



# **Portable dna sequencing: impacting the response to emerging and re- emerging viral pathogens on the ground**

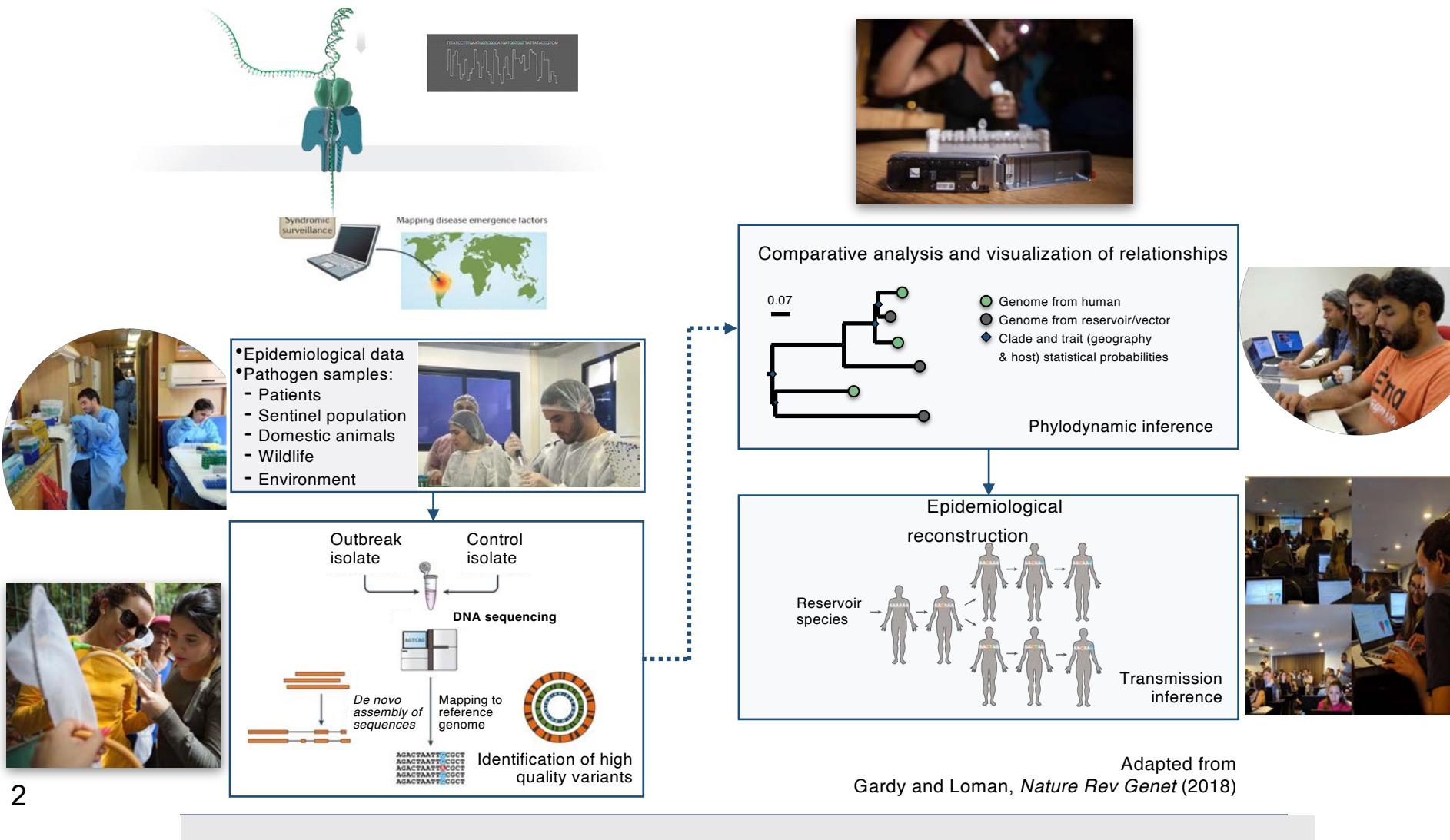
Marta Giovanetti

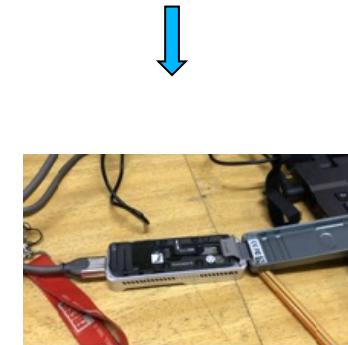
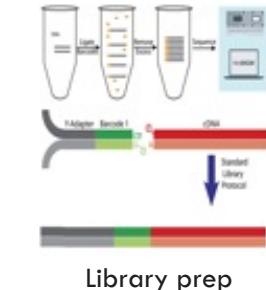
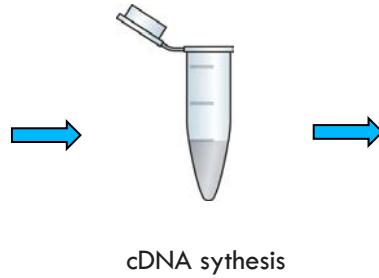
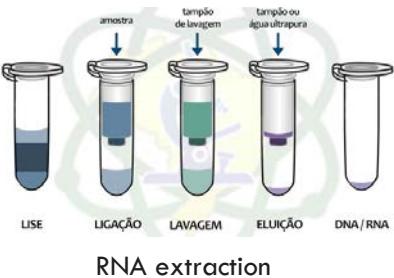
Fundação Oswaldo Cruz

University Campus Bio-Medico

✉ giovanetti.marta@gmail.com

 Mittenavoig





Phylogenetic Inference

Alignment

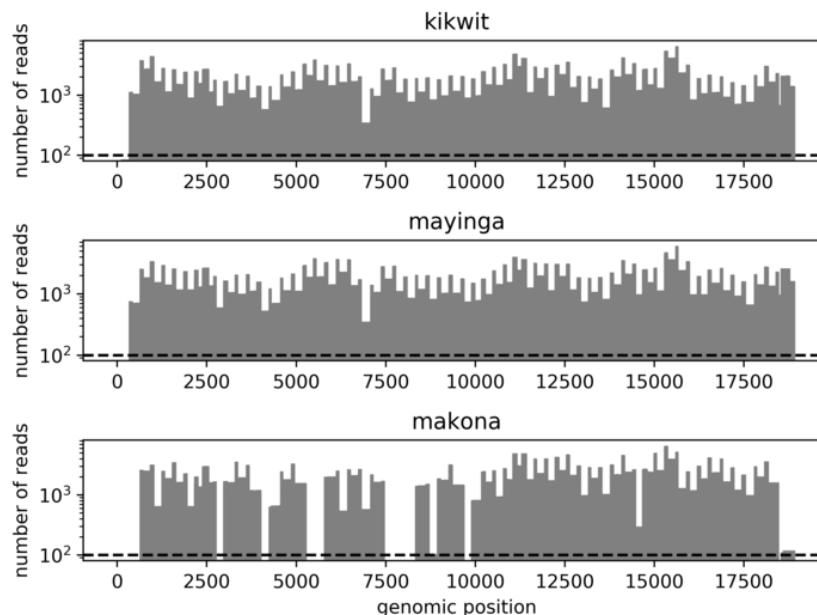
A sequence alignment diagram showing two DNA strands with matching bases highlighted in red. The top strand has positions 1, 30, and 40 indicated. The bottom strand has positions 1, 10, 20, 30, and 40 indicated.



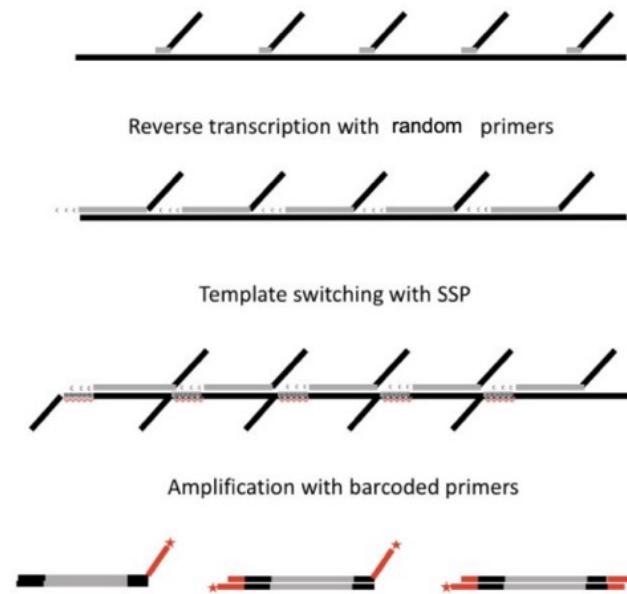
Assembling

# New sequencing protocols

## Targeted



## Untargeted



## Multiplex tiling PCR

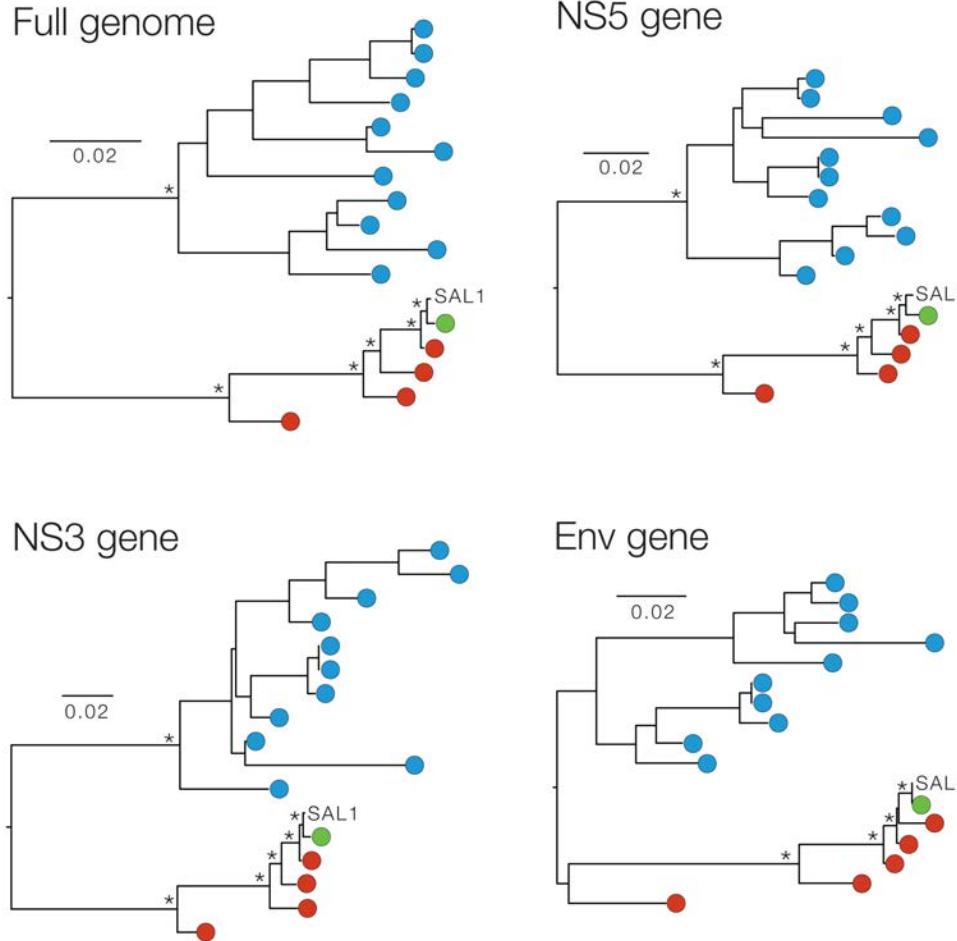
Validated on Ebola, Nipah, polio, rabies, Zika, chikungunya, dengue, and many others by community

## RNA metagenomics

Josh Quick, Ingra Morales

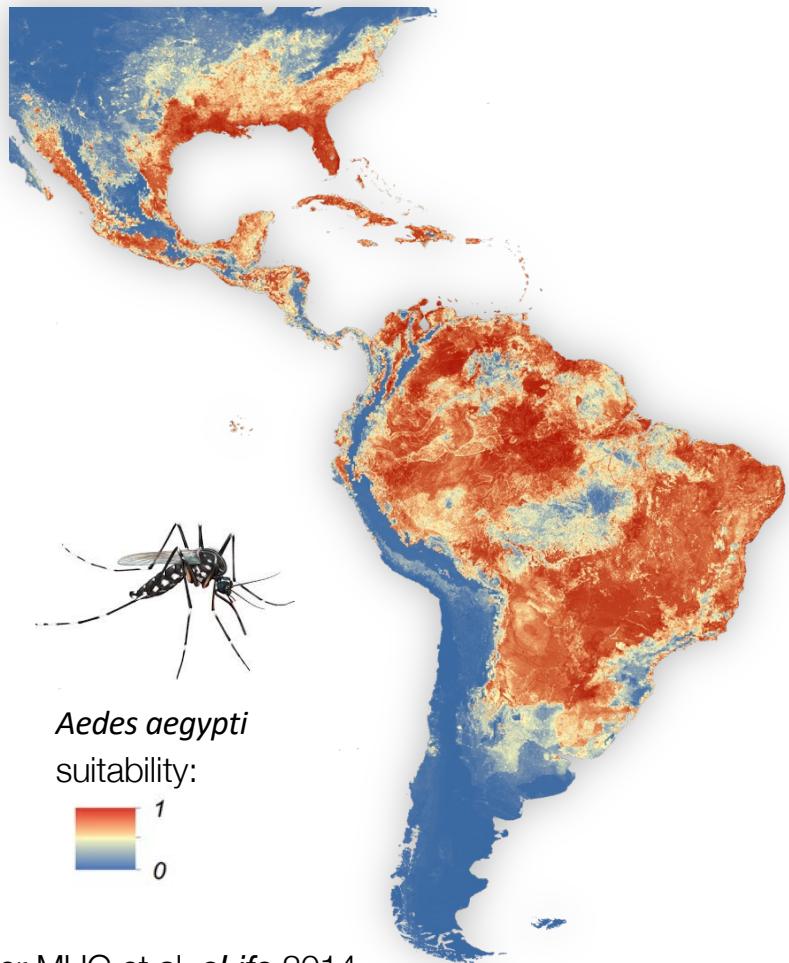
**When everything started?**

# ZIKV complete genome from Salvador, Bahia

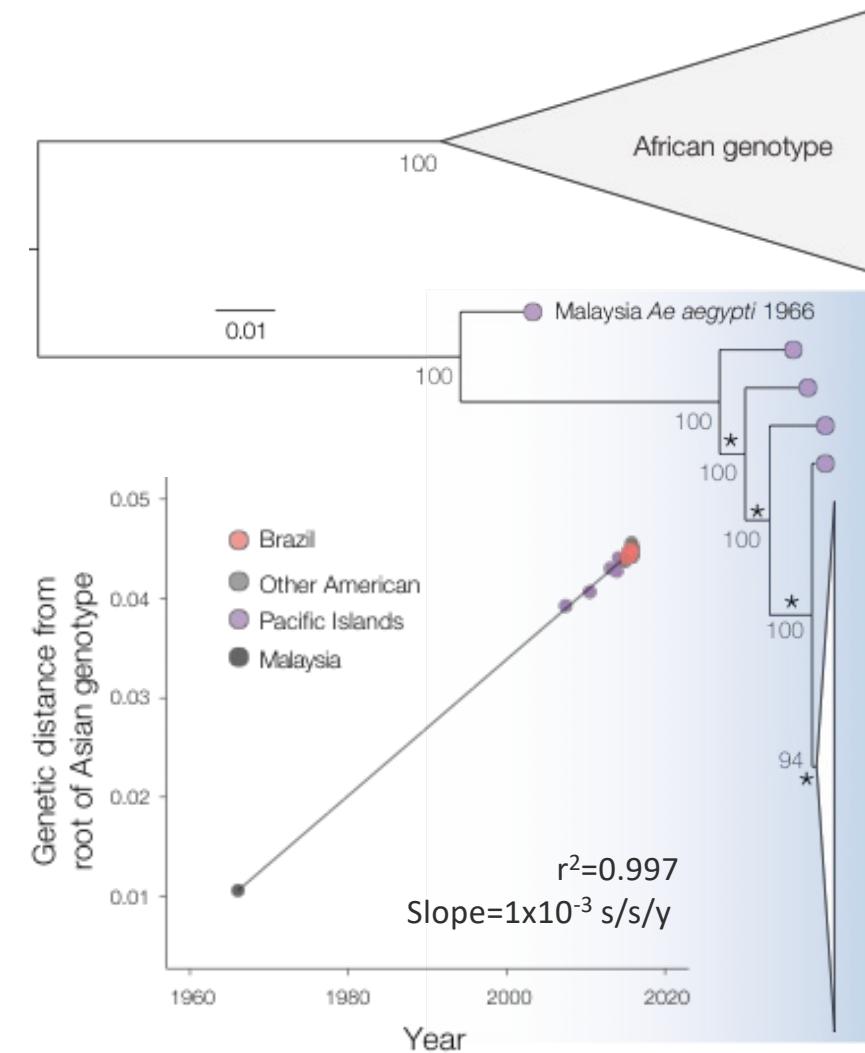


Giovanetti M et al. IGE 2016

# Emergence of ZIKV as a function of local transmission regime, viral pathogenesis



Kraemer MUG et al. *eLife* 2014



Faria et al. *Science* March 2016

# Genetic characterization of ZIKV in different body fluids of a newborn with a severe congenital syndrome

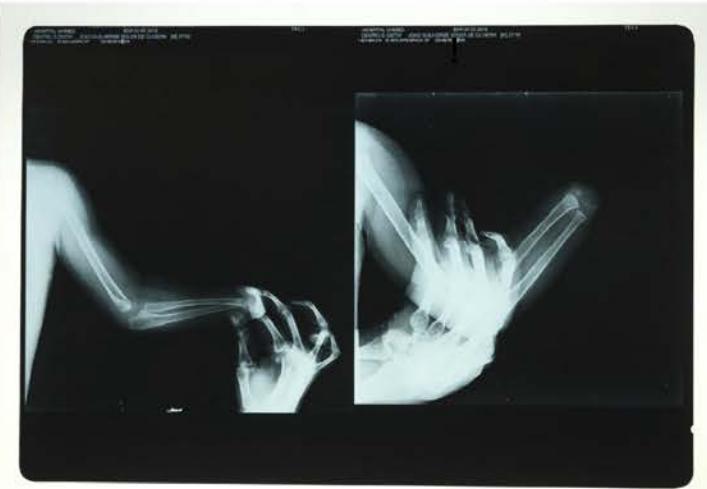
A



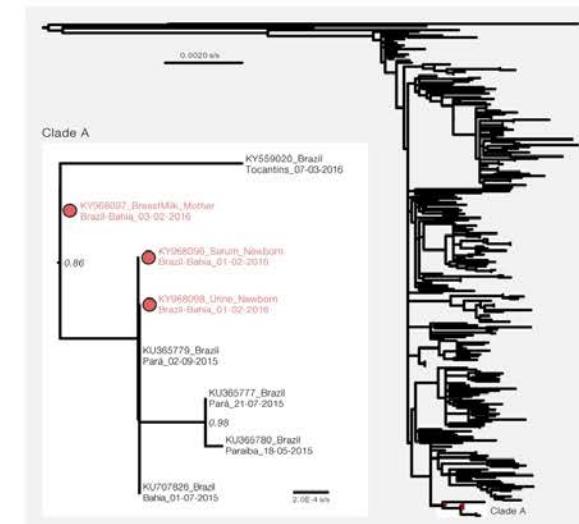
C.



