Low
The complexity of the workflow	Moderate	Moderate to Low	Low
Coverage of the genome	High	Moderate with high uniformity	Low, with variable uniformity
Sequence depth	Low	High	High
The amount of starting material	High	Moderate to Low	Low
Sensitivity to the target	Low	High	High
Sensitivity to the background	High	Low	Low
Susceptibility to mutational effect	Low	High	High
Applications			
Track transmission	Yes	Yes	Yes
Identification of novel pathogen	Yes	No	No
Identification of co-infections and complex disease	Yes	Only Illumina respiratory panels	No
Identification of new mutations	Yes	Yes	No

Bacterial and viral Genome Sequencing



Aplicaciones basadas en la preparación de librería

RNA Structure	30
Selective 2'-Hydroxyl Acylation Analyzed by Primer Extension Sequencing (SHAPE-Seq)	31
Parallel Analysis of RNA Structure (PARS-Seq)	32
Fragmentation Sequencing (FRAG-Seq)	33
CXXC Affinity Purification Sequencing (CAP-Seq)	34
Alkaline Phosphatase, Calf Intestine-Tobacco Acid Pyrophosphatase Sequencing (CIP-TAP)	36
Inosine Chemical Erasing Sequencing (ICE)	38
m6A-Specific Methylated RNA Immunoprecipitation Sequencing (MeRIP-Seq)	39
 Low-Level RNA Detection	40
Digital RNA Sequencing	42
Whole-Transcript Amplification for Single Cells (Quartz-Seq)	43
Designed Primer-Based RNA Sequencing (DP-Seq)	44
Switch Mechanism at the 5' End of RNA Templates (Smart-Seq)	45
Switch Mechanism at the 5' End of RNA Templates Version 2 (Smart-Seq2)	47
Unique Molecular Identifiers (UMI)	49
Cell Expression by Linear Amplification Sequencing (CEL-Seq)	51
Single-Cell Tagged Reverse Transcription Sequencing (STRT-Seq)	52

Sequencing Methods Review

A review of publications featuring Illumina® Technology

Aplicaciones basadas en la preparación de librería

RNA Transcription	5
Chromatin Isolation by RNA Purification (ChIRP-Seq)	7
Global Run-on Sequencing (GRO-Seq)	9
Ribosome Profiling Sequencing (Ribo-Seq)/ARTseq™	12
RNA Immunoprecipitation Sequencing (RIP-Seq)	15
High-Throughput Sequencing of CLIP cDNA library (HITS-CLIP) or	17
Crosslinking and Immunoprecipitation Sequencing (CLIP-Seq)	17
Photoactivatable Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP)	19
Individual Nucleotide Resolution CLIP (iCLIP)	22
Native Elongating Transcript Sequencing (NET-Seq)	24
Targeted Purification of Polysomal mRNA (TRAP-Seq)	25
Crosslinking, Ligation, and Sequencing of Hybrids (CLASH-Seq)	26
Parallel Analysis of RNA Ends Sequencing (PARE-Seq) or	27
Genome-Wide Mapping of Uncapped Transcripts (GMUCT)	27
Transcript Isoform Sequencing (TIF-Seq) or	29
Paired-End Analysis of TSSs (PEAT)	29

Sequencing Methods Review

A review of publications featuring Illumina® Technology

Aplicaciones basadas en la preparación de librería

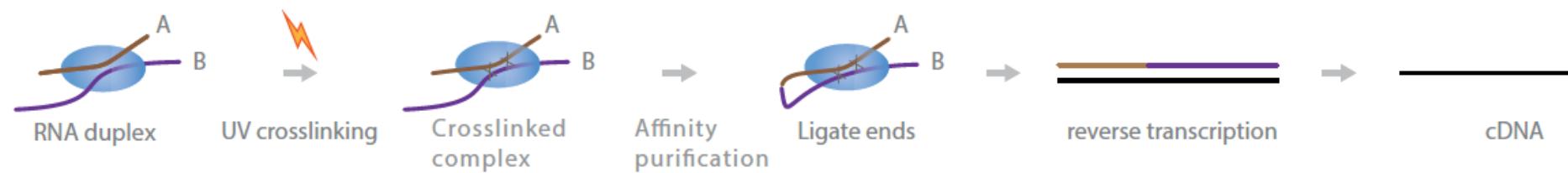
DNA Methylation	63
Bisulfite Sequencing (BS-Seq)	65
Post-Bisulfite Adapter Tagging (PBAT)	70
Tagmentation-Based Whole Genome Bisulfite Sequencing (T-WGBS)	72
Oxidative Bisulfite Sequencing (oxBS-Seq)	73
Tet-Assisted Bisulfite Sequencing (TAB-Seq)	74
Methylated DNA Immunoprecipitation Sequencing (MeDIP-Seq)	76
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Methyl-Binding-Domain–Capture (MBDCap) Sequencing	79
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DNase I Hypersensitive Sites Sequencing (DNase-Seq)	85
MNase-Assisted Isolation of Nucleosomes Sequencing (MAINE-Seq)	88
Chromatin Immunoprecipitation Sequencing (ChIP-Seq)	91
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Assay for Transposase-Accessible Chromatin Sequencing (ATAC-Seq)	96
Chromatin Interaction Analysis by Paired-End Tag Sequencing (ChIA-PET)	97
Chromatin Conformation Capture (Hi-C/3C-Seq)	99
Circular Chromatin Conformation Capture (4-C or 4C-Seq)	101
Chromatin Conformation Capture Carbon Copy (5-C)	104

Sequencing Methods Review

A BÜRSCHI publications featuring Illumina® Technology

CROSSLINKING, LIGATION, AND SEQUENCING OF HYBRIDS (CLASH-SEQ)

Crosslinking, ligation, and sequencing of hybrids (CLASH-Seq) maps RNA-RNA interactions¹⁸. In this method RNA-protein complexes are UV crosslinked and affinity-purified. RNA-RNA hybrids are then ligated, isolated, and reverse-transcribed to cDNA. Deep sequencing of the cDNA provides high-resolution chimeric reads of RNA-RNA interactions.



