Standardization of next generation sequencing methods for the quality control of live-attenuated vaccines

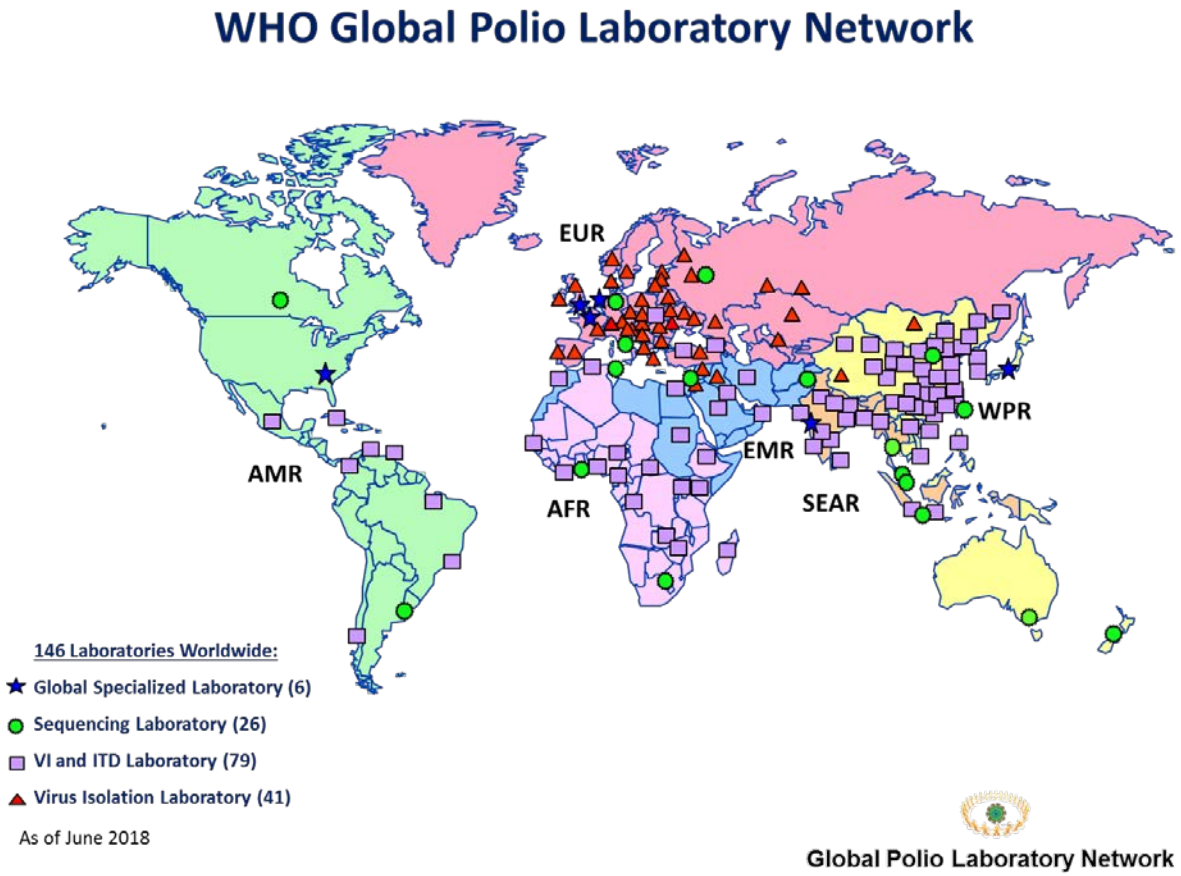
Workshop on Standards for NGS Detection of Viral Adventitious Agents in Biologics/Biomanufacturing.
18-19 September 2019

Javier Martin

Medicines & Healthcare products Regulatory Agency

The National Institute for Biological Standards and Control

A key player in the Global Polio Eradication Initiative



NIBSC is…

… **WHO Collaborative Centre for Biological Standardization**

… **WHO global Specialized Polio Laboratory**
(only 6 such labs in the world)

… **WHO Collaborating Centre for Reference & Research on Poliomyelitis**
(only 5 such centres in the world)

Sabin live-attenuated OPV strains

Type 1

Mahoney
(healthy children)

24 in vivo
43 in vitro

LS-c

5 in vitro
3 plaque purifications

LSc2ab

Type 2

P712
(healthy children)