B



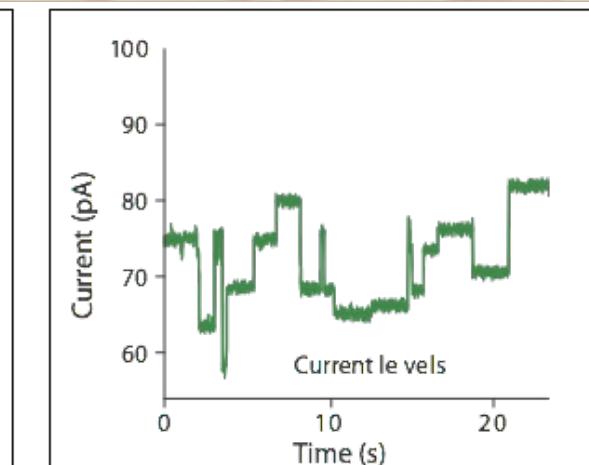
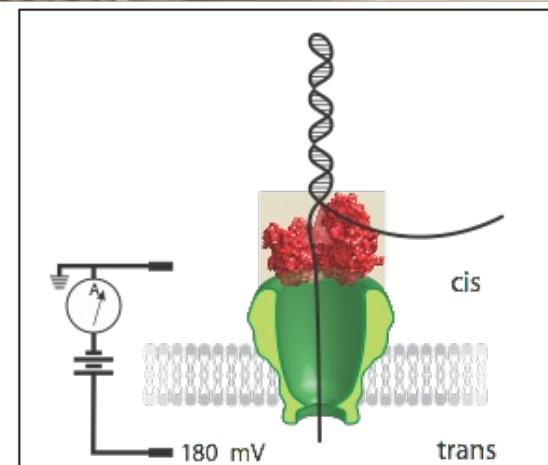
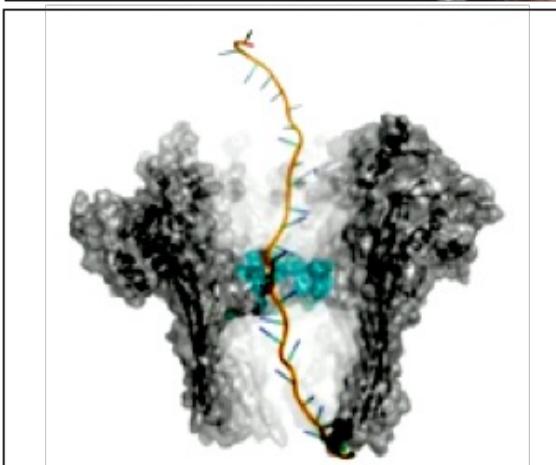
D.



**Previous MinION work on Ebola virus:** Quick J, Loman N, et al. *Nature* 2016

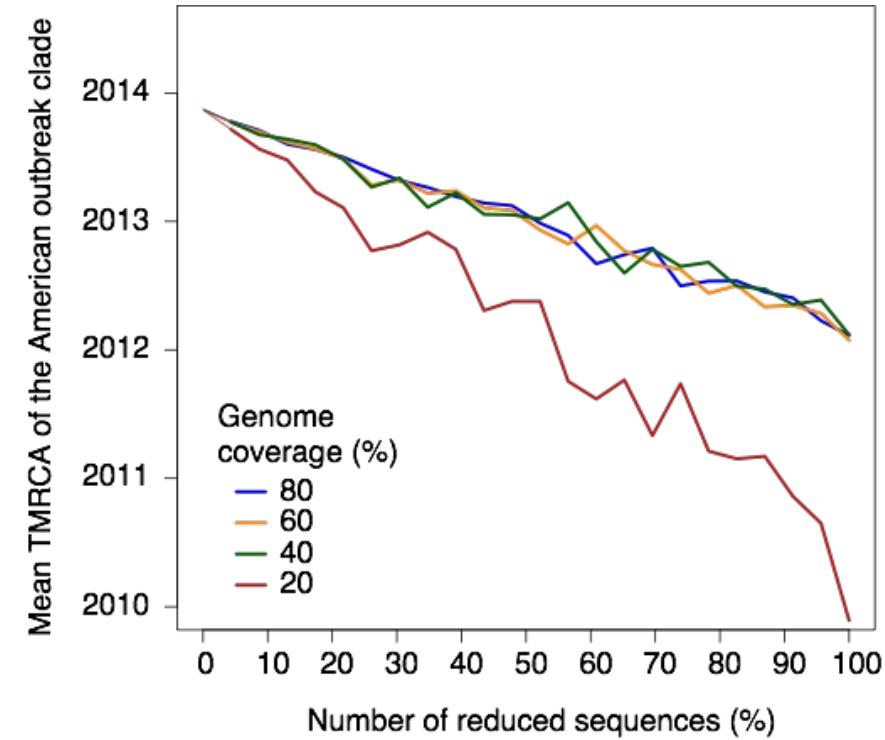
**Current MinION ZiBRA protocol:** Quick J et al. *Nature Protocols* May 2017 *in press*

**MinION work on Zika virus in Brazil:** Faria et al. *Nature* May 2017 *in press*

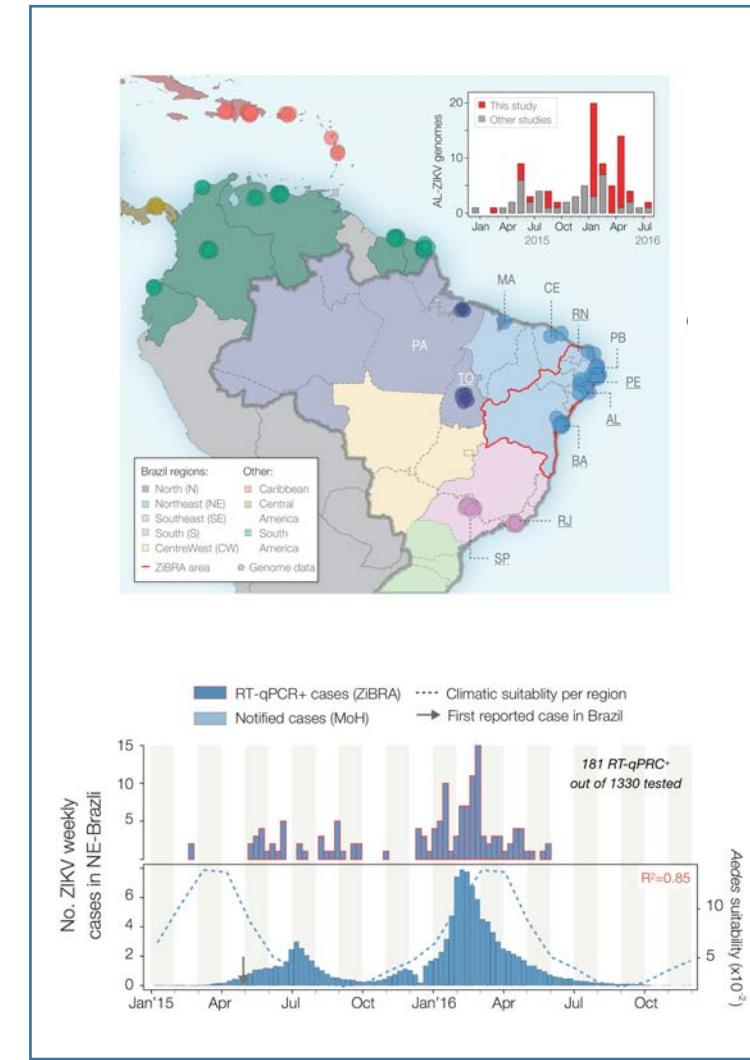
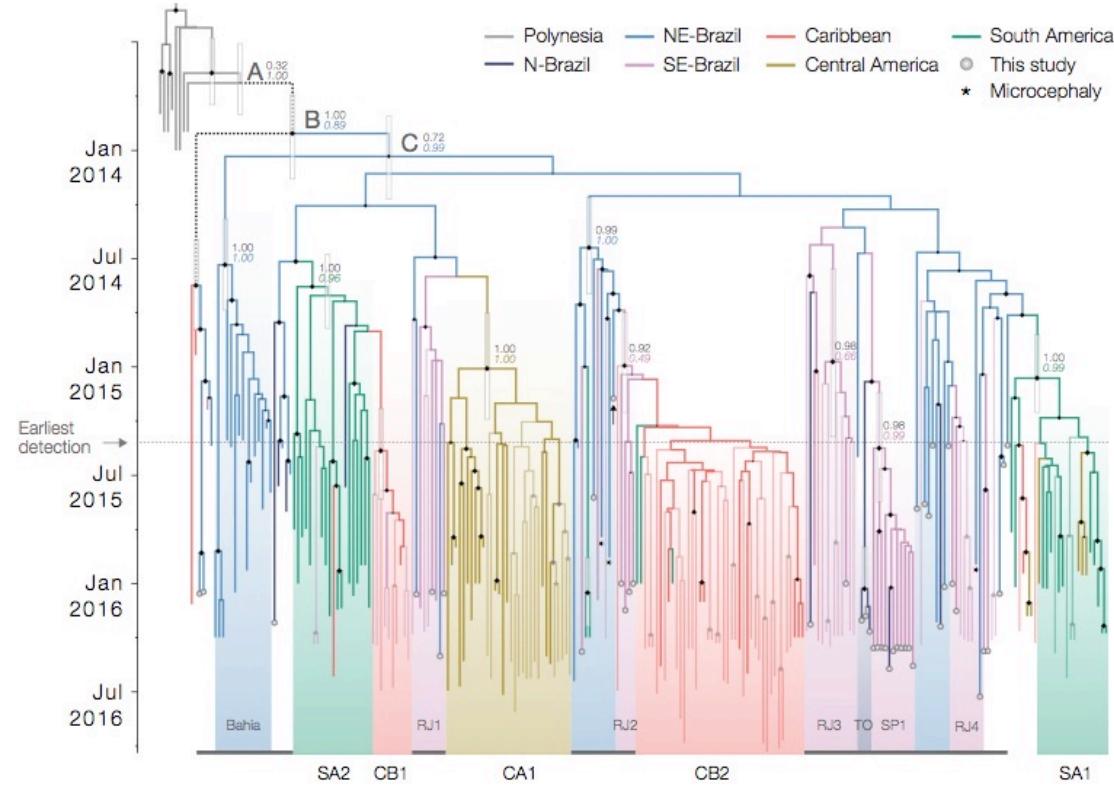


## Full-length genomes contain higher resolution compared to partial sequences

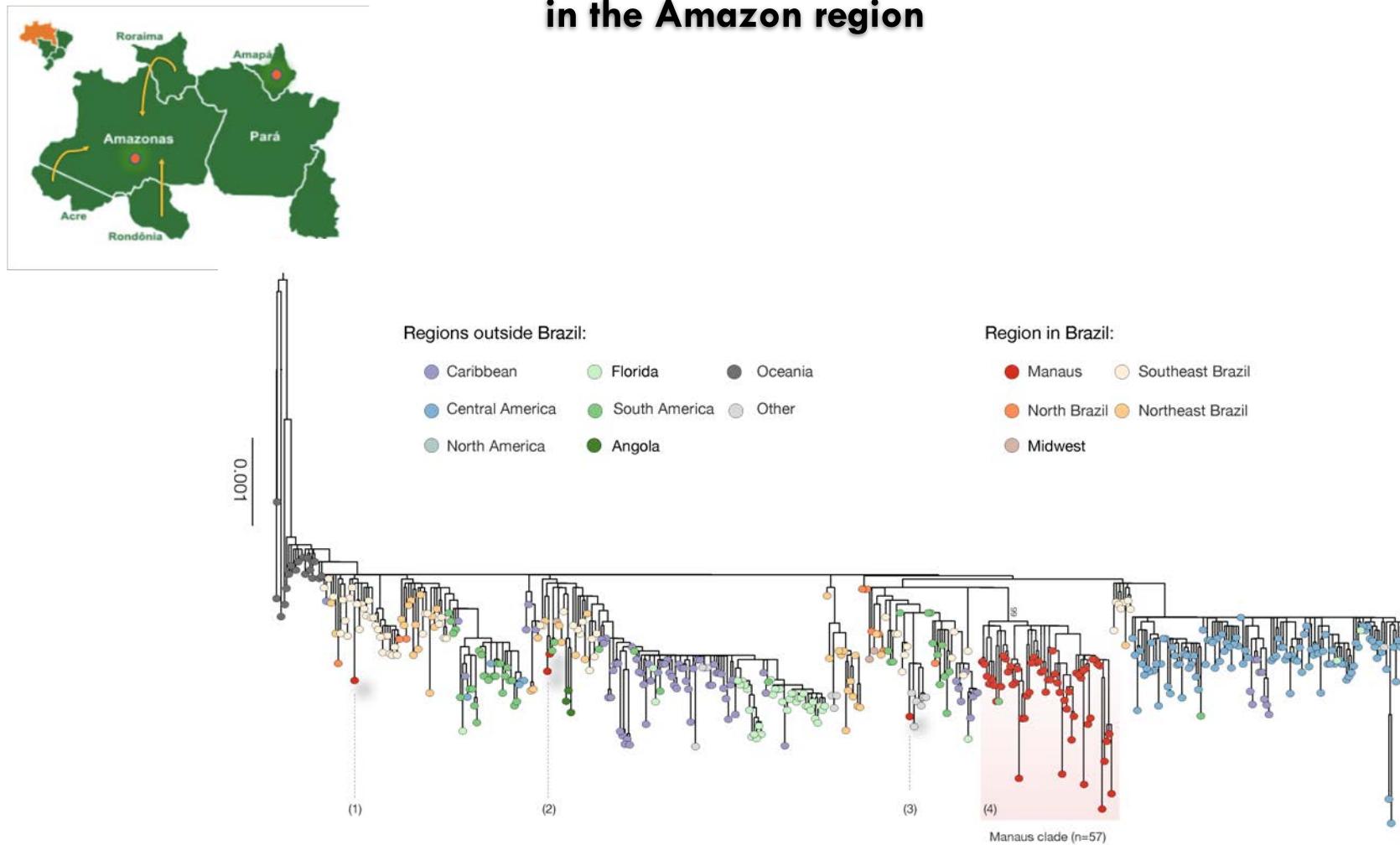
- Identification of mutations associated with transmission or disease severity
- Increased phylogenetic resolution is obtained when:
  - Full-length instead of partial sequences are generated
  - Sampling interval between collections are wider (not always possible)



## 54 Zika virus complete genomes obtained during a «road trip» in northeastern Brazil



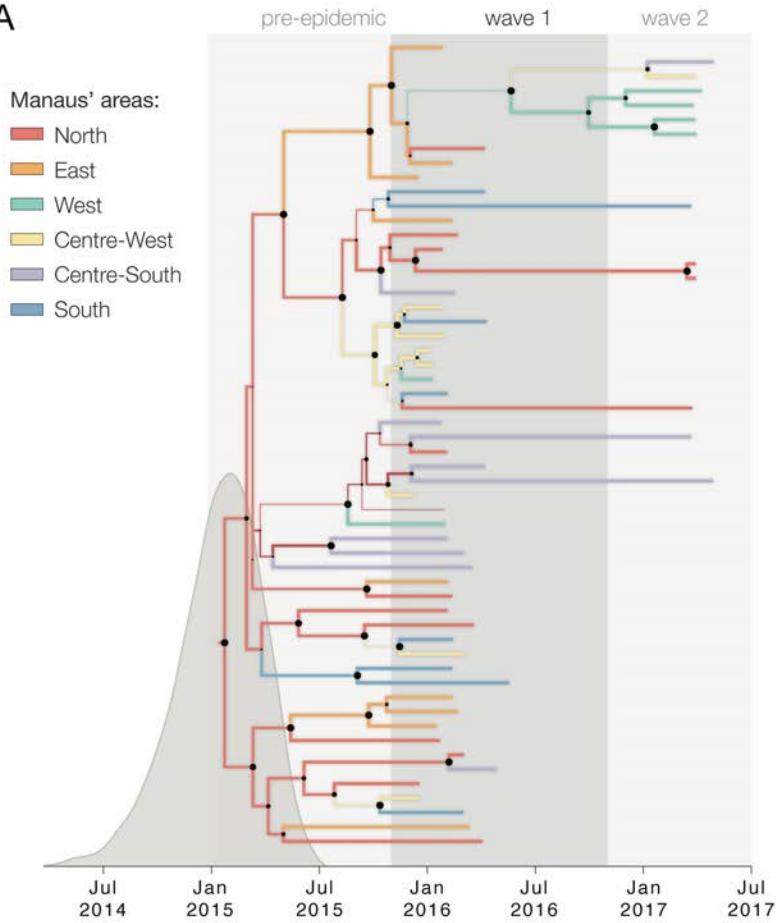
# Genomic and epidemiological surveillance of Zika virus in the Amazon region