Pros	Cons
<ul style="list-style-type: none">Maps RNA-RNA interactionsPerformed <i>in vivo</i>	<ul style="list-style-type: none">Hybrid ligation may be difficult between short RNA fragments

CHROMATIN ISOLATION BY RNA PURIFICATION (CHIRP-SEQ)

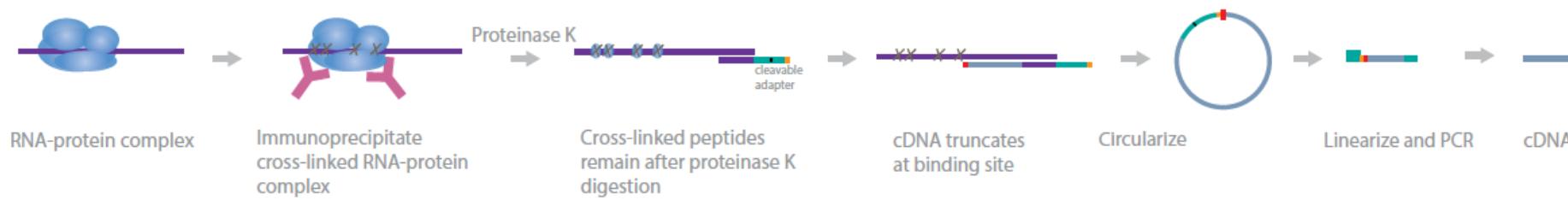
Chromatin isolation by RNA purification (ChIRP-Seq) is a protocol to detect the locations on the genome where non-coding RNAs (ncRNAs), such as long non-coding RNAs (lncRNAs), and their proteins are bound⁷. In this method, samples are first crosslinked and sonicated. Biotinylated tiling oligos are hybridized to the RNAs of interest, and the complexes are captured with streptavidin magnetic beads. After treatment with RNase H the DNA is extracted and sequenced. With deep sequencing the lncRNA/protein interaction site can be determined at single-base resolution.



Pros	Cons
<ul style="list-style-type: none">• Binding sites can be found anywhere on the genome• New binding sites can be discovered• Specific RNAs of interest can be selected	<ul style="list-style-type: none">• Nonspecific oligo interactions can lead to misinterpretation of binding sites• Chromatin can be disrupted during the preparation stage• The sequence of the RNA of interest must be known

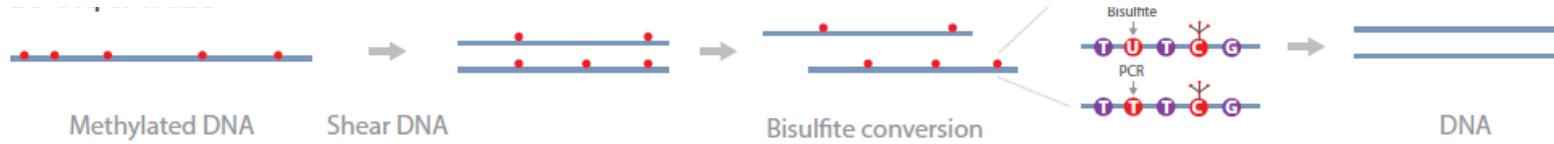
INDIVIDUAL NUCLEOTIDE RESOLUTION CLIP (iCLIP)

Individual nucleotide resolution CLIP (iCLIP) maps protein-RNA interactions similar to HITS-CLIP and PAR-CLIP¹⁵. This approach includes additional steps to digest the proteins after crosslinking and to map the crosslink sites with reverse transcriptase. In this method specific crosslinked RNA-protein complexes are immunoprecipitated. The complexes are then treated with proteinase K, as the protein crosslinked at the binding site remains undigested. Upon reverse transcription, cDNA truncates at the binding site and is circularized. These circularized fragments are then linearized and PCR-amplified. Deep sequencing of these amplified fragments provides nucleotide resolution of protein-binding site.

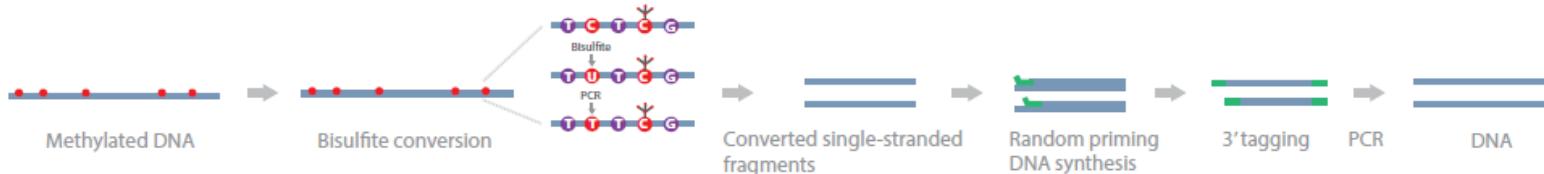


Pros	Cons
<ul style="list-style-type: none">Nucleotide resolution of protein-binding siteAvoids the use of nucleasesAmplification allows the detection of rare events	<ul style="list-style-type: none">Antibodies not specific to target will precipitate nonspecific complexesNon-linear PCR amplification can lead to biases affecting reproducibilityArtifacts may be introduced in the circularization step