3 in vitro
4 plaque purifications
1 chimpanzee passage

P712Ch

3 plaque purifications

P712Ch2ab

Type 3

Leon
(fatal case)

20 in vivo
8 in vitro

Leon (Sabin)

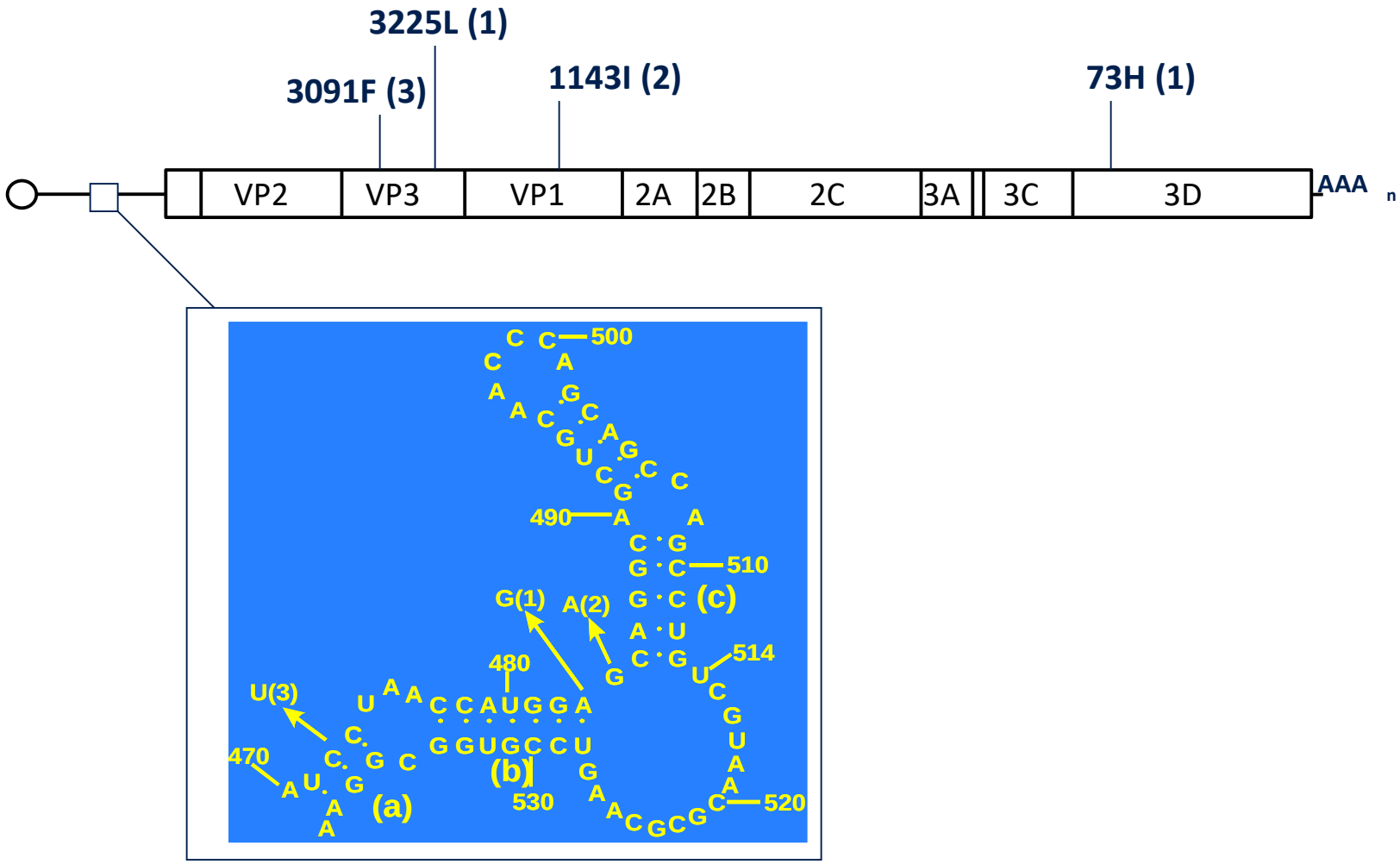