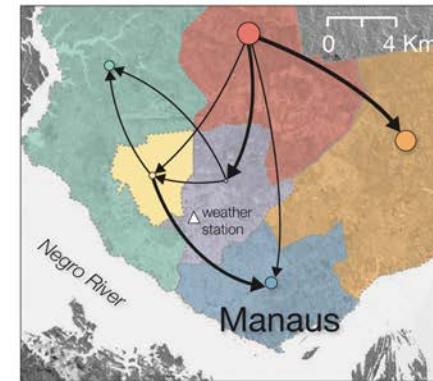
Giovanetti et al. *Cell Reports* 2020

# Genomic and epidemiological surveillance of Zika virus in the Amazon region

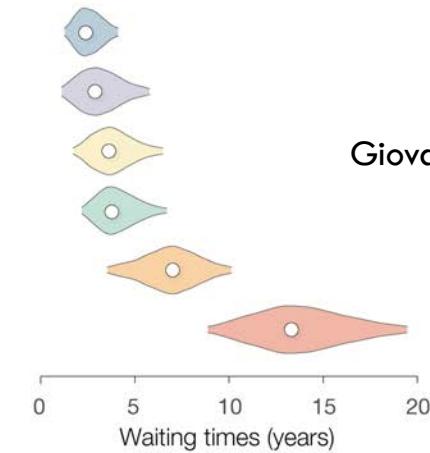
A



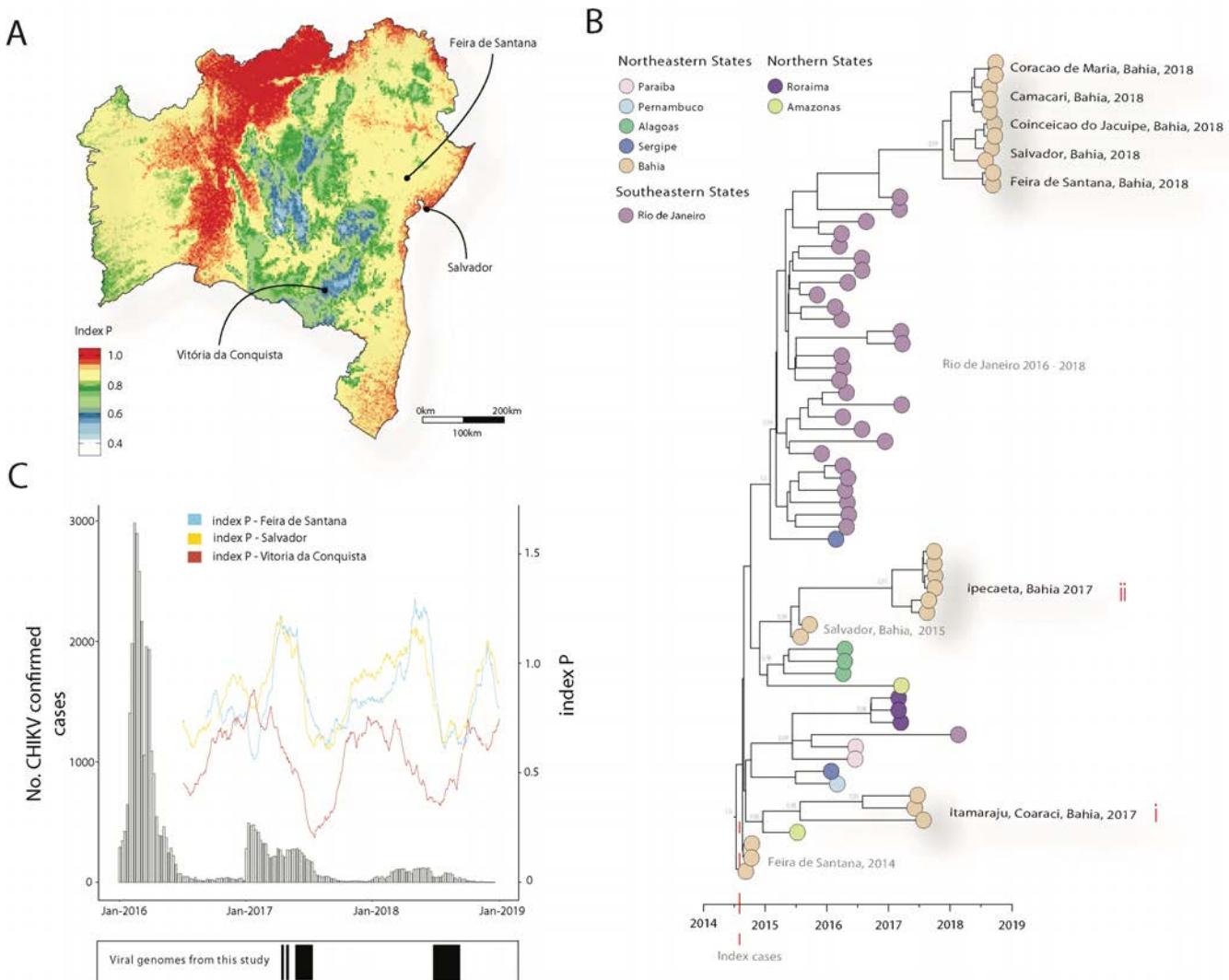
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C

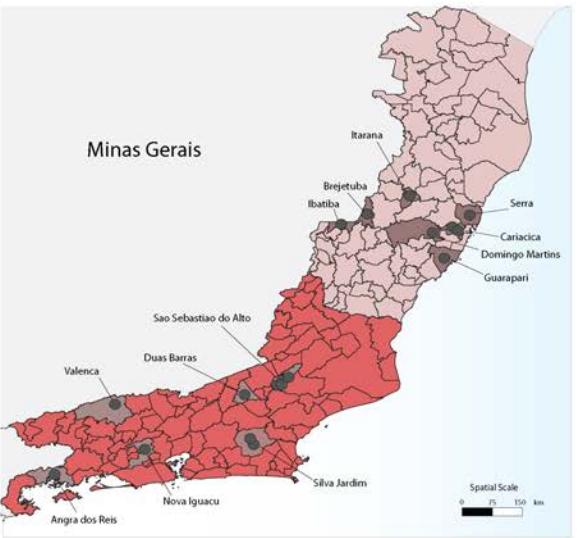


# Return of the founder Chikungunya virus to its place of introduction into Brazil is revealed by genomic characterization of exanthematic disease cases



# Yellow fever virus re-emergence and spread in Southeast Brazil, 2016-2019

A

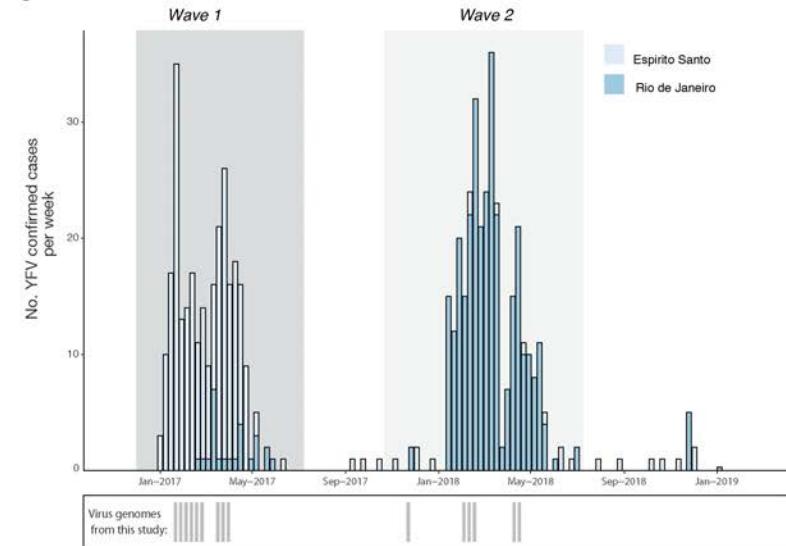


Spatial area  
under investigation:



● genomes from this study

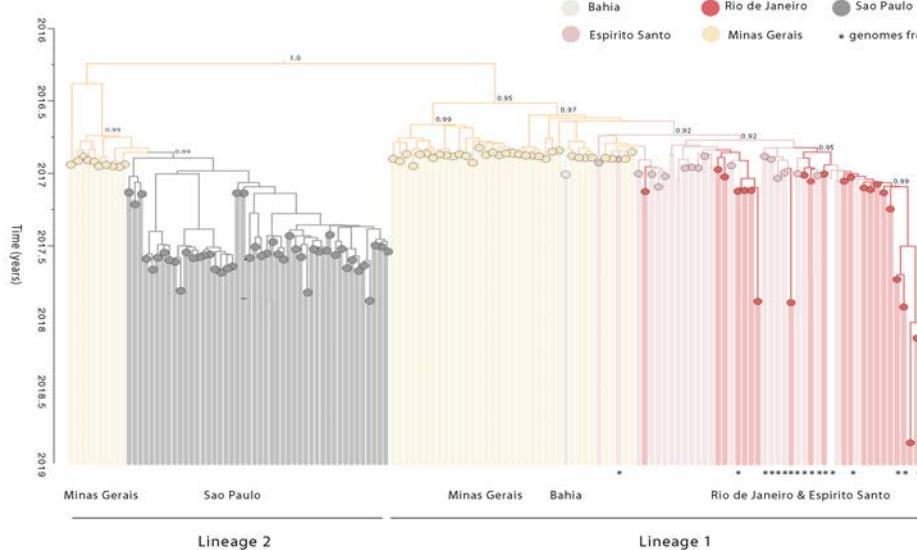
B



Sampling Location

Bahia  
Espirito Santo  
Minas Gerais  
Sao Paulo  
Rio de Janeiro

\* genomes from this study



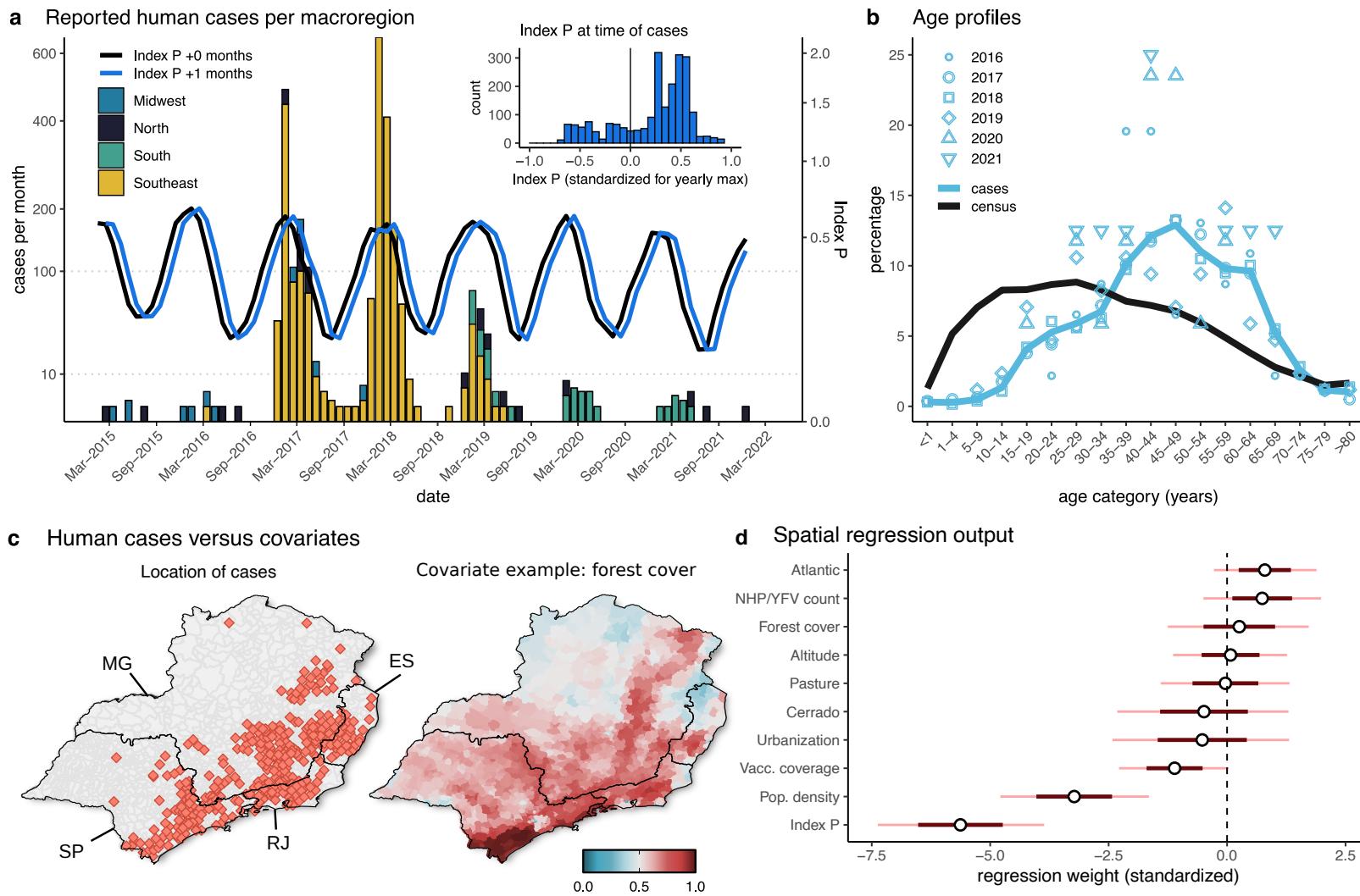
Giovanetti et al. Journal of virology 2019

**Genomic epidemiology sheds light on the recent spatio-temporal dynamics  
of Yellow Fever virus and the spatial corridor that fueled its ongoing  
emergence in southern Brazil**

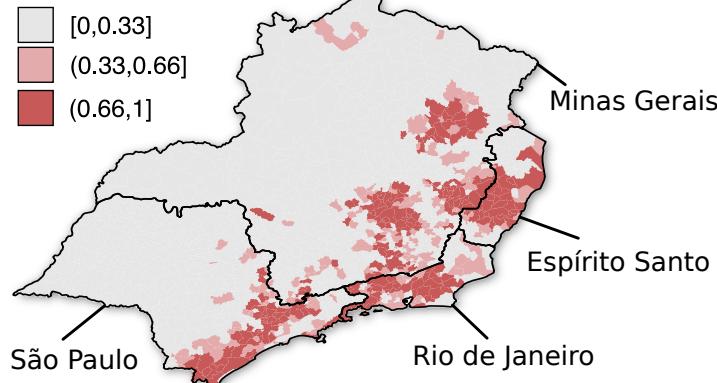
Marta Giovanetti, Francesco Pinotti, Camila Zanluca, Vagner Fonseca, Taishi Nakase, Andrea C. Koishi, Marcel Tscha, Guilherme Soares, Gisiane Gruber Dorl, Antônio Ernesto M.L Marques, Renato Sousa, Talita Emile Ribeiro Adelino, Joilson Xavier, Carla de Oliveira, Sandro Patroca da Silva, Natalia Rocha Guimaraes, Hegger Fritsch, Maria Angélica Mares-Guia, Flavia Levy, Pedro Henrique Passos, Vinicius Leme da Silva, Luiz Augusto Pereira, Ana Flávia Mendonça, Isabel Luana de Macêdo, Davi Emanuel Ribeiro de Sousa, Gabriela Rodrigues de Toledo Costa, Marcio Botelho de Castro, Felipe Campos de Melo Iani, Maira Alves Pereira, Karina Ribeiro Leite Jardim Cavalcante,  Andre Ricardo Ribas de Freitas, Carlos Frederico Campelo de Albuquerque, Eduardo Marques Macário, Marlei Pickler Debiasi dos Anjos, Rosane Campanher Ramos, Aline Alves Scarpellini Campos, Adriano Pinter, Marcia Chame, Livia Abdalla, Irina Nastassja Riediger, Sérvio Pontes Ribeiro, Ana Isabel Bento,  Tulio de Oliveira, Carla Freitas, Noely Fabiana Oliveira de Moura, Allison Fabri, Cintia Damasceno Dos Santos Rodrigues, Carolina Cardoso Dos Santos, Marco Antonio Barreto de Almeida, Edmilson dos Santos, Jader Cardoso, Douglas Adriano Augusto, Eduardo Krempser, Luís Filipe Mucci, Renata Rispoli Gatti, Sabrina Fernandes Cardoso, João Augusto Brancher Fuck, Maria Goretti David Lopes, Ivana Lucia Belmonte, Gabriela Mayoral Pedroso da Silva, Maiane Regina Ferreira Soares, Marilia de Melo Santos de Castilhos, Joseana Cardoso de Souza e Silva, Alceu Bisetto Junior, Emanuelle Gemin Pouzato, Laurina Setsuko Tanabe, Daniele Akemi Arita, Ricardo Matsuo, Josiane dos Santos Raymundo, Paula Cristina Linder Silva, Ana Santana Araújo Ferreira Silva, Sandra Samila, Glauco Carvalho, Rodrigo Stabeli, Wildo Navegantes, Luciano Andrade Moreira, Alvaro Gil A. Ferreira, Guilherme Garcia Pinheiro, Bruno Tardelli Diniz Nunes, Daniele Barbosa de Almeida Medeiros, Ana Cecília Ribeiro Cruz, Rivaldo Venâncio da Cunha, Wes Van Voorhis, Ana Maria Bispo de Filippis, Maria Almiron, Edward C. Holmes, Daniel Garkauskas Ramos, Alessandro Romano, José Lourenço, Luiz Carlos Junior Alcantara, Claudia Nunes Duarte dos Santos

**doi:** <https://doi.org/10.1101/2023.01.13.23284525>

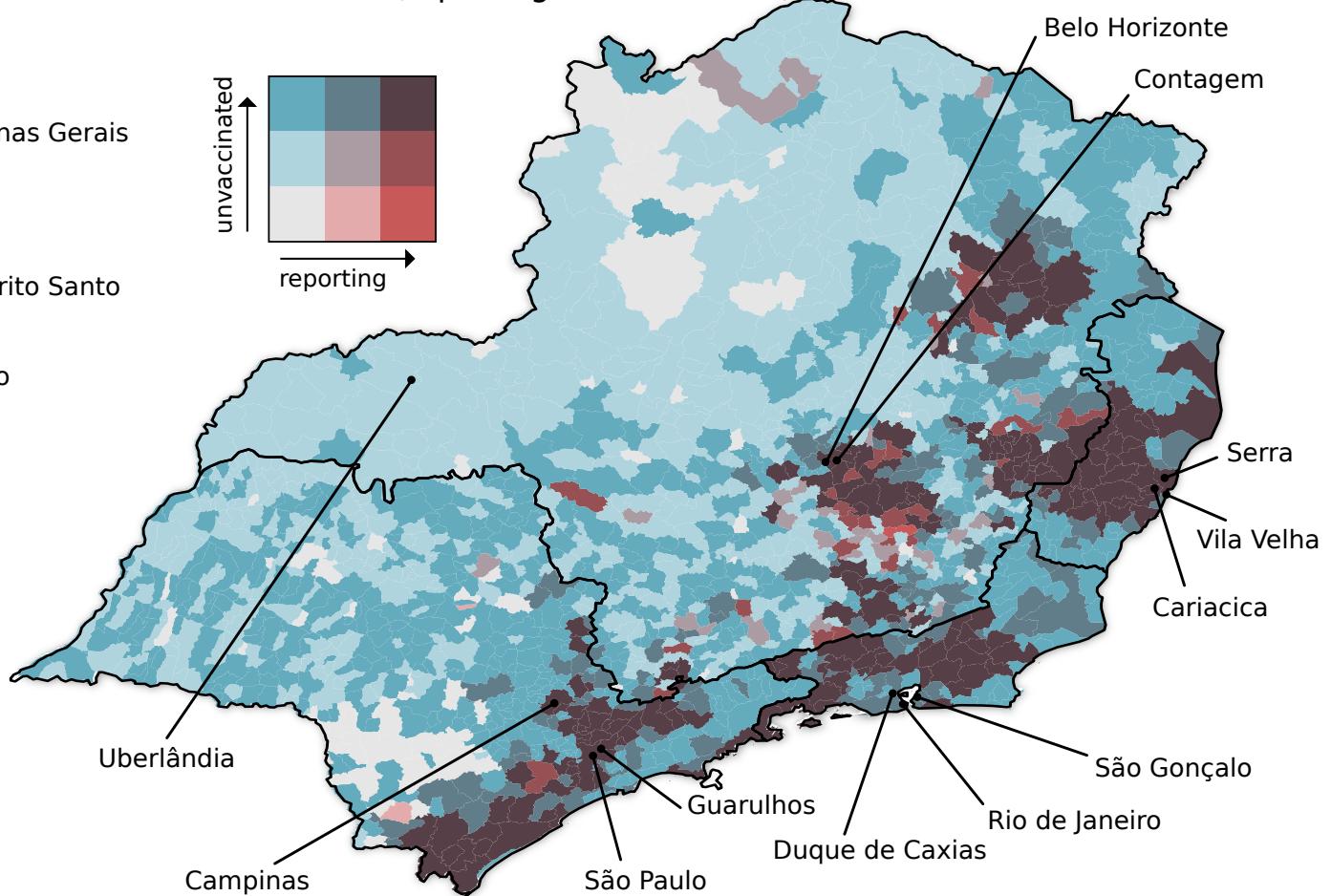
**doi:** <https://doi.org/10.1101/2023.01.13.23284525>