BISULFITE SEQUENCING (BS-SEQ)



EpiGnome Methyl-Seq



Pros	Cons
BS-Seq or WGBS <ul style="list-style-type: none"> <i>CpG and non-CpG methylation throughout the genome is covered at single-base resolution</i> <i>5mC in dense, less dense, and repeat regions are covered</i> 	<ul style="list-style-type: none"> <i>Bisulfite converts unmethylated cytosines to thymidines, reducing sequence complexity, which can make it difficult to create alignments</i> <i>NPs where a cytosine is converted to thymidine will be missed upon bisulfite conversion</i> <i>Bisulfite conversion does not distinguish between 5mC and 5hmC</i>

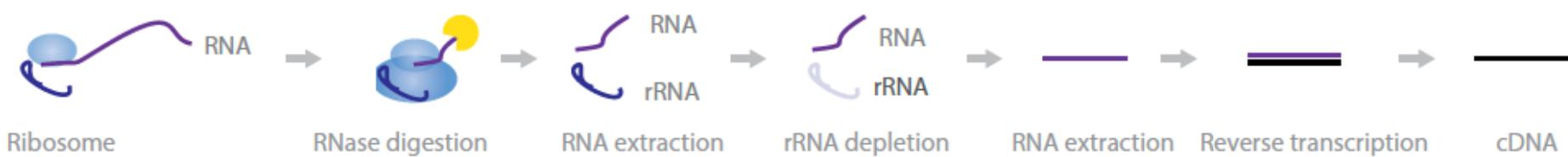
EpiGnome

- Pre-library bisulfite conversion*
- Low input gDNA (50 ng)*
- Uniform CpG, CHG, and CHH coverage*
- No fragmentation and no methylated adapters*
- Retention of sample diversity*
- Bisulfite converts unmethylated cytosines to thymidines, reducing sequence complexity, which can make it difficult to create alignments*
- SNPs where a cytosine is converted to thymidine will be missed upon bisulfite conversion*
- Bisulfite conversion does not distinguish between 5mC and 5hmC*

BULSCNII
High duplicate percentage

RIBOSOME PROFILING SEQUENCING (RIBO-SEQ)/ARTSEQ™

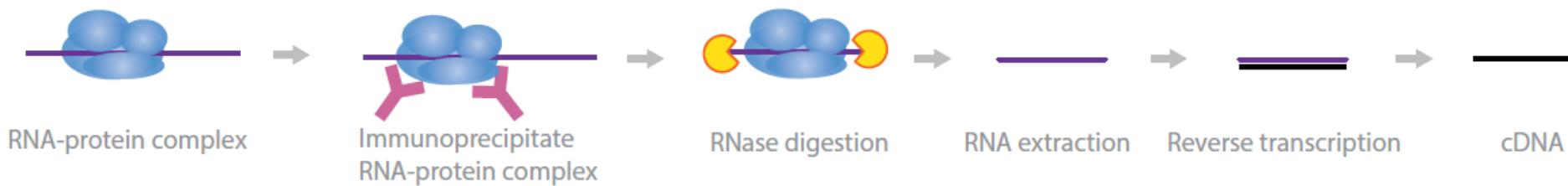
Active mRNA Translation Sequencing (ARTseq), also called ribosome profiling (Ribo-Seq), isolates RNA that is being processed by the ribosome in order to monitor the translation process¹⁰. In this method ribosome-bound RNA first undergoes digestion. The RNA is then extracted and the rRNA is depleted. Extracted RNA is reverse-transcribed to cDNA. Deep sequencing of the cDNA provides the sequences of RNAs bound by ribosomes during translation. This method has been refined to improve the quality and quantitative nature of the results. Careful attention should be paid to: (1) generation of cell extracts in which ribosomes have been faithfully halted along the mRNA they are translating *in vivo*; (2) nuclease digestion of RNAs that are not protected by the ribosome followed by recovery of the ribosome-protected mRNA fragments; (3) quantitative conversion of the protected RNA fragments into a DNA library that can be analyzed by deep sequencing¹¹. The addition of harringtonine (an alkaloid that inhibits protein biosynthesis) causes ribosomes to accumulate precisely at initiation codons and assists in their detection.



Pros	Cons
<ul style="list-style-type: none">• Reveals a snapshot with the precise location of ribosomes on the RNA• Ribosome profiling more closely reflects the rate of protein synthesis than mRNA levels• No prior knowledge of the RNA or ORFs is required• The whole genome is surveyed• Can be used to identify protein-coding regions	<ul style="list-style-type: none">• Initiation from multiple sites within a single transcript makes it challenging to define all ORFs• Does not provide the kinetics of translational elongation

RNA IMMUNOPRECIPITATION SEQUENCING (RIP-SEQ)