34 in vitro

Leon LP34

3 plaque purifications

Leon $12a_1b$

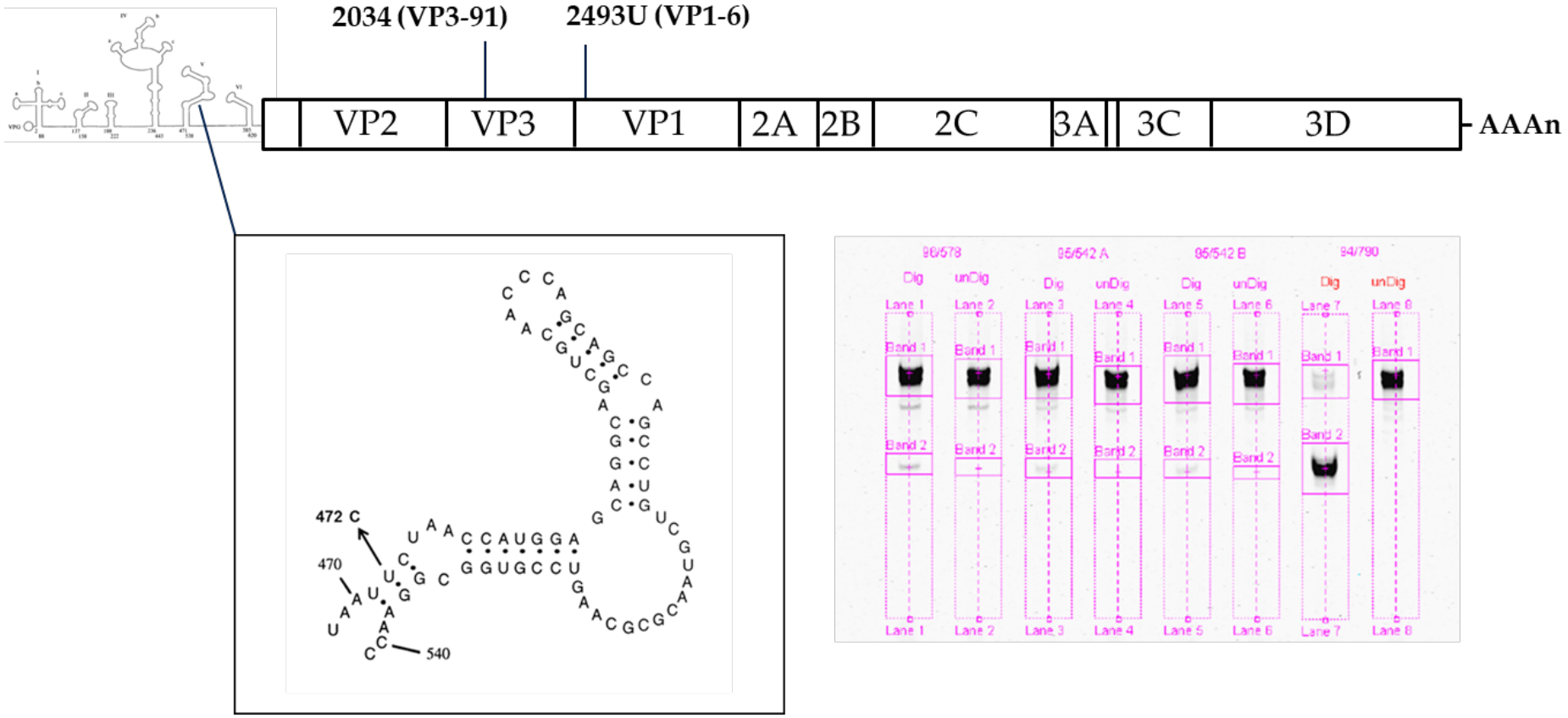
Determinants of attenuation in Sabin poliovirus OPV strains