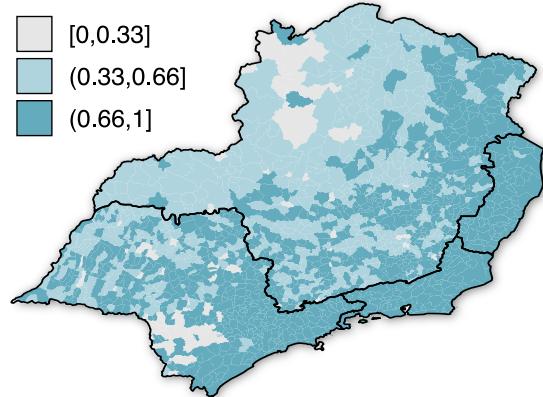
**a** Probability of reporting

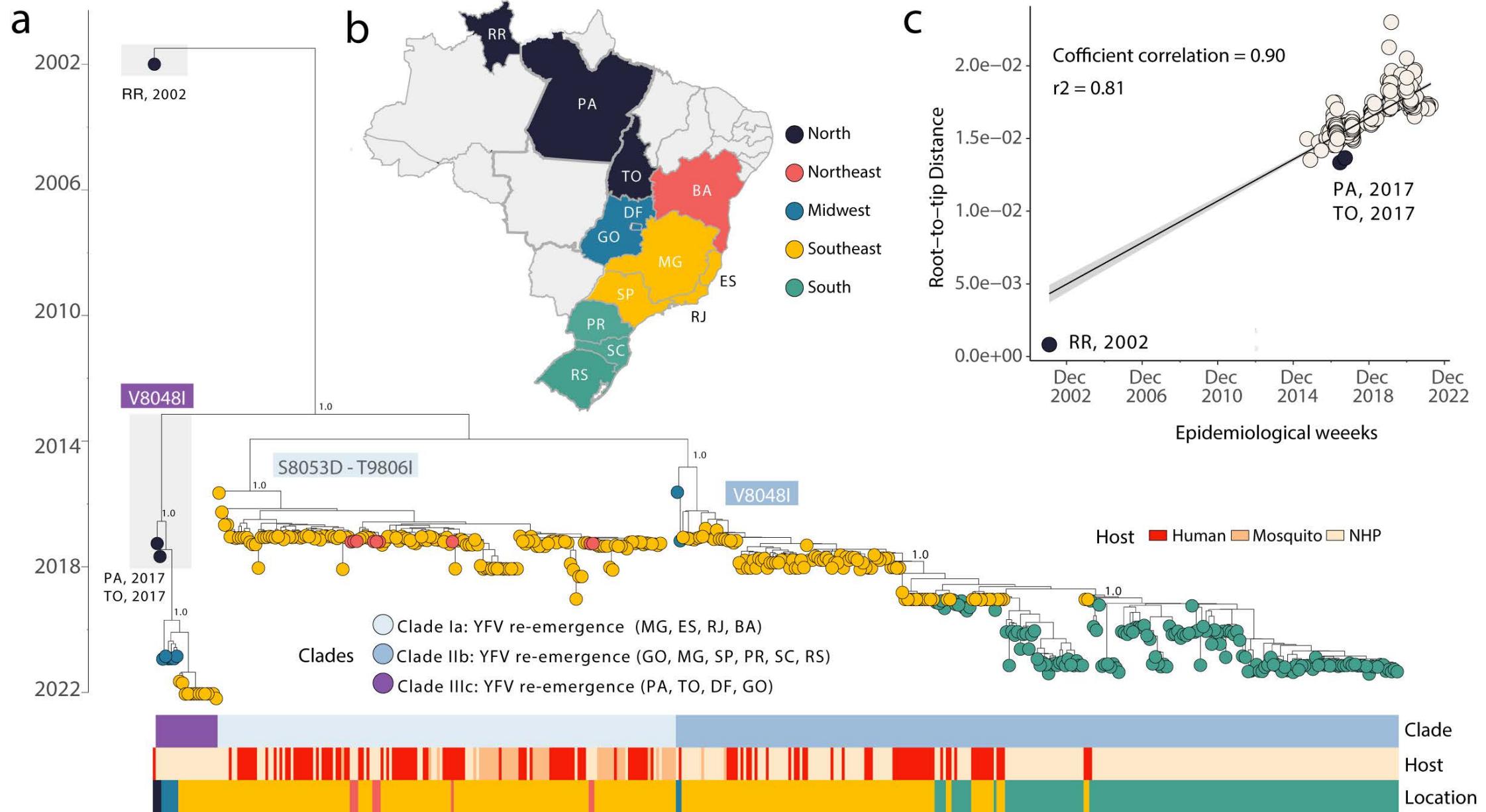


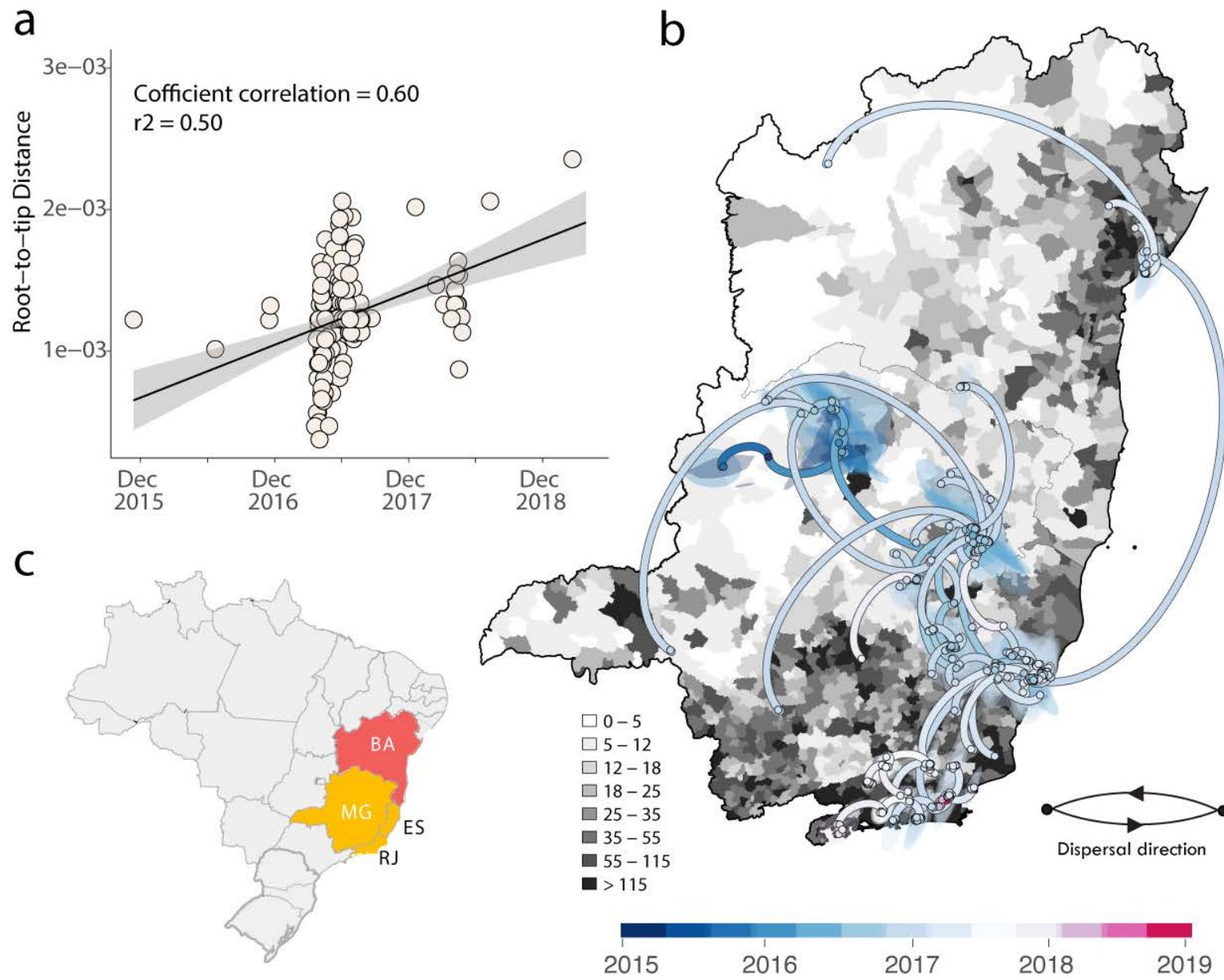
**c** Bivariate distribution (reporting, unvaccinated)

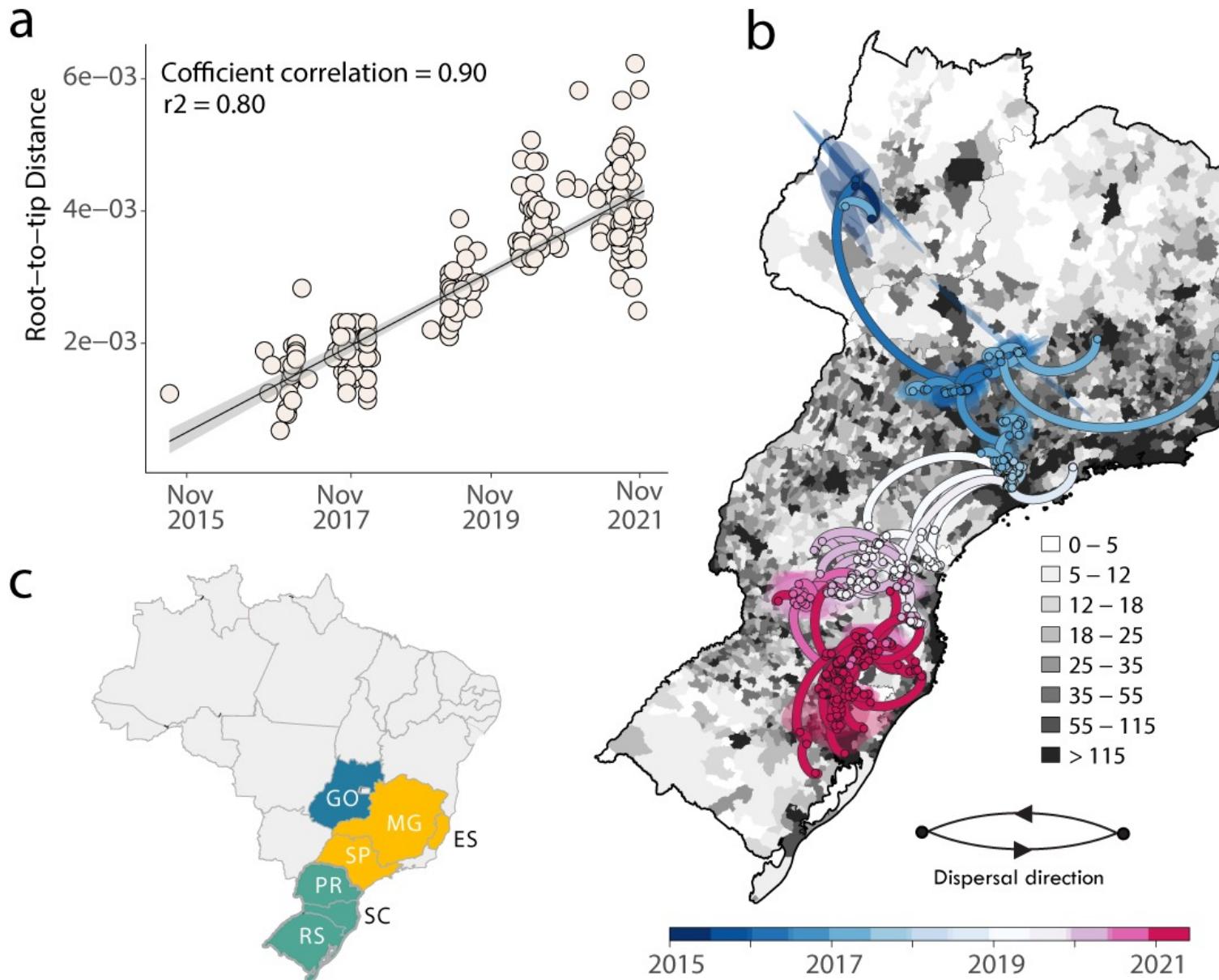


**b** Proportion unvaccinated









# Mobile Real Time Genomic Surveillance in Midwest Brazil

ZIKV, CHIKV, YFV, SLEV DENV, MAY, ORO, WNV



Entomologia



Sequenciamento

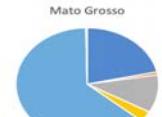


Bioinformática



## Brazil

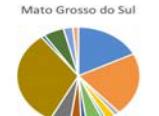
Mato Grosso  
Total: 1097 mosquitos  
Cidade: Cuiabá  
Nº de espécies: 19



Aedes vexans  
Aedes vexans

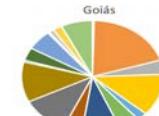
Total de mosquitos: 2.760

Mato Grosso do Sul  
Total: 209 mosquitos  
Cidades: Coxim, Campo Grande e Chapadão do Sul  
Nº de espécies: 17

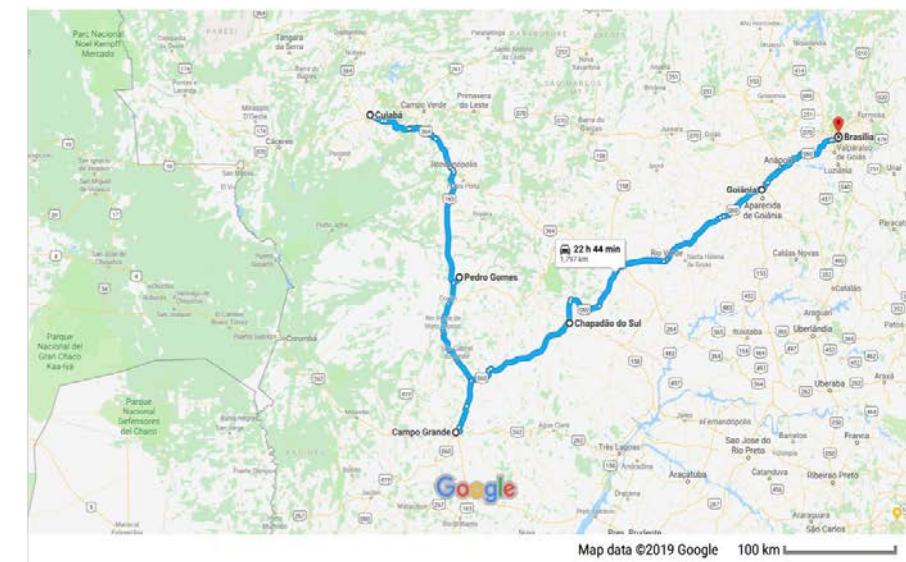


Aedes vexans  
Aedes vexans

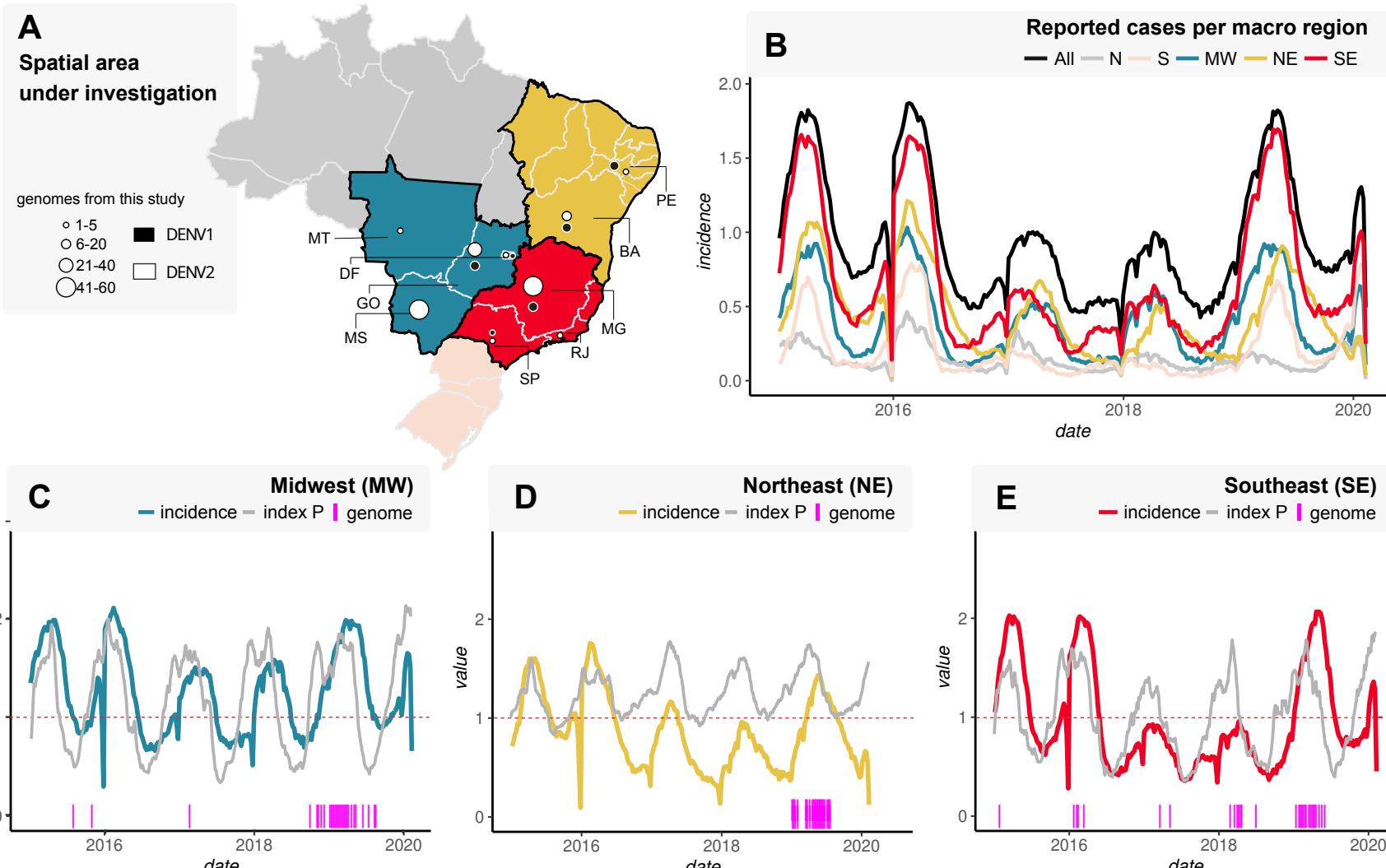
Goiás  
Total: 1454 mosquitos  
Cidades: St. Antônio de Goiás, Aparecida de Goiânia, Trindade e Cachoeira Dourada  
Nº de espécies: 18