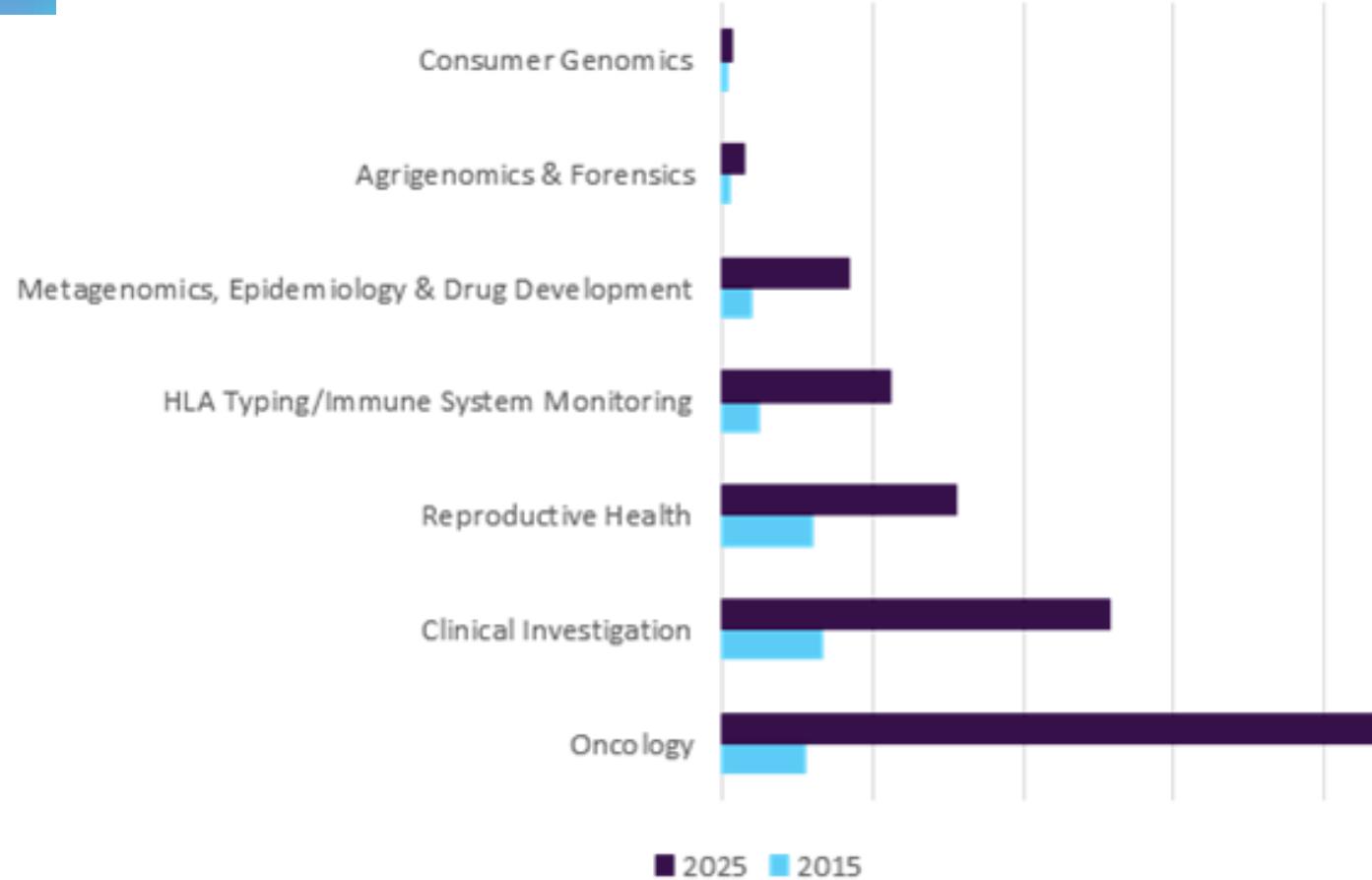
RNA immunoprecipitation sequencing (RIP-Seq) maps the sites where proteins are bound to the RNA within RNA-protein complexes¹². In this method, RNA-protein complexes are immunoprecipitated with antibodies targeted to the protein of interest. After RNase digestion, RNA covered by protein is extracted and reverse-transcribed to cDNA. The locations can then be mapped back to the genome. Deep sequencing of cDNA provides single-base resolution of bound RNA.



Pros	Cons
<ul style="list-style-type: none">Maps specific protein-RNA complexes, such as polycomb-associated RNAsLow background and higher resolution of binding site due to RNase digestionNo prior knowledge of the RNA is requiredGenome-wide RNA screen	<ul style="list-style-type: none">Requires antibodies to the targeted proteinsNonspecific antibodies will precipitate nonspecific complexesLack of crosslinking or stabilization of the complexes may lead to false negativesRNase digestion must be carefully controlled