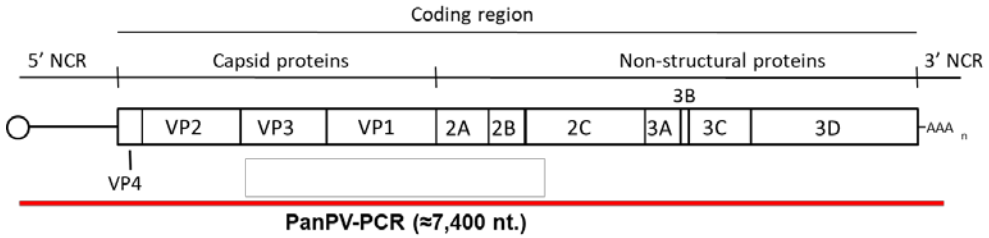
Safety testing of OPV

OPV Production	Testing		
	Until 2002	*2002-2013*	*2013 onwards*
Master Seed	MNVT	MNVT	MNVT
↓	↓	↓	↓
Working Seed	MNVT	MNVT	TgmNVT
↓	↓	↓	↓
Monovalent Single Harvest	-	MAPREC	MAPREC
↓	↓	↓	↓
Monovalent Pooled Harvest	MNVT	TgmNVT MAPREC	TgmNVT MAPREC
↓			
Final Bulk Vaccine (mOPV, bOPV, tOPV)			

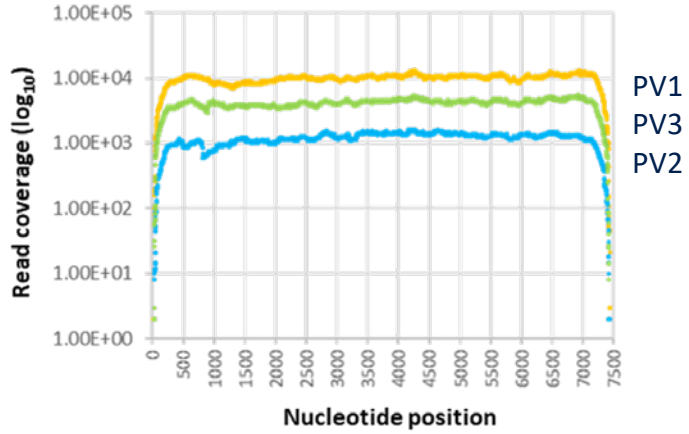
Mutation Analysis by PCR and Restriction Enzyme Cleavage

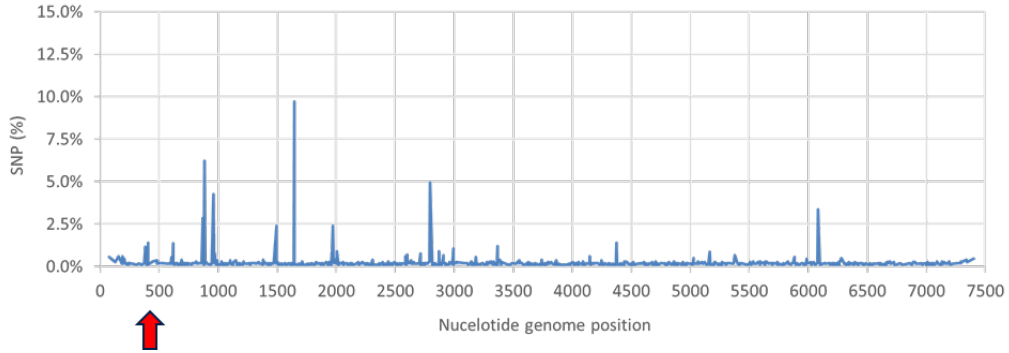


Next Generation Sequencing (NGS) analysis of OPV strains



Samples analyzed	OPV sample
RNA extraction	High Pure Viral RNA kit (Roche)
cDNA-PCR synthesis	Whole-genome Pan-PV PCR
Library preparation	Nextera XT reagents
NGS	MiSeq Nextera™ XT kit v2
Data analysis	**Geneious R10** *Mapping to Sabin reference* *SNP calling*





Standardization of NGS analysis for the quality control of OPV

Outline of Collaborative Study

- Objectives:
  - Phase One
    - Validate NGS as an alternative to MAPREC for type 3 OPV
    - 5’ UTR neurovirulent mutation measured by MAPREC – U472C in Sabin 3
  - Phase Two
    - Validate NGS as an alternative to MAPREC for type 1 and 2 OPV
    - Evaluate genetic consistency of mutational profiles of vaccines in the entire viral genome
- Eight participants from Europe (n=6), USA (n=1) and Japan (n=1) including manufacturers (n=4) and control labs (n=4)
- Study Protocol:
  - Determine 472C content in each vaccine sample by NGS in 5 independent determinations (PCR or RNA)
  - Use in-house NGS protocols
  - Use in-house bioinformatics tools
  - Provide raw sequence files
  - Raw data re-analyzed at NIBSC and compared to MAPREC data