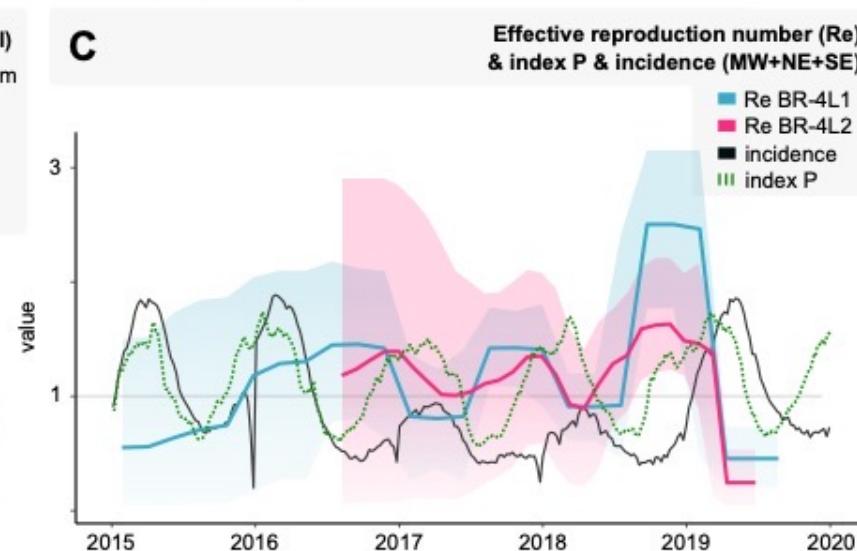
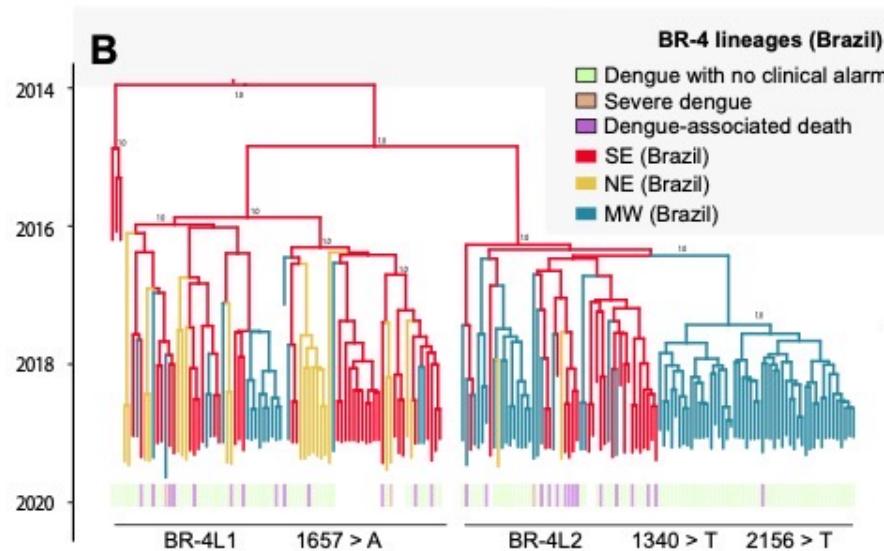
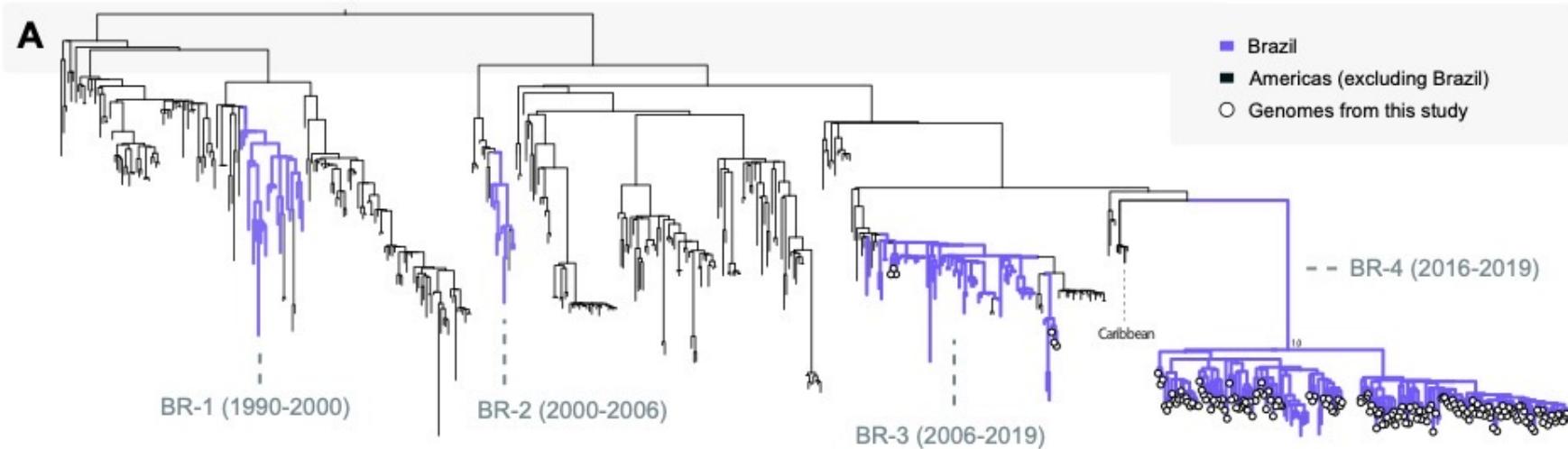


Aedes vexans  
Aedes vexans

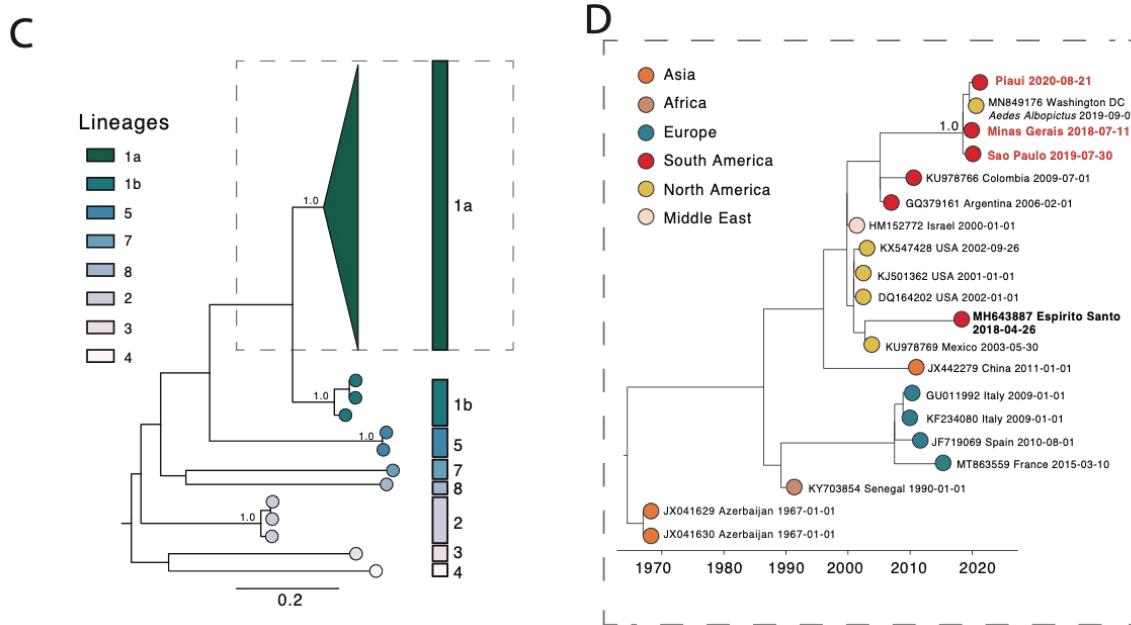
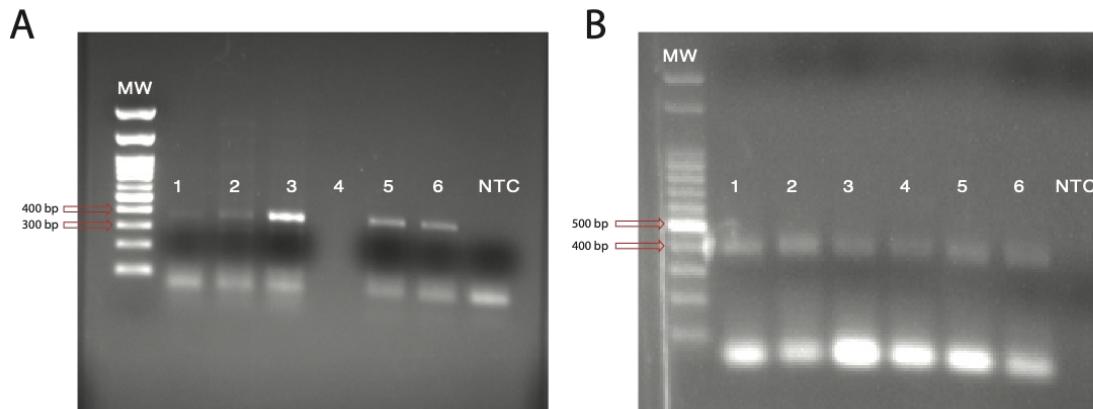


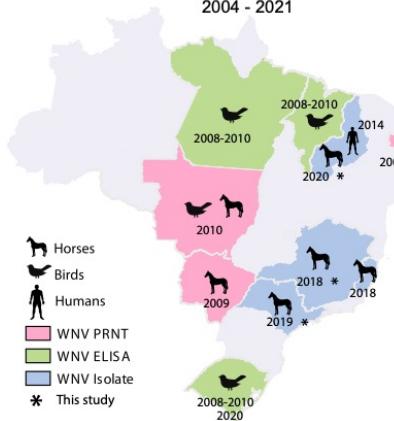
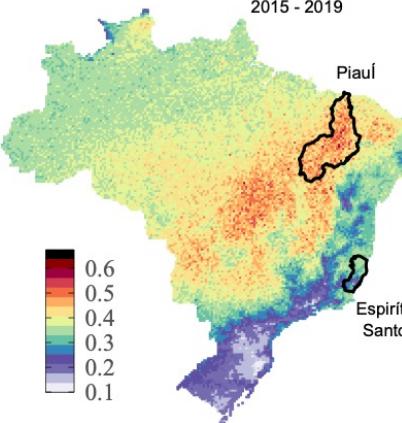
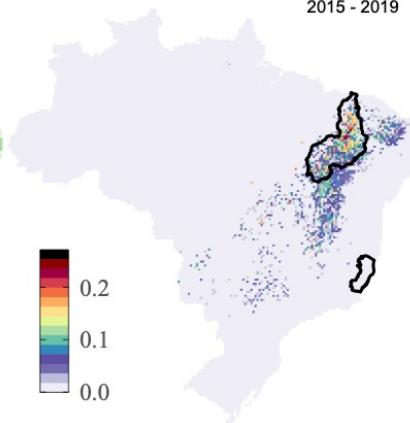
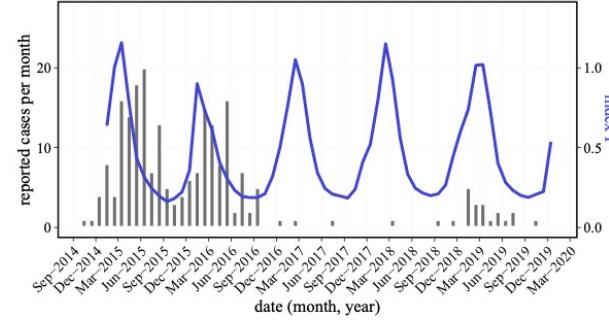
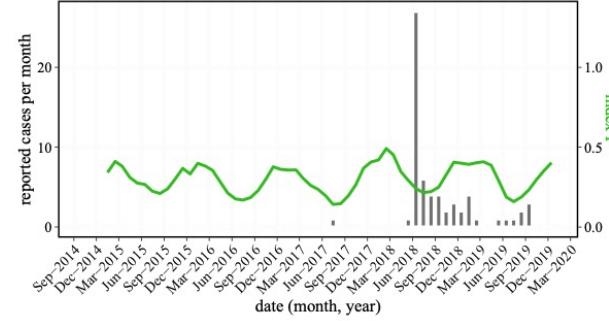
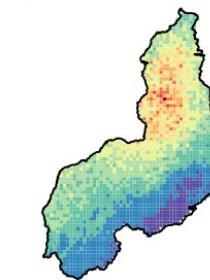
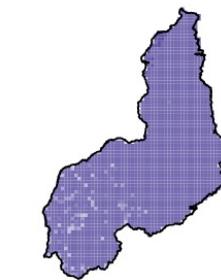
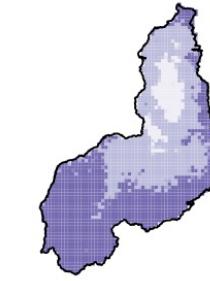
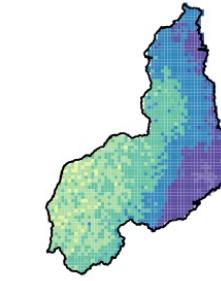
# Portable sequencing in the field and the classroom: a retrospective examination of the circulation of DENV1 and DENV2 in Brazil





# West Nile virus in Brazil

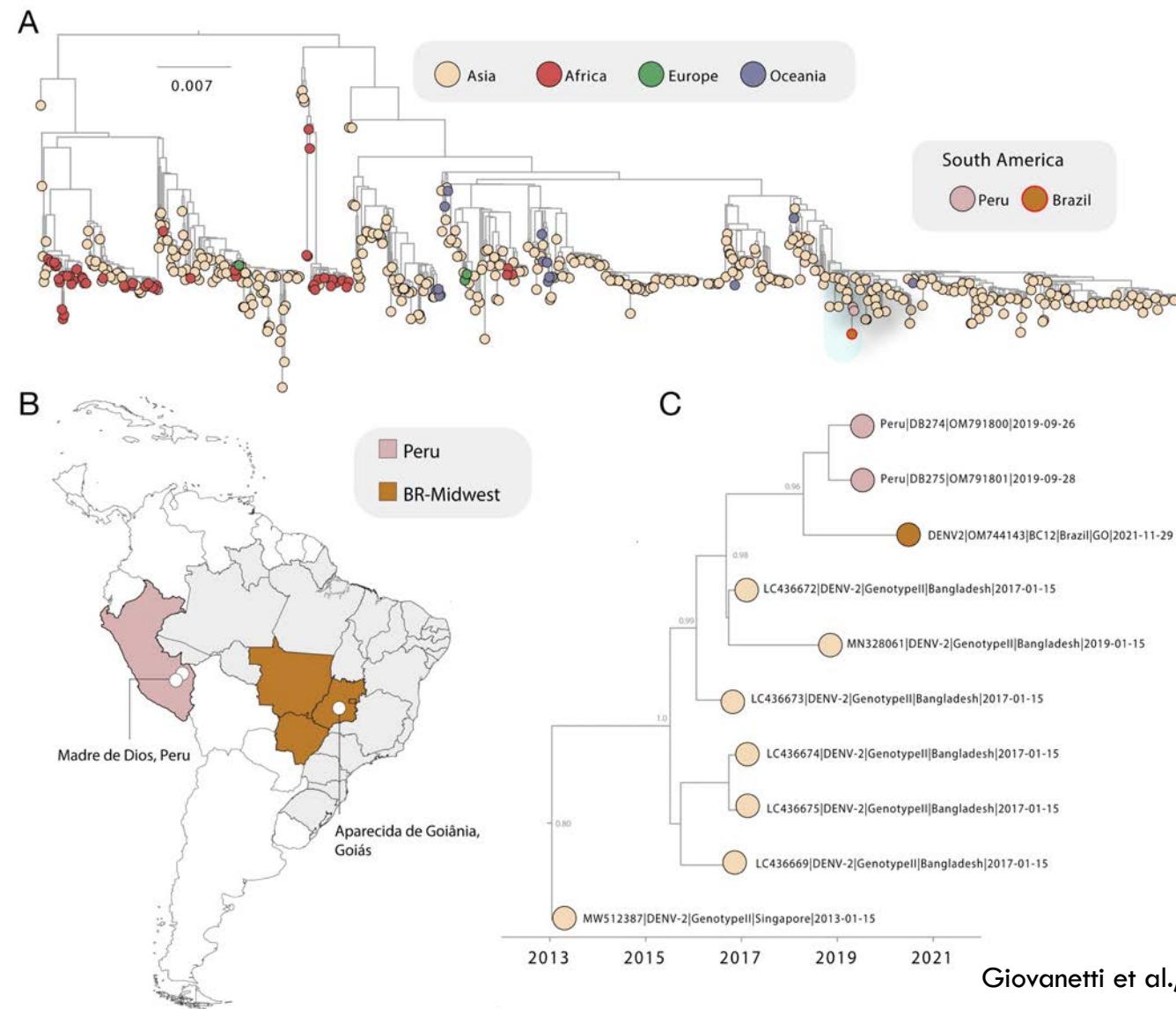


**A** Historic data on WNV  
2004 - 2021**B** Mean WNV suitability (P)  
2015 - 2019**C** Proportion of months with P>1  
2015 - 2019**D** State of Piauí**E** State Espírito Santo**F** March 2016**G** June 2016**H** September 2016**I** December 2016