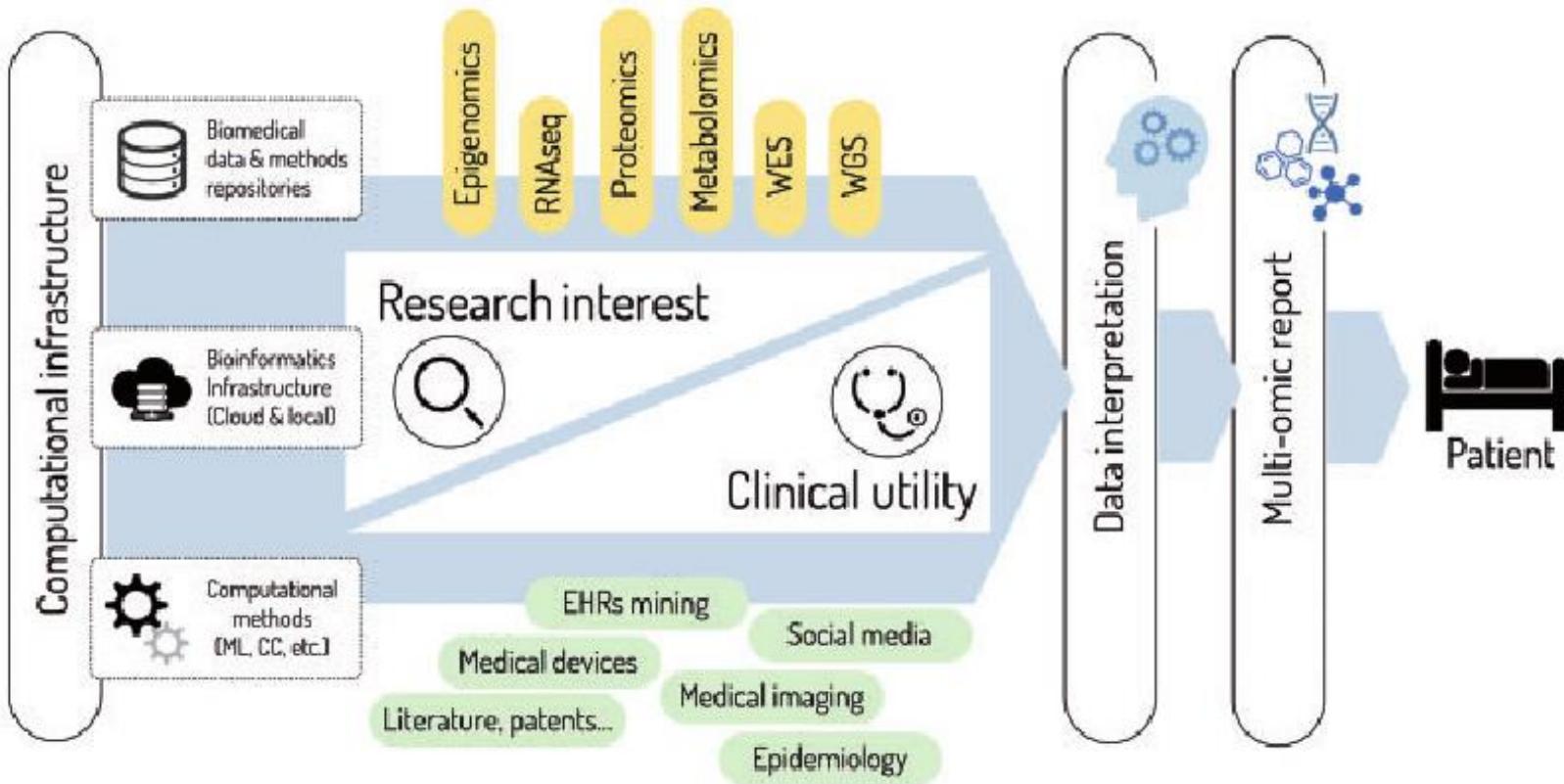


Global next generation sequencing market by applications, 2015 & 2025 (USD Million)



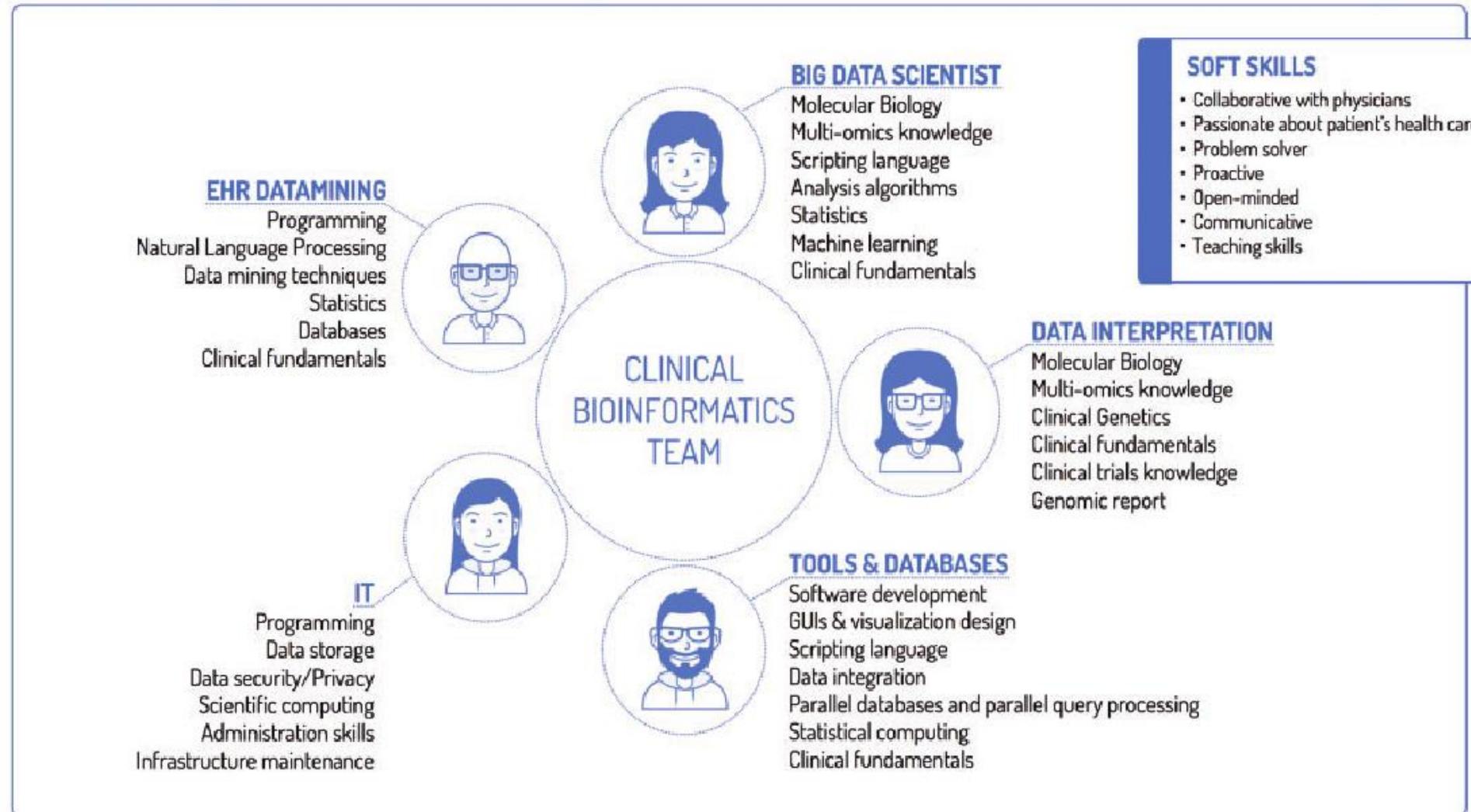
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Precision Medicine workflow: from data to patient care