Collaborative Study OPV samples (PV3)

Sample Code	Vaccine	Description	Result		
			MAPREC	MNVT	TgmNVT
3A	05/146	SO+3	Pass	Pass	Pass
3B	96/578	High MAPREC ref	-	-	-
3C	93/636	SO+3	Fail	Fail	Fail (20/20)
3D	POL3	SO+3	Pass	Pass	Pass
3E	96/572	Low MAPREC ref	-	-	-
3F	97/676	RSO3	Fail	Fail	Pass
3G	98/660	RSO3	Pass	Pass	Pass
3H	98/650	RSO3	Pass	Pass	Pass
3I	96/578	High MAPREC ref	-	-	-
3J	96/568	SO+3	Fail	Fail (1/2)	Fail (11/14)
3K	H2328	cDNA-derived	Pass	Pass	Pass

Numbers in brackets indicate number of tests passing or failing over total number of tests performed in different labs if more than one test was performed

Results - MAPREC (U472C) values summary

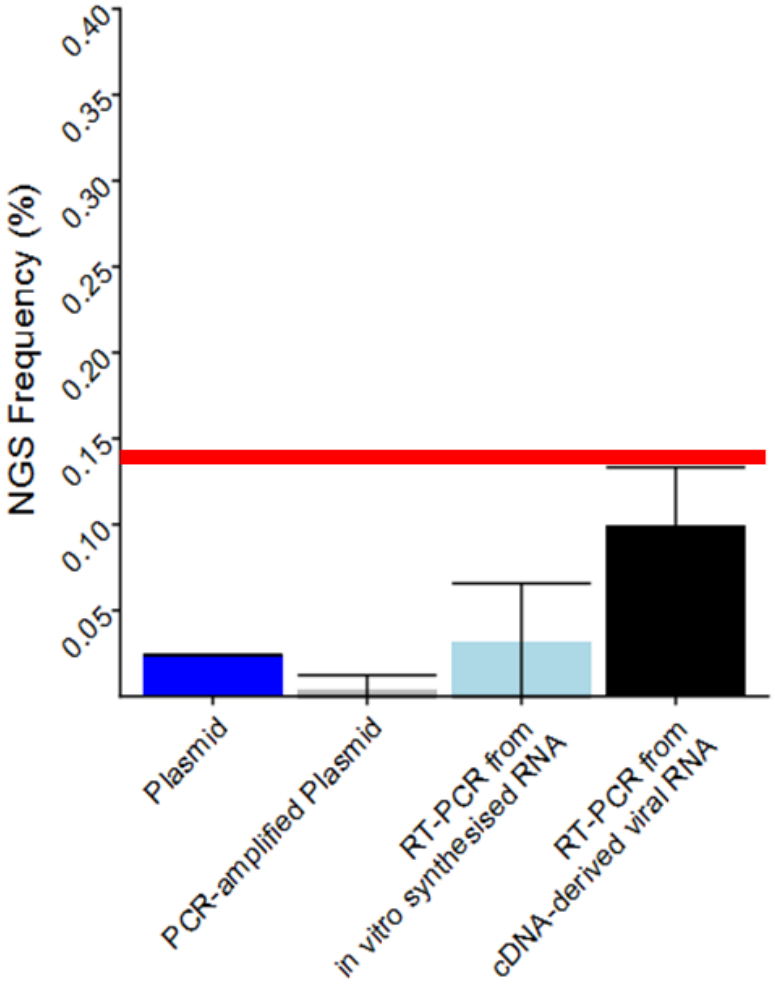
Sample	Mean	SD	Min	Max	Outcome
3K	0.09	0.09	0.00	0.22	Pass
3G	0.35	0.07	0.24	0.41	Pass
3A	0.50	0.11	0.38	0.63	Pass
3D	0.67	0.07	0.60	0.78	Pass
3E	0.75	0.12	0.60	0.89	Pass
3H	0.91	0.23	0.65	1.20	Pass
3I	1.14	0.14	1.06	1.39	Fail
3B	1.18	0.15	0.98	1.33	Fail
3F	1.56	0.22	1.33	1.82	Fail
3J	1.64	0.12	1.55	1.84	Fail
3C	3.21	0.21	2.94	3.42	Fail