## Emergence of Dengue Virus Serotype 2 Cosmopolitan Genotype, Brazil

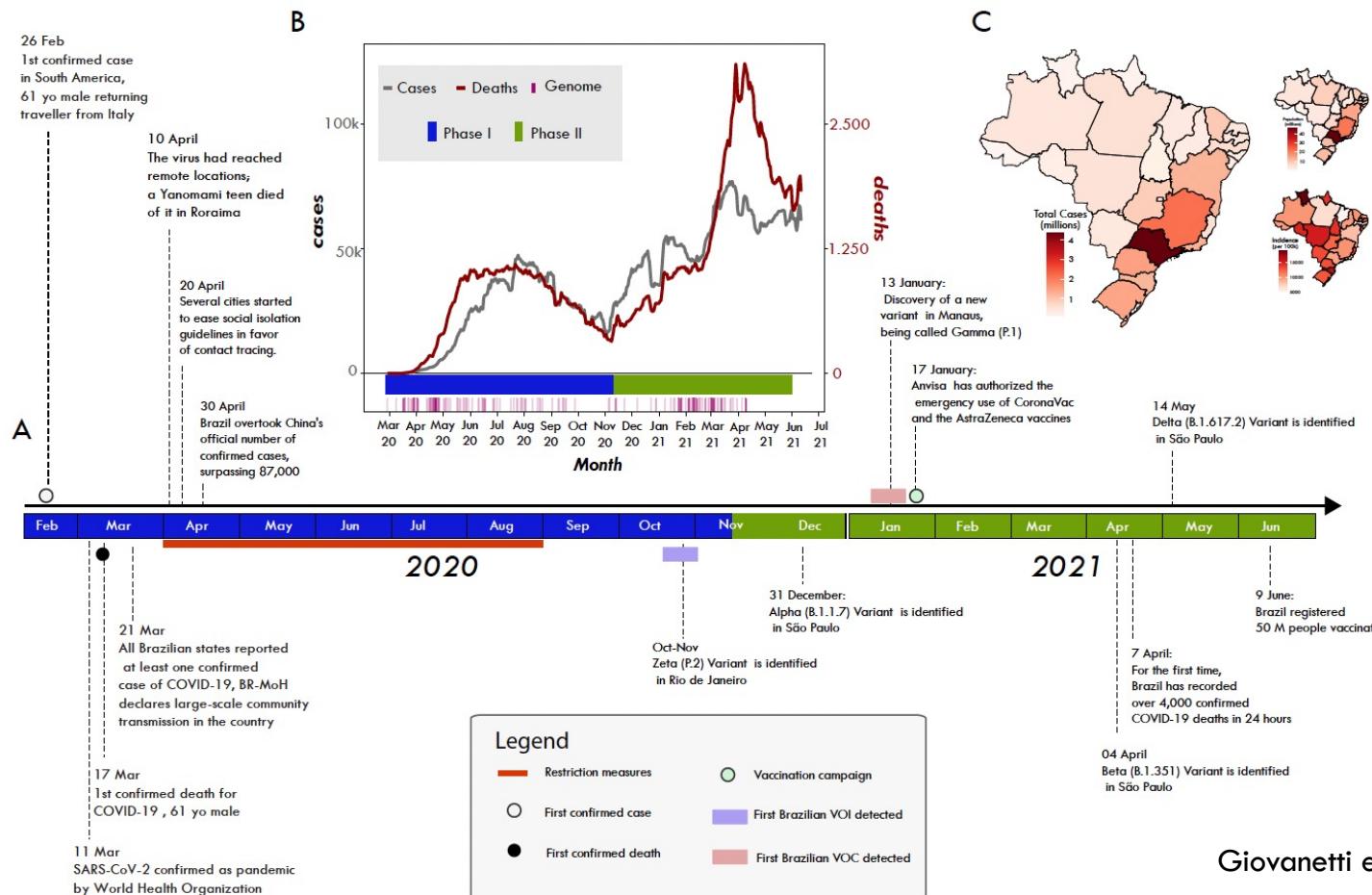
Marta Giovanetti,<sup>1</sup> Luiz Augusto Pereira, Gilberto A. Santiago, Wagner Fonseca, Maria Paquita García Mendoza, Carla de Oliveira, Laise de Moraes, Joilson Xavier, Stephane Tosta, Hegger Fristch, Emerson de Castro Barbosa, Evandra Strazza Rodrigues, Dana Figueroa-Romero, Carlos Padilla-Rojas, Omar Cáceres-Rey, Ana Flávia Mendonça, Fernanda de Bruycker Nogueira, Rivaldo Venancio da Cunha, Ana Maria Bispo de Filippis, Carla Freitas, Cassio Roberto Leonel Peterka, Carlos Frederico Campelo de Albuquerque, Letícia Franco, Jairo Andrés Méndez Rico, Jorge L. Muñoz-Jordán,<sup>1</sup> Vinícius Lemes da Silva,<sup>1</sup> Luiz Carlos Junior Alcantara<sup>1</sup>

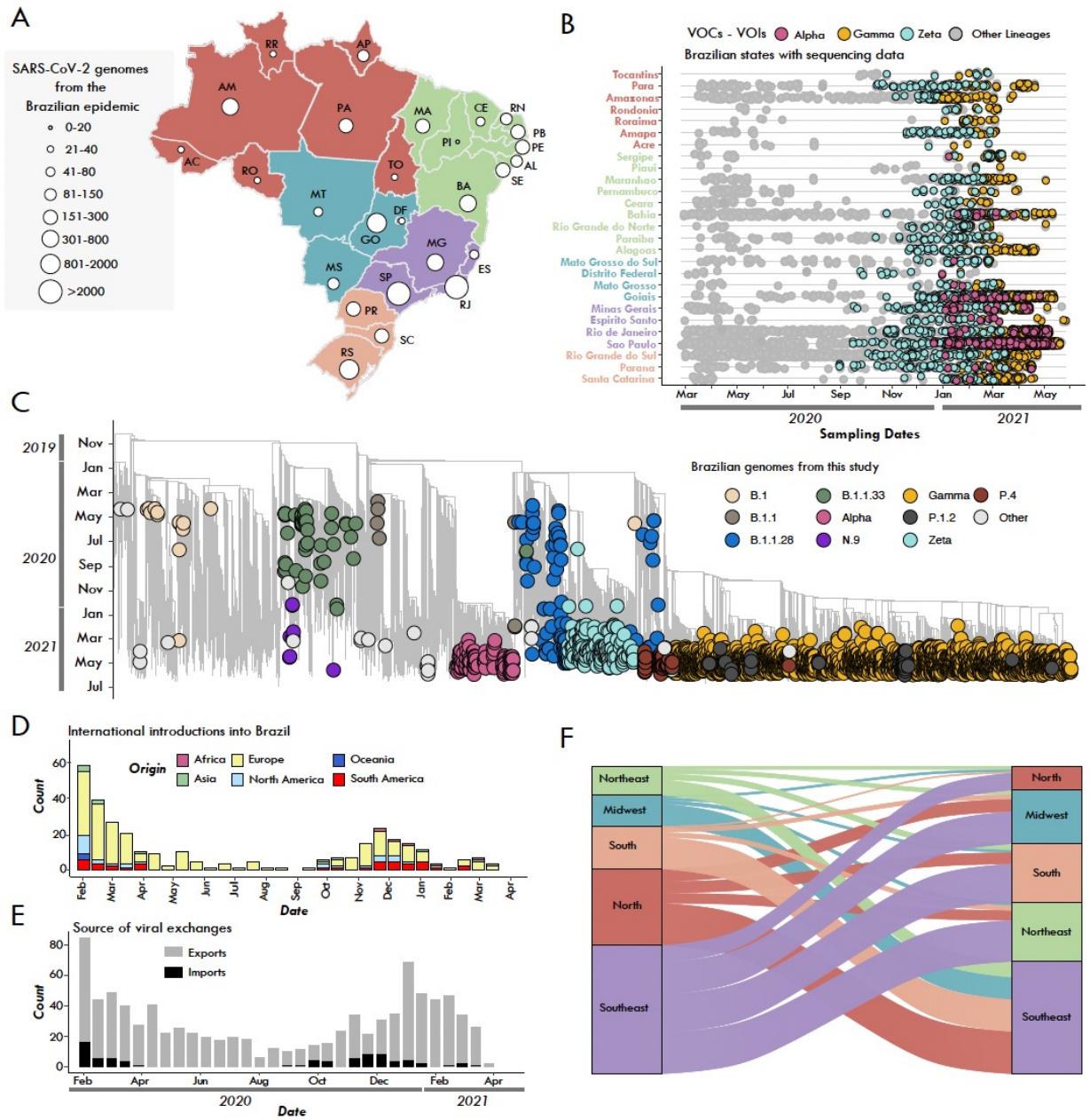


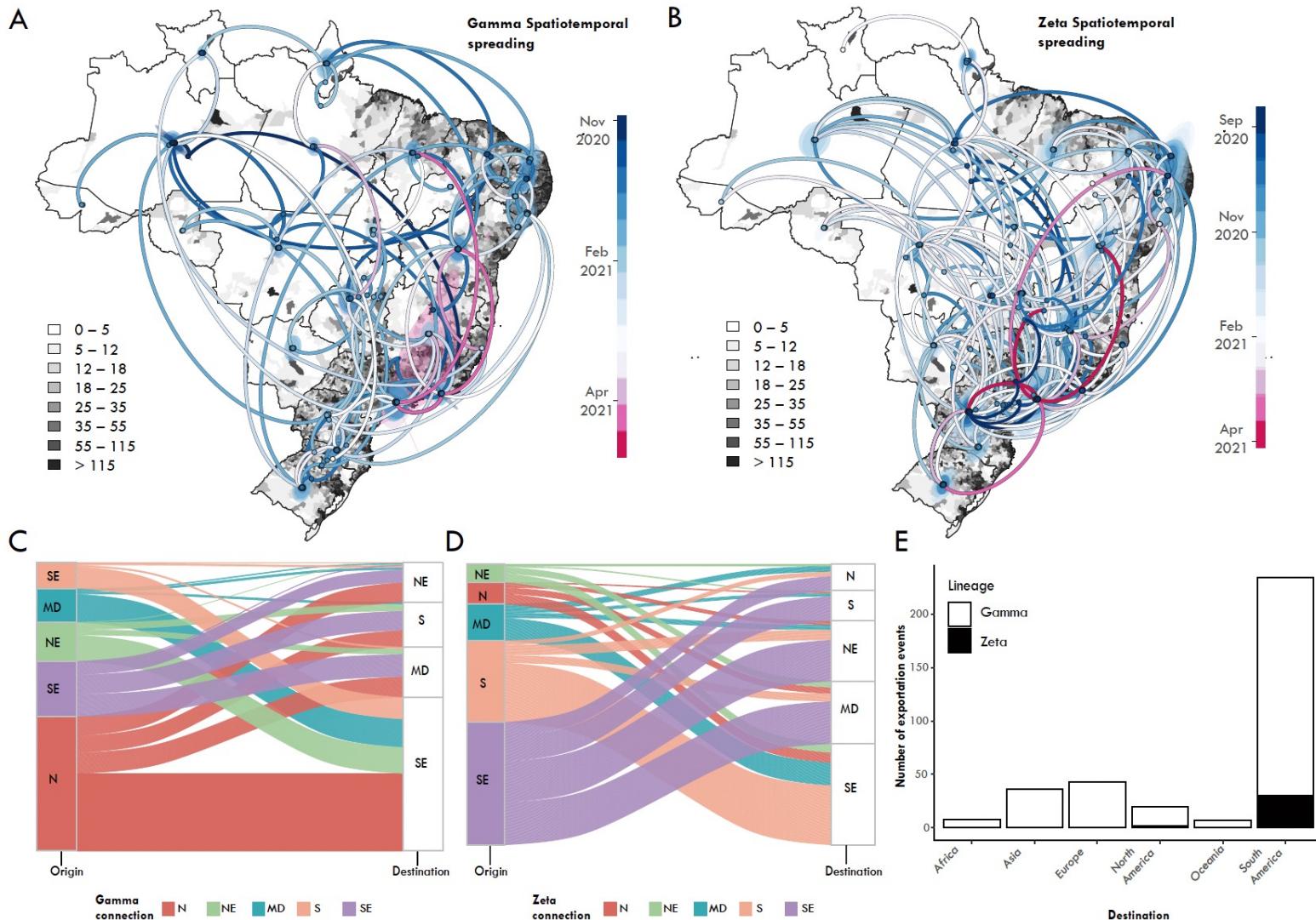


OPEN

# Genomic epidemiology of the SARS-CoV-2 epidemic in Brazil







# Workflow

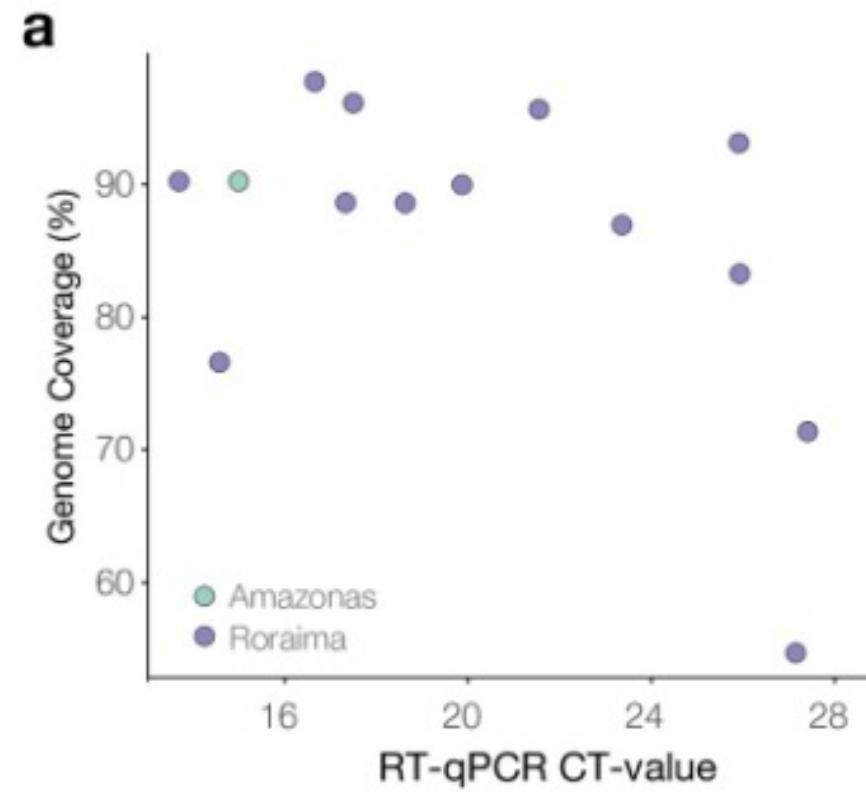
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- Sample selection
- Extraction
- cDNA synthesis
- Multiplex PCR
- Nanopore sequencing

# Sample selection



- Samples with a positive molecular assay
- Viral load
  - ✓ Complete genome



Fonte: Naveca et al (2019)

# Sample selection



- Spatio-temporal distribution
- Clinical relevant features
- Metadata availability



AH2	X	✓	f <sub>x</sub>	No														
A	B	C	D	E	F	G	H	I	J	K	L	M	N	O				
1	ID	Virus	Status_genome	Original_Lab	Original_Lab_ID	Original_Lab_CT	Sample_Type	Host	State	Municipality	Lat_Mun	Long_Mun	Lat_state	Long_state	Collection_Date			
2	OPAS 01	DENV2	Sequenced	Lacen-MG	00025/19 NS1	28	Serum	Human	MG	BETIM	-19,9688	-44,1946	-18,1890	-43,9400	07/02/2019			
3	OPAS 02	DENV2	Sequenced	Lacen-MG	00034/19 PFH	37	Serum	Human	MG	UBERABA	-19,6018	-47,9696	-18,1890	-43,9400	18/03/2019			

AH2	X	✓	f <sub>x</sub>	No										
P	Q	R	S	T	U	V	W	X	Y	Z				
1	Onset_date	Age	Sex	rural or urban	neighborhood	MG_cDNA Date	MG_Sequencing PCR Date	PCR cycle number	Negative Control	Qubit_ngPerULPoolA	Qubit_ngPerULPoolB			
2	07/02/2019	77	Male	Urban	BUENO FRANCO	11/08/2019	11/08/2019	37	CN02	28,8	19			
3	15/03/2019	80	Male	Urban	QUINTA DA BOA ESPERANÇA	11/08/2019	11/08/2019	37	CN02	23,4	20,4			

AN1	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	
1	MG_Barcod_Number	MG_Library_Number	MG_Flowcell_Number	febre	cefaleia	mialgia	exantema	artralgia intensa	dor retroorbital	vomitos	pre_disease	Denv_class	
2	NB 01	Library 2	FAK65290	Yes	No	Yes	No	No	No	No	Yes	Fatal case	
3	NB 02	Library 2	FAK65290	Yes	No	No	No	No	No	No	Yes	Fatal case	

# Primal Scheme

## A PRIMAL SCHEME

Scheme name

Email

Fastq  
choose file No file chosen  
One or more viral reference genomes in FASTA format

Amplicon length  
 400

Overlap  
 75

**Generate my scheme!**

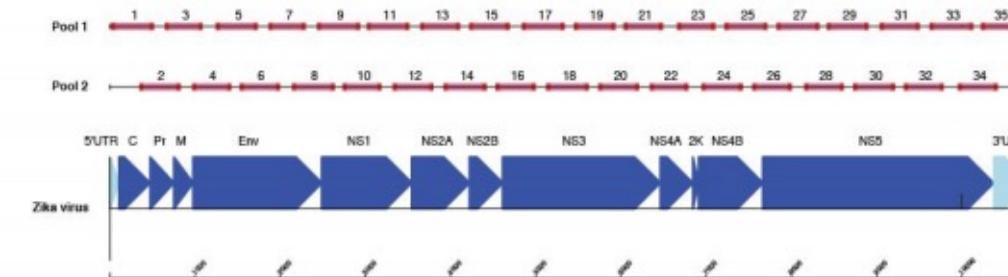
Want to try it out but no genomes to hand?  
Download CHIKV\_demo.fas