Gómez-López et al., Brief Bioinf 2017, 1-15

Clinical bioinformatics laboratory profile



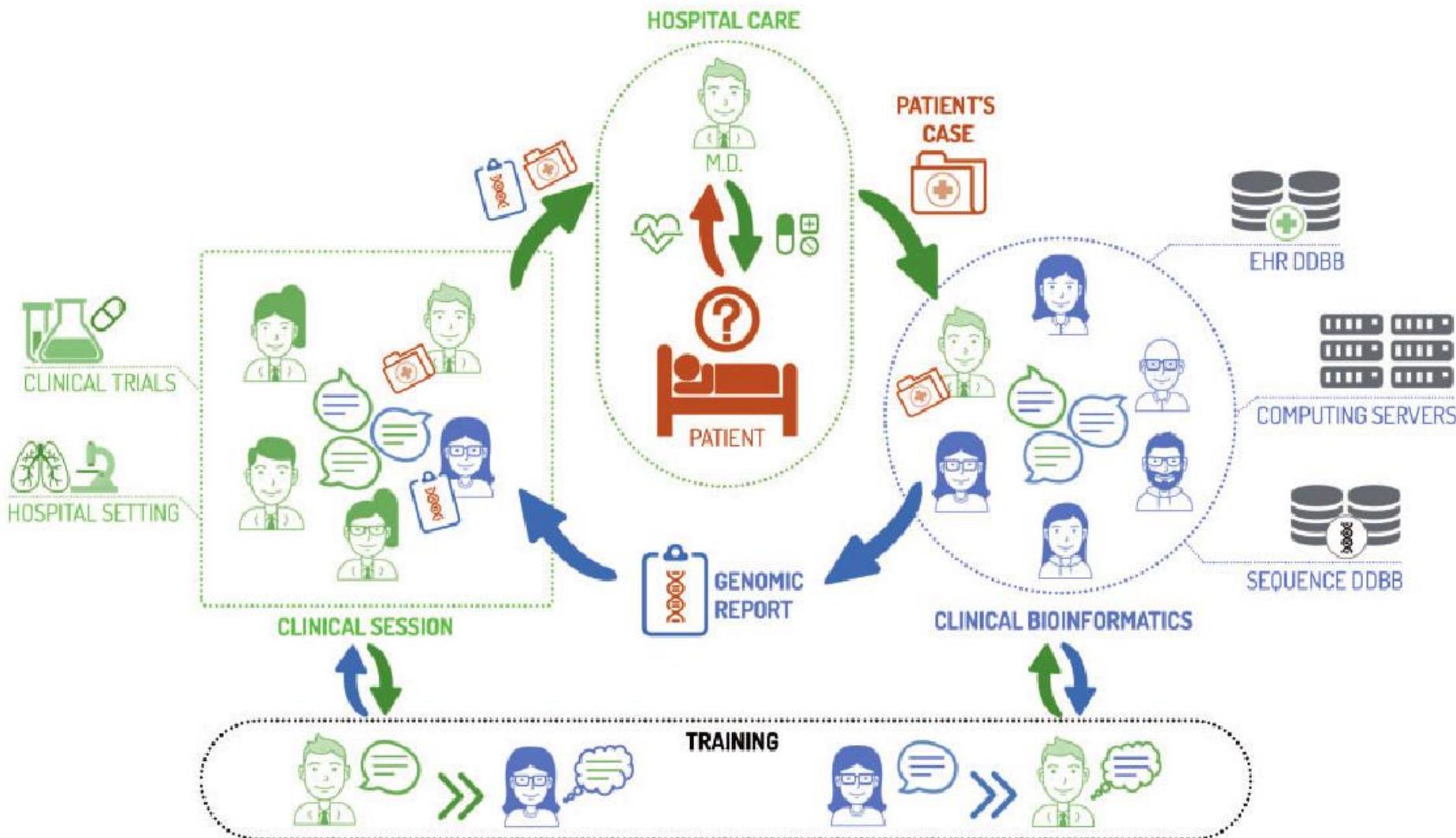
Skills for Clinical Bioinformaticians

Box 1. Fundamental technical skills for clinical bioinformaticians

1. Informatics
 - Experience in UNIX command line.
 - A basic programming language (i.e. Python). R as a useful language for handling statistics.
 - Knowledge of big data environments.
2. Life sciences
 - Understand the different types of biological data and databases.
 - Comprehend HTP data analysis methods.
 - Multi-omics data integration and interpretation.
3. Clinical scenario
 - Be familiar with EHRs, clinical terminology and medical procedures and protocols.
 - Get to know medical genomics: diagnosis, predictive and prognosis biomarkers.
 - Understand clinical trial design and monitoring.