- Low intra-lab variability
- Low within-assay variability
- Values between 0.09 and 3.21

NGS laboratory methods

Lab	RNA Extraction	PCR Strategies	PCR Purification	Quantity	NGS Strategies	In-House Analysis
1	QIAmp Viral RNA Mini Kit	Two-step RT-PCR using SuperScript IV first stand RT synthesis. RT primers polio specific (Neverov and Chumakov 2010)	QIAquick PCR purification kit	Qubit	Miseq platform, Nextera XT kit, Illumina cartridges, Single operator	fastqc, trimmomatic, bwa, samtools, bcftools, Q Score > 20
2	MagJET Viral DNA and RNA Kit	Two-step RT-PCR using SuperScript III first stand RT synthesis, KAPA Hifi Hotstart ReadyMix for PCR, polio specific primers amplifying nt 186- nt 864	Abgene storage plate	Qubit	Miseq platform, Nextera XT kit, two operators	CLC Genomic Workbench (Qiagen)
3	QIAmp Viral RNA Mini Kit	Two-step RT-PCR using AccuScript RT,Herculase Taq DNA polymerase, polio specific primers, Two PCRs: nt 223-nt 919 (Short), nt 1- nt-7432 (Whole genome sequencing: WGS)	Agentcourt AMPure XP Beads	Qubit and qPCR	Miseq platform, Nextera XT kit, Miseq Reagent Nano Kit V2	CLC Genomic Workbench (Qiagen)
4	Roche HighPure Viral RNA Extraction Kit	One-Step RT-PCR using SuperScript III with Platinum Taq DNA Polymerase, polio specific primers, WGS	Agentcourt AMPure XP Beads	Qubit	Miseq platform, Nextera XT V2 kit, two operators	Geneious, v 10 Biomatters
5	Details Not Provided	Details Not Provided	Details Not Provided	Details Not Provided	Details Not Provided	Details Not Provided
6	Roche HighPure Viral RNA Extraction Kit	Instead of PCR, RNA libraries prepared using NEBNEXT Ultra II RNA Library Prep Kit For Illumina	Agentcourt AMPure XP Beads	Qubit and BioAnalyzer	Miseq platform, Nextera XT kit, Two operators	CLC Genomic Workbench (Qiagen)
7	MagNA Pure LC total nucleic acid isolation kit and Kingfisher System	Two-Step RT-PCR ,RT: Full length cDNA, PCR: 2 overlapping PCRs covering whole genome nt 1-nt 3797 + nt 3734 - nt 7432, Superscript III + Long Range	Agentcourt AMPure XP Beads	Qubit and BioAnalyzer	Miseq platform, Nextera XT V2 kit, two operators	CLC Genomic Workbench (Qiagen)
8	MagNA Pure	RT PCR NEB Next Ultra Directional RNA Library Prep Kit (Illumina), Random Priming Approach: First strand: ProtoScript II RT. NEBNext Q5 Hot Start HiFi PCR Master Mix, WGS	Agentcourt AMPure XP Beads	BioRad Droplet Digital PCR	Illumina HiSeq4000 platform, Cartridges: 300 cycle	Trimmomatic, FastQA, FASTQFilter, FASTQ-Screen, BWA, ea-utils, bamtools, samtools, bamcount, base quality >= Q30