**B**

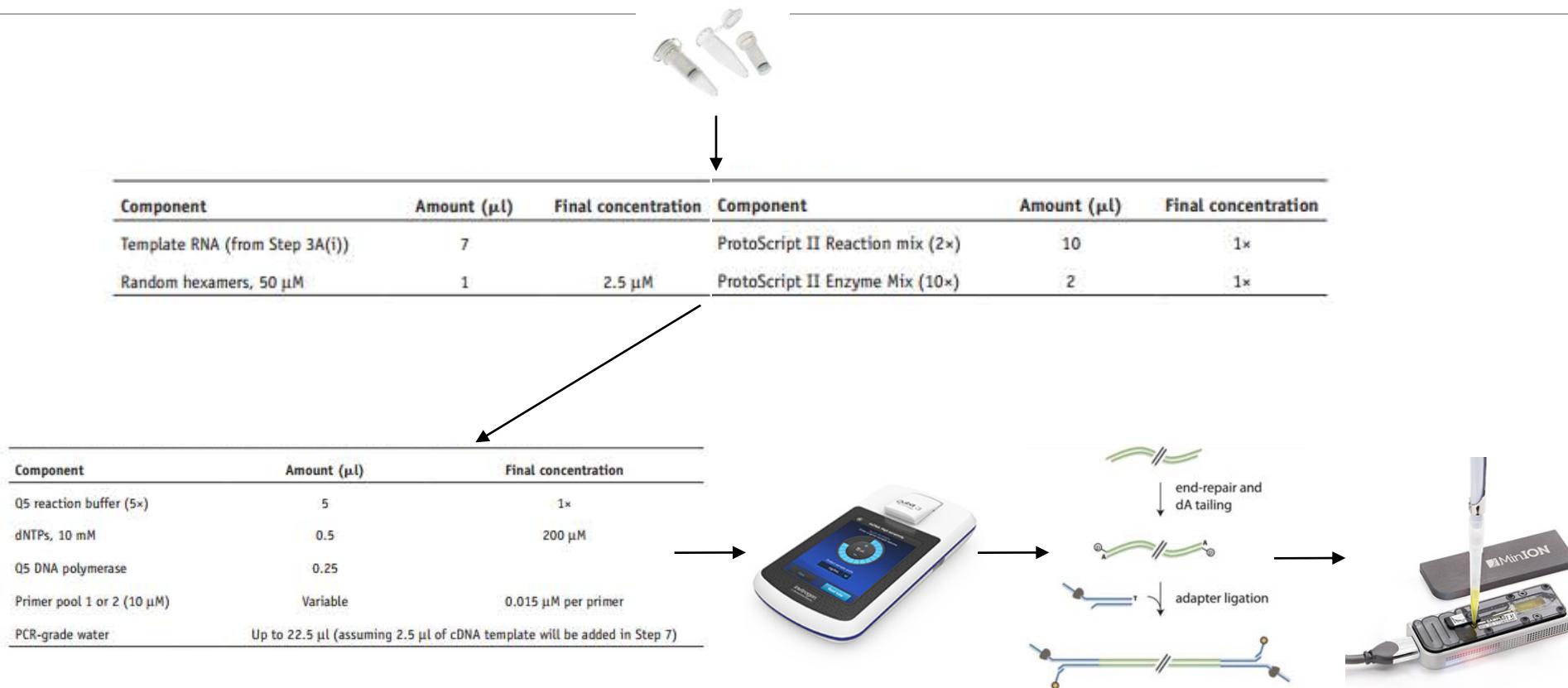
Job Name: CHIKV  
FASTQ file: [virusdata/CHIKV/CHIKV\\_demo.fas](#)  
Amplicon length: 400  
Overlap: 75 [Download as CSV](#)

**Primer Table**

Region No.	Pos.	Left Primer Name	Left Primer Sequence	Right Primer Name	Right Primer Sequence
1	1	400_3_LEFT_3	CTTTTGTAGGGCCCTGCGAGT	400_3_RIGHT_3	AGGGCCATCACTCTTETNAT
2	3	400_3_LEFT_3	TGTGGACAGGAGTCNNCTC	400_3_RIGHT_3	CGGGCAATTTCGGGGGGTGG
3	1	400_3_LEFT_3	GTCAGGTTGGGTTGGTCAACCA	400_3_RIGHT_3	TWAGGCGGSSGGCATCCCTTA
4	1	400_4_LEFT_4	TGGGTTGGCTGCTTAAGGGGG	400_4_RIGHT_4	CTTAAAGGTTGTCCTTGGGT
5	1	400_3_LEFT_2	TGTATGCAATGAACTGGGGATC	400_3_RIGHT_2	GTCTGAACGGGGTTCACAN
6	3	400_6_LEFT_3	AGAGAGGCTCTTAACTGCACTT	400_6_RIGHT_3	GGGGCTTGAGGGTTCATTT
7	1	400_7_LEFT_3	TGAGACGGGAGGAGGAGAT	400_7_RIGHT_3	TTGGAGGCTGGGGGGGGGGTT
8	1	400_8_LEFT_3	GGAGGCTGGGGGGGGGTTT	400_8_RIGHT_3	GGGAGAGGGGGGGGAAATTTTA
9	1	400_9_LEFT_3	TGCAAGAAAGAAGAATTTGAT	400_9_RIGHT_3	GGCAAGTAACCTTCCAGATG
10	1	400_10_LEFT_3	ATCTGAGCTGGGGGGGGTGTG	400_10_RIGHT_3	GGTTTGTTGAGGGGGGGGGTG

**C**

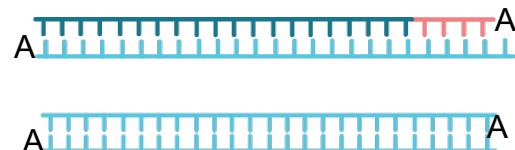
# Workflow for tiling amplicon sequencing



## Step by step...

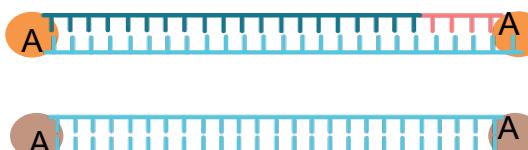
End repairs 3'

Leave all the fragments the same and allow the connection of barcodes and adapters



Barcodes

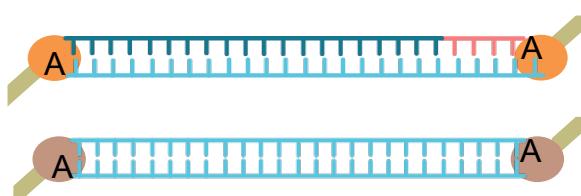
Facilitates sample identification – each sample has its barcode



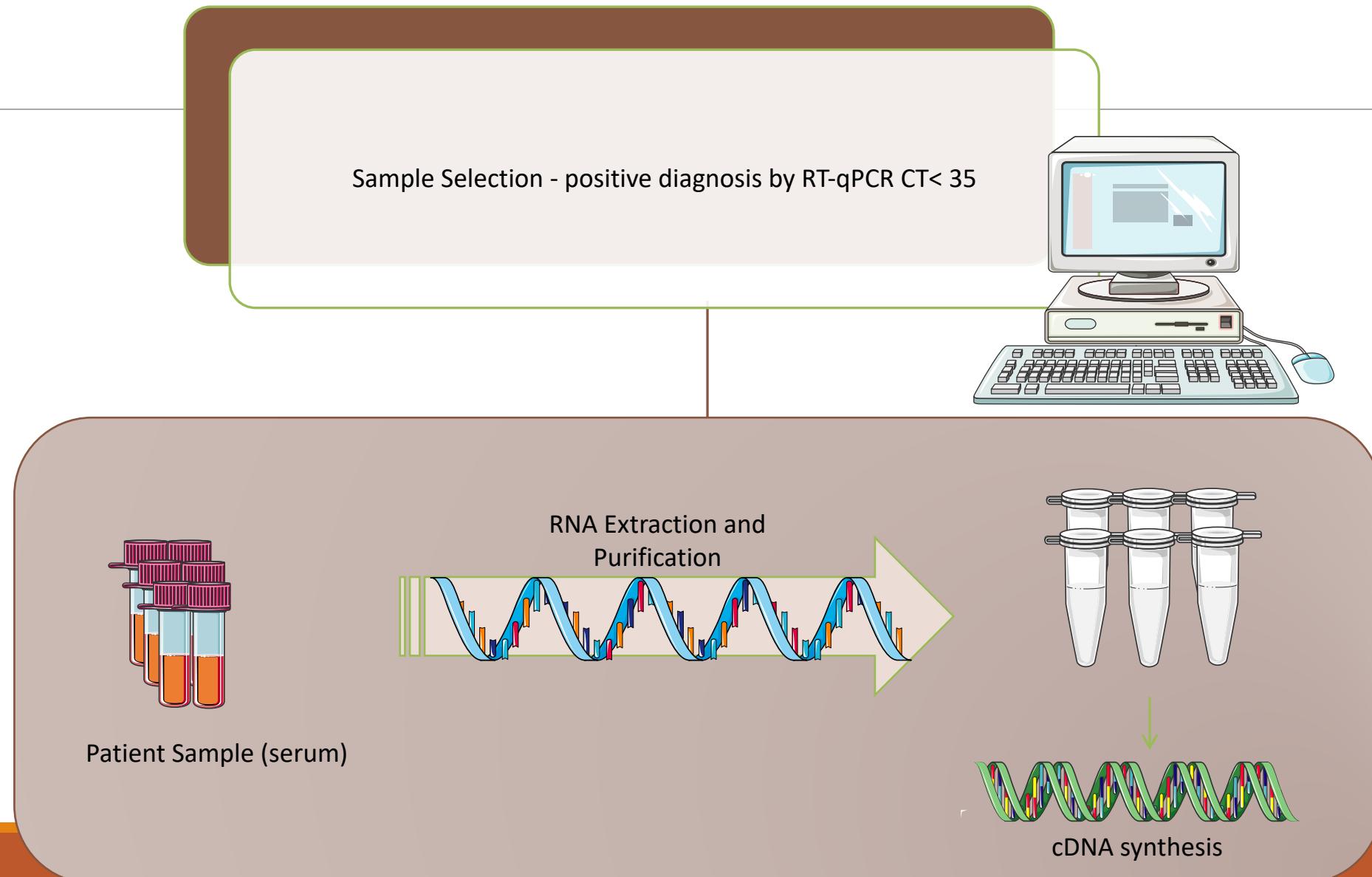
---

Adapters

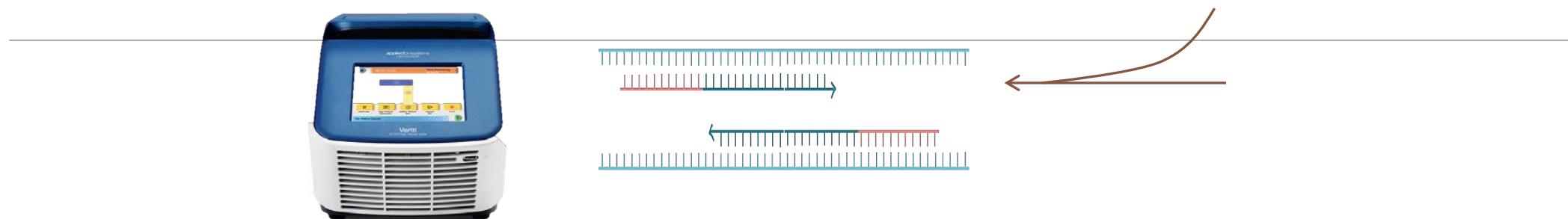
Motor proteins that help anchor the fragments to the flowcell nanopore



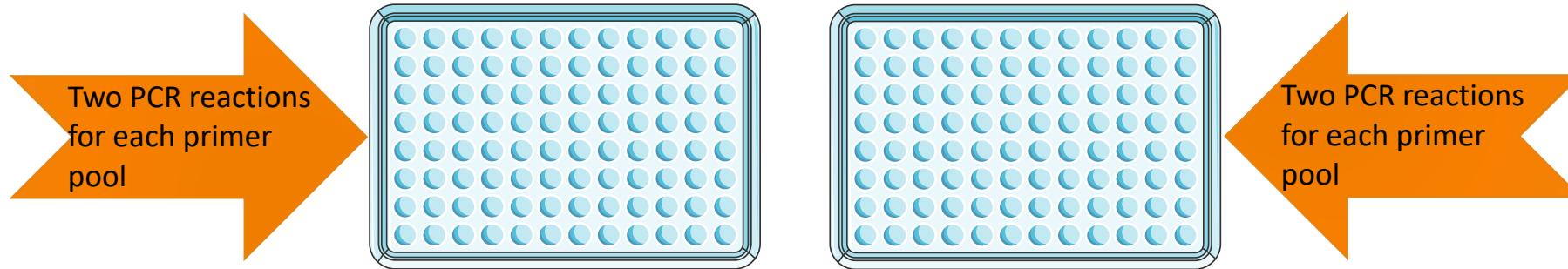
## Direct sequencing



## Sequencing PCR



**Pool A e B (independently)**



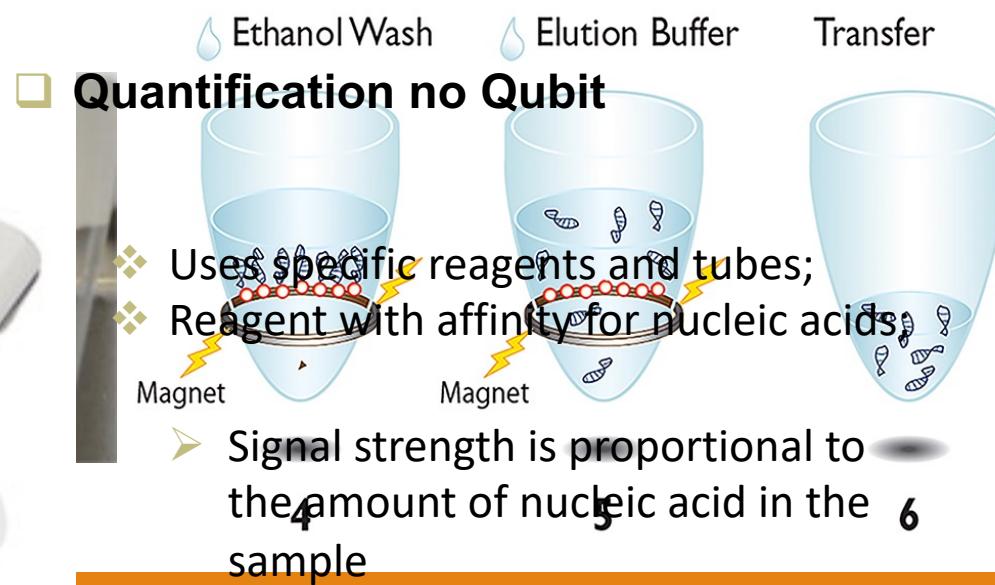
Ex: Primers Denv 1

Pool A - Primers Forward e Reverse 1, 3, 5, 7,... 15

Pool B - Primers Forward e Reverse 2, 4, 6, 8,... 14



## Purification and Quantification of samples



## Sample normalization

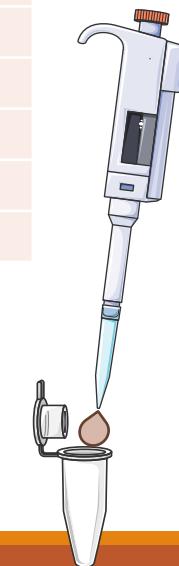
- Put all samples at the same concentration

---

- For arbovirus we need to normalize all samples at 40ng in 20ul

Ex:

Sample	ng/ul	Sample volume	NFW volume	Final Volume
1 too low		Will not be submitted to NGS		
2	5	8,00	12,00	20
3	10	4,00	16,00	
4	15	2,67	17,33	
5	30	1,33	18,67	
6	44	0,91	19,09	
7	60	0,67	19,33	
8 too high		Dilute and quantify again		



# End Prep Repair

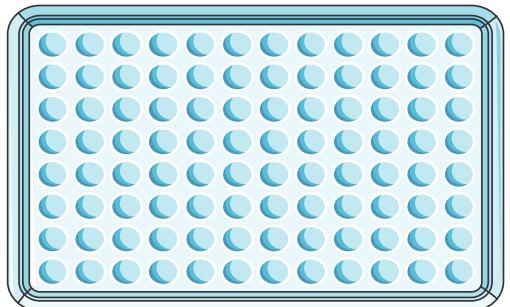
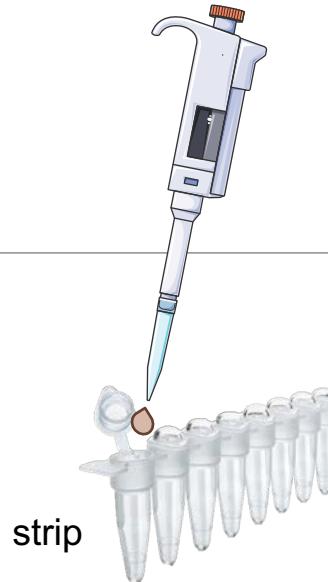
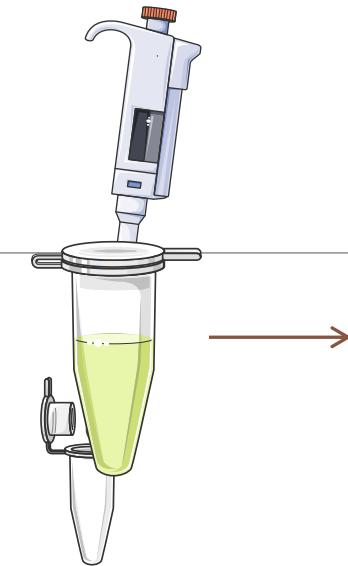
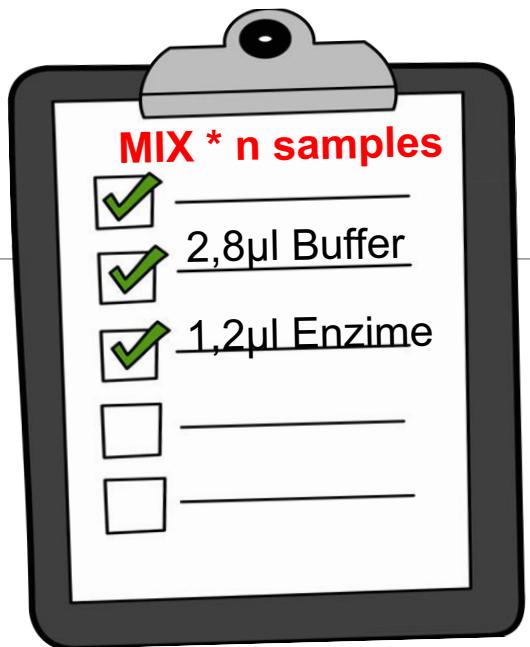
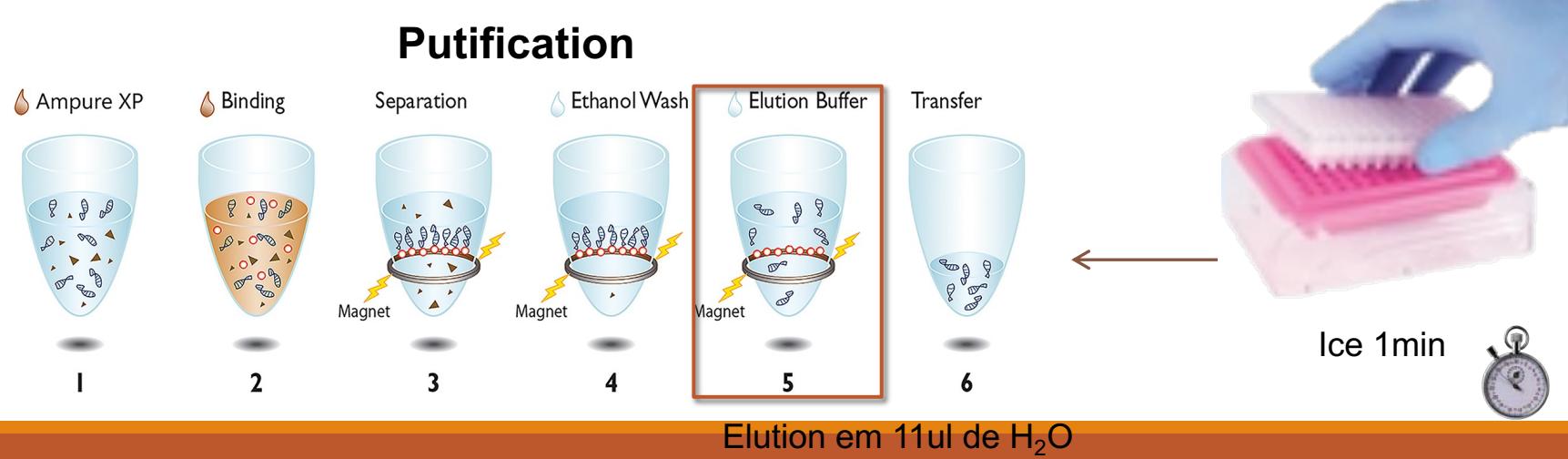
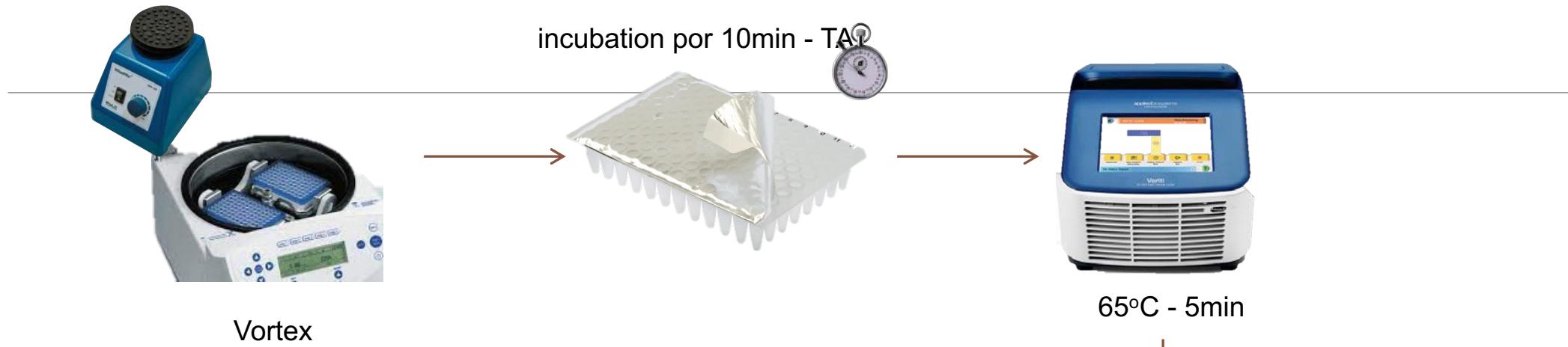