Gómez-López et al., Brief Bioinf 2017, 1-15

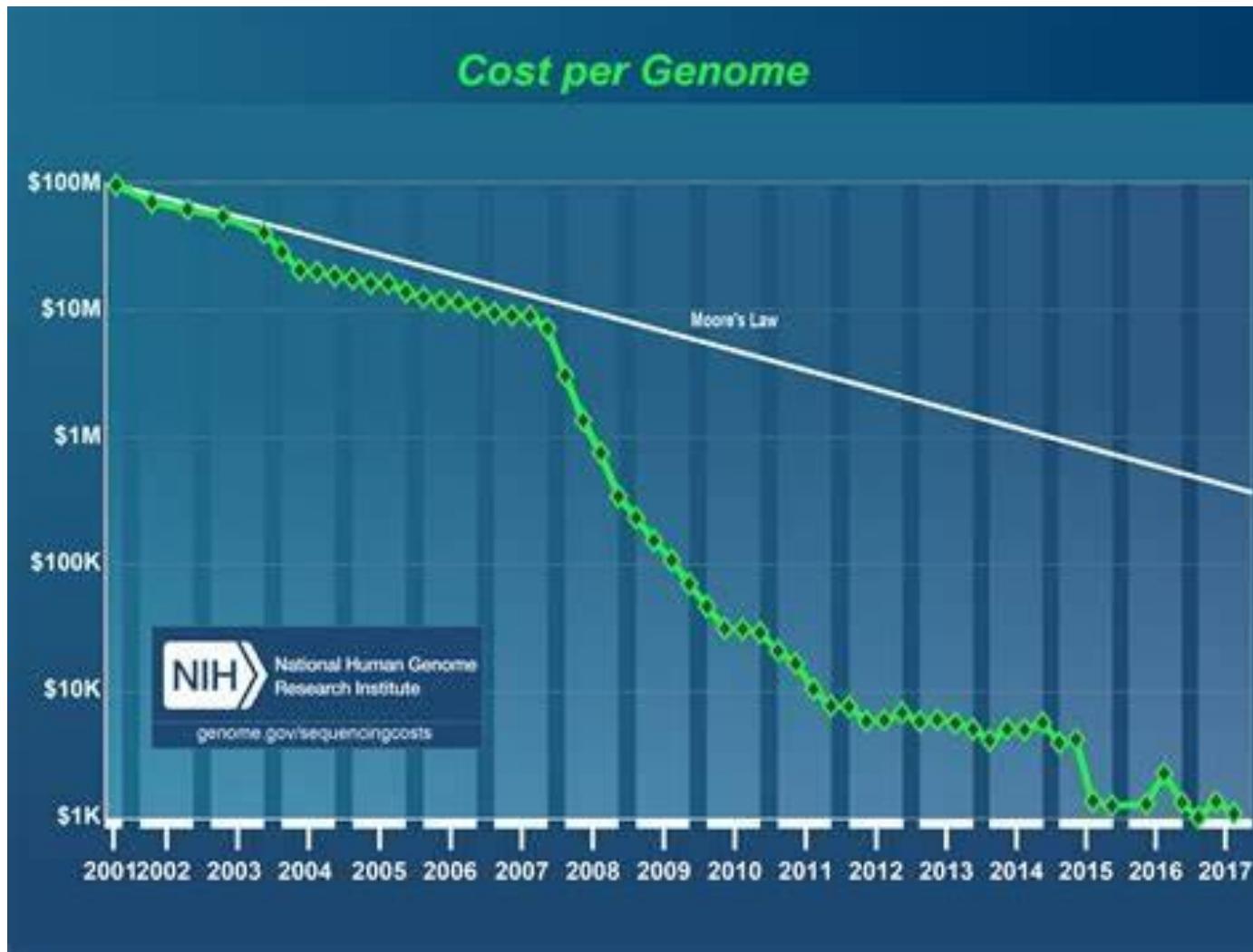
Precision Medicine Workflow in Hospitals



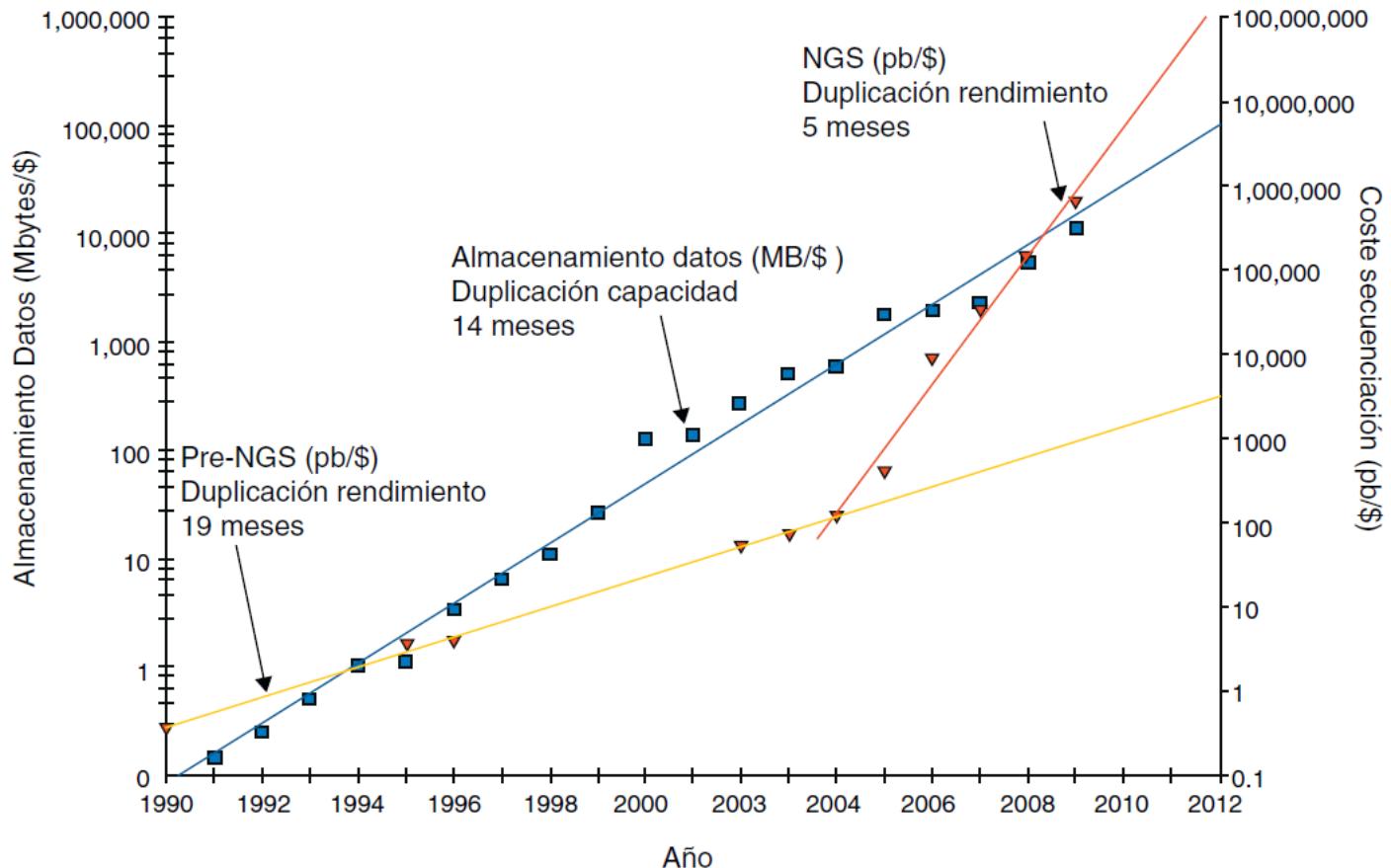
Retos de la Bioinformática en NGS

- Tecnología que evoluciona muy rápido
 - nuevos formatos de ficheros
 - nuevas aplicaciones
 - nuevos análisis
- Coste de la secuenciación disminuye
 - el embudo es el análisis de datos
- Adquisición de secuenciador debe ir ligado
 - a la compra de computo y contratación
 - de bioinformático

Coste actual de la secuenciación



Costes del almacenamiento vs secuenciación



Adaptada de Stein, Genome Biology 2010, 11:207
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Retos de la Bioinformática en NGS

- Necesidades de computo
 - ficheros de gran volumen (10Gb)
 - elevado uso de CPU y/o memoria
 - software no comercial en SO Unix
- Necesidades son dependientes de proyecto
 - No es lo mismo secuenciar un genoma 500Gb que 50 genomas 25Tb
- Si el proyecto es la aplicación en clínica
 - Las necesidades de almacenamiento aumentan por número de pacientes y por tiempo

Retos de la Bioinformática en NGS

- Desarrollo de BD curadas (confianza = reference)
- Algoritmos que resuelvan el problema biológico planteado.
- Necesidades de Bioinformáticos
Análisis de los datos

Softwares comerciales en Bioinformática y NGS

Table I: Examples, features and comparisons of some commonly used commercial bioinformatics software suites

Software	Company	Cost (USD) ^a	Free trial (days)	Platform ^b	NGS analyses ^c	Evolutionary analyses ^d	Database searching ^e	Plug-ins	Workflows	Teaching suitability
Avadis NGS	Strand Scientific Intelligence	\$4500	20	M, W, L	✓	✗	✗	✗	✓	✗
CLC Genomics Workbench	CIC bio, Qiagen	\$5500	30	M, W, L	✓	✓	✓	✓	✓	✓
CodonCode Aligner	CodonCode	\$720	30	M, W	✓	✓	✗	✗	✗	✓
Genamics Expression	Genamics	\$295	30	W	✗	✓	✓	✓	✗	✗
Geneious	Biomatters	\$795	14	M, W, L	✓	✓	✓	✓	✓	✓
Full Lasergene Suite	DNASTAR	\$5950	30	M, W	✓	✓	✓	✓	✓	✓
MacVector & Assembler	MacVector	\$300	21	M	✓	✓	✓	✗	✗	✓
NextGENe	Softgenetics	\$4049	35	W	✓	✗	✗	✗	✗	✗
Sequencher	Gene Codes	\$2500	30	M, W	✓	✓	✓	✓	✗	✓
VectorNTI Advance	Life Technologies	\$600	30	W	✗	✓	✓	✗	✓	✓

Softwares en Bioinformática y NGS

- Tecnología que evoluciona muy rápido
 - nuevos formatos de ficheros
 - nuevas aplicaciones
 - nuevos análisis
 - nuevos algoritmos
- Software en continuo desarrollo (Unix)

**Gracias por la
atención
Preguntas ???**



Isabel Cuesta

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isabel.cuesta@isciii.es