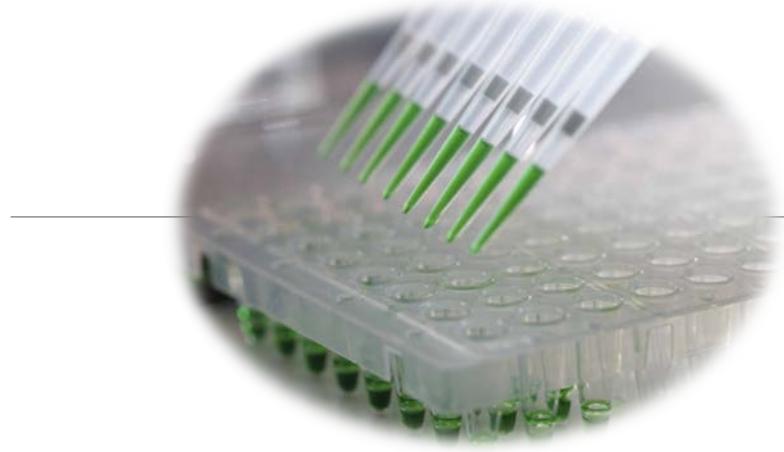
Plate with purified and  
standardized A and B amplicons  
(40ng in 20ul)

Transfer 20µl of  
purified  
amplicon

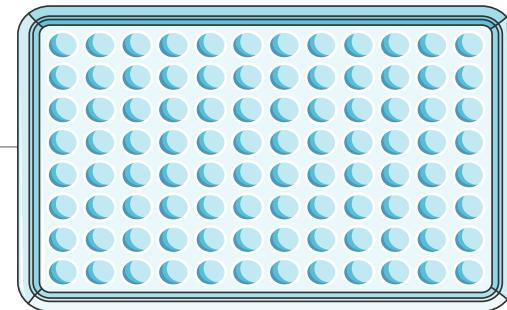
## End Prep Repair



## Barcodes



12,5 $\mu$ l  
Blunt T/A



Purified Plate after End Prep  
Reaction (10 $\mu$ l)

Incubate for 30min - RT



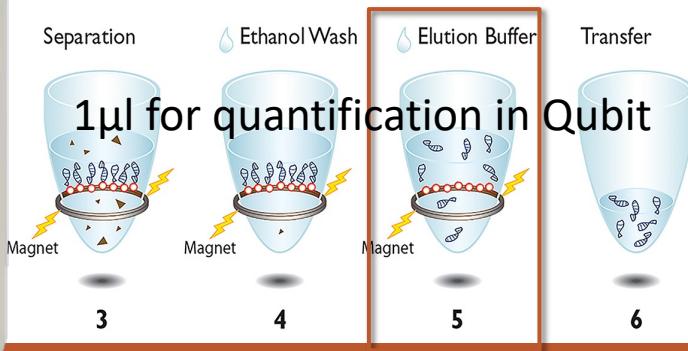
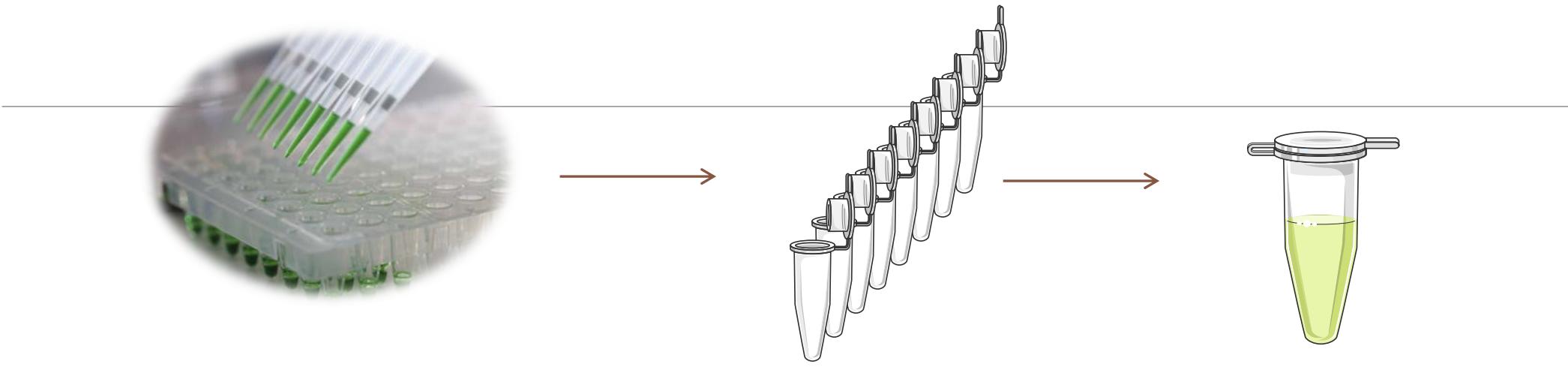
Vortex

Stop the reaction at 65°C -  
15min + ice 1min



Distribute 2.5 $\mu$ l of barcode

## Pool + Purification

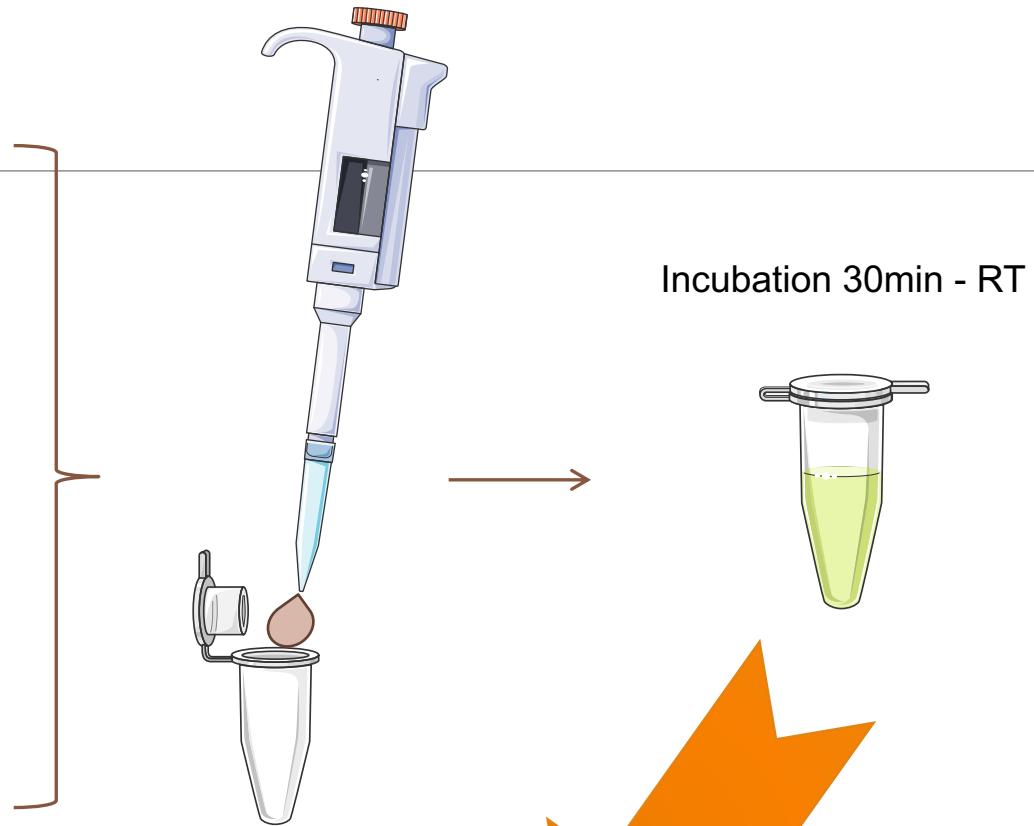


Purification with  
25µl of beads per  
sample (1:1 V/V)  
of beads

Elution in 46µl H<sub>2</sub>O



# Adaptor



Purification with beads 1:1

## Library Purification



**2 washes with SFB – homogenize and repeat**



## Library Dilution



Library dilution to 30ng in 12 $\mu$ l

ex: quantification = 10ng/ $\mu$ l  
➤ 3 $\mu$ l of our library  
➤ 9 $\mu$ l of EB

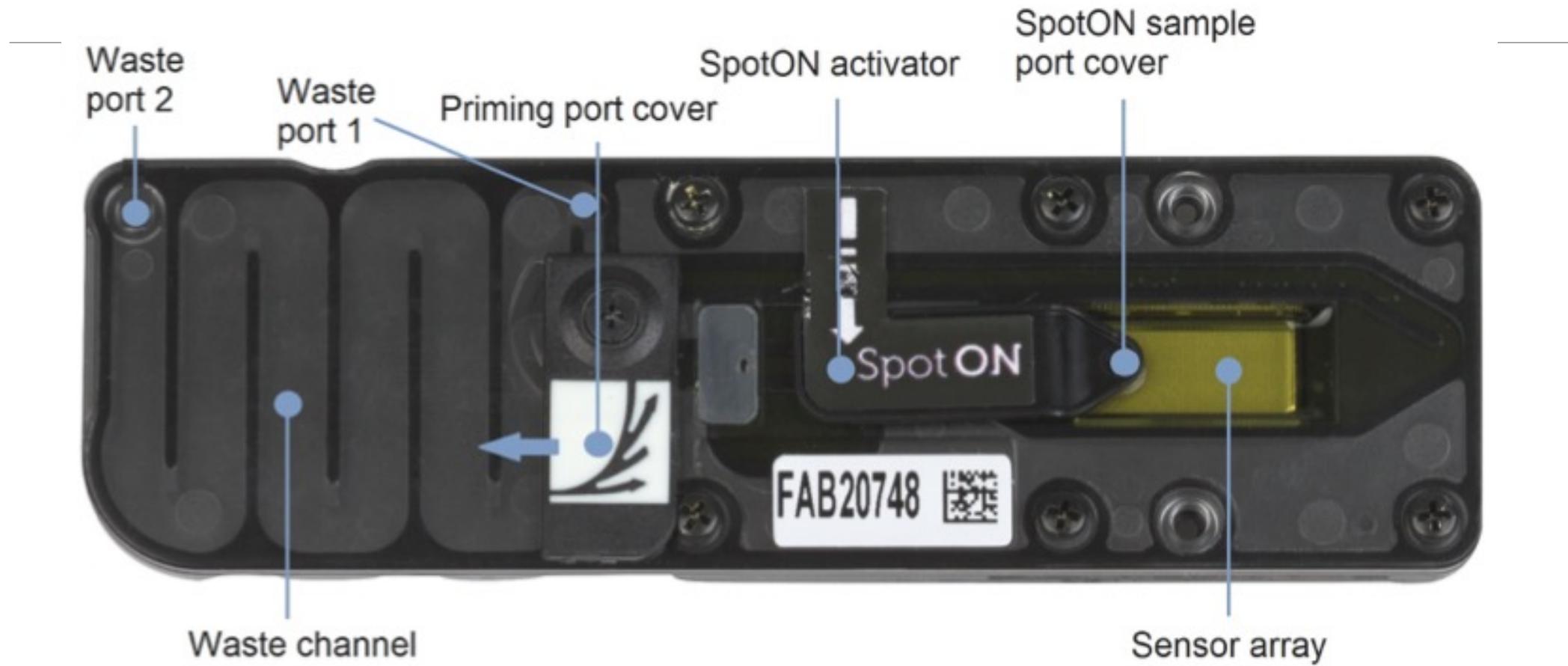


Load flowcell with running buffer and library

Running Buffer= FLT + FB

Diluted Library+ SQB + LB

## Flowcell



## Samples from infected individuals

Clinical  
Virology  
PCR, Genomics



## Pathogen genome sequences

Alignment Methods  
Sequence Evolution  
Models



## Genetic distances

Recombination  
Analysis

Phylogenetic Methods



## Phylogeny

(time scale = genetic distance)

Molecular Clock Models



## Phylogeny

(time scale = years)

Coalescent Theory



Population  
dynamics

Selection  
Analysis

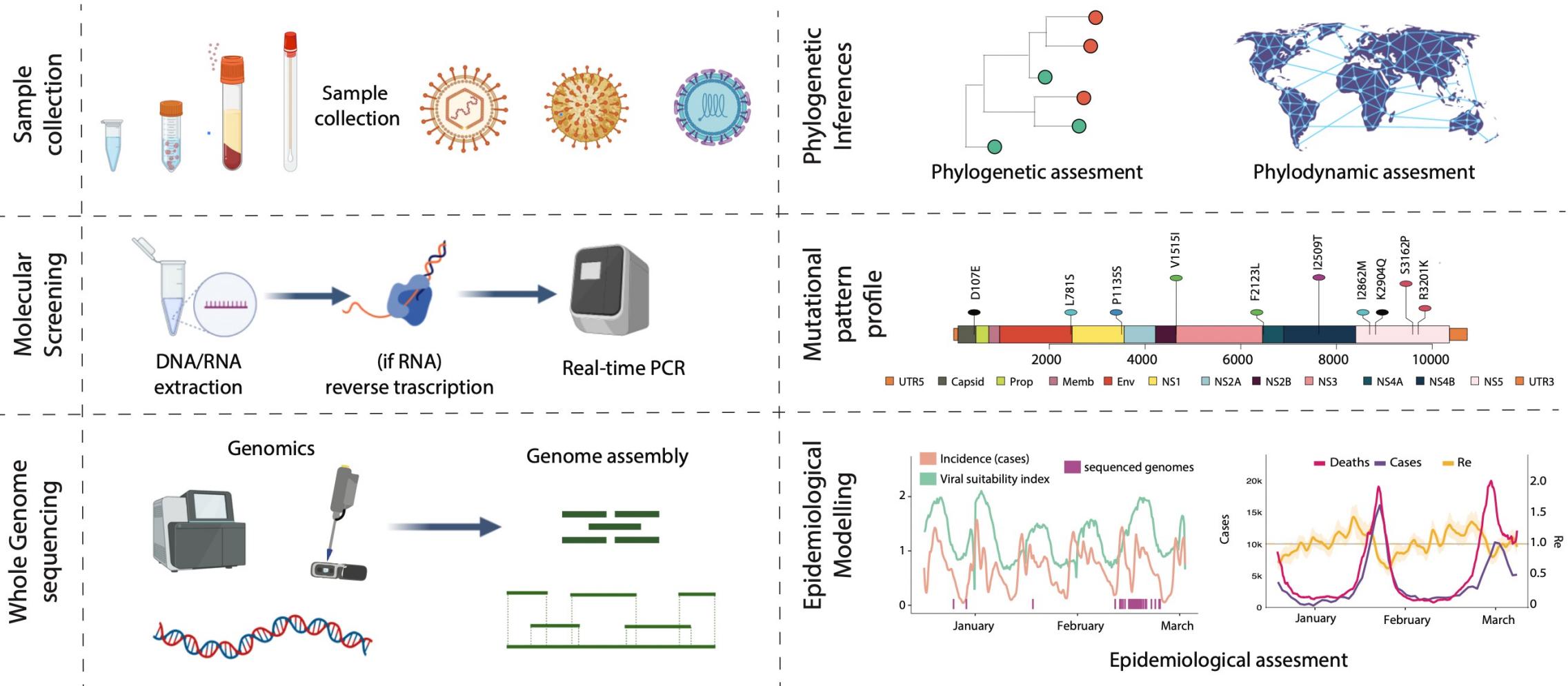


Adaptation  
Dynamics

Phylogeographic models



Spatial  
dynamics



Public  
Engagement

Education

Open Access

Gender

Ethics



**Making Research and Innovation with Education**

# Integrated training



Data sharing  
Real-time sequencing  
Sample handling  
Phylogenetics  
BEAST  
Case study of sequencing rabies in the field  
Outbreak Scenario & Molecular Epidemiology

Lab-in-a-suitcase  
NextStrain

# Integrated training