NGS analysis of OPV strains - background error



1) Plasmid sequenced directly

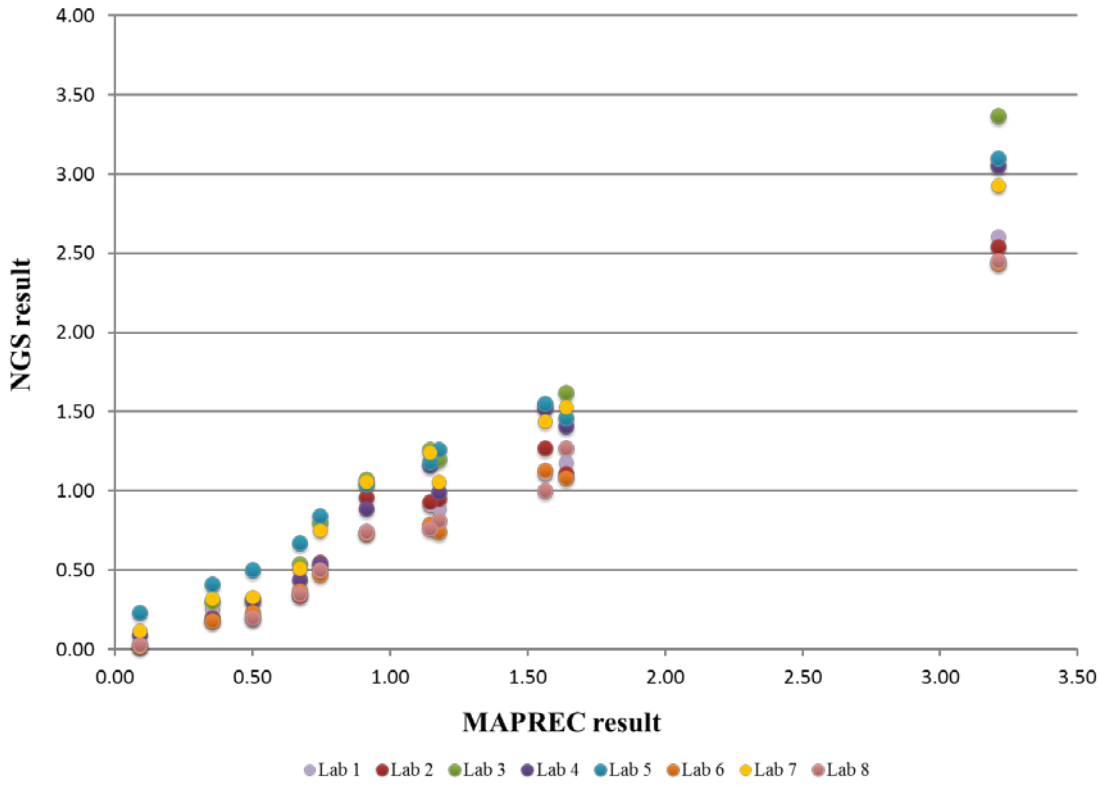
2) Plasmid in One-Step RT-PCR reaction

3) Transcription of RNA from plasmid → Transcribed RNA in One-Step RT-PCR reaction

4) Transcription of RNA from plasmid → Transfection of RNA into susceptible cells → Recover virus → Extract RNA → Extracted RNA in One-Step RT-PCR reaction

NGS vs MAPREC (U472C) - correlation

Analysis	Sample	Labs	Mean	Min	Max	Variance Components (as SD)		
						Intra-Lab	Inter-Lab	Total
In-House	3K	7	0.08	0.03	0.18	0.01	0.06	0.06
In-House	3G	7	0.24	0.18	0.36	0.05	0.06	0.08
In-House	3A	8	0.28	0.18	0.41	0.04	0.08	0.09
In-House	3D	8	0.40	0.26	0.53	0.06	0.08	0.11
In-House	3E	8	0.63	0.46	0.86	0.16	0.11	0.20
In-House	3H	8	0.87	0.68	1.09	0.10	0.14	0.17
In-House	3I	8	0.98	0.70	1.38	0.09	0.21	0.22
In-House	3B	8	0.98	0.73	1.20	0.10	0.16	0.19
In-House	3F	8	1.30	0.99	1.66	0.17	0.22	0.28
In-House	3J	8	1.33	1.05	1.69	0.13	0.22	0.25
In-House	3C	8	2.80	2.40	3.58	0.23	0.40	0.47
NIBSC	3K	8	0.07	0.02	0.23	0.02	0.07	0.08
NIBSC	3G	7	0.26	0.18	0.41	0.07	0.08	0.10
NIBSC	3A	8	0.30	0.20	0.50	0.07	0.09	0.11
NIBSC	3D	8	0.45	0.34	0.67	0.10	0.11	0.15
NIBSC	3E	8	0.62	0.47	0.84	0.15	0.13	0.20
NIBSC	3H	8	0.90	0.73	1.07	0.14	0.13	0.19
NIBSC	3B	8	0.99	0.74	1.26	0.13	0.20	0.24
NIBSC	3I	8	1.02	0.76	1.26	0.11	0.17	0.21
NIBSC	3F	8	1.32	1.00	1.55	0.16	0.21	0.26
NIBSC	3J	8	1.33	1.08	1.62	0.16	0.19	0.25
NIBSC	3C	8	2.81	2.44	3.37	0.20	0.34	0.39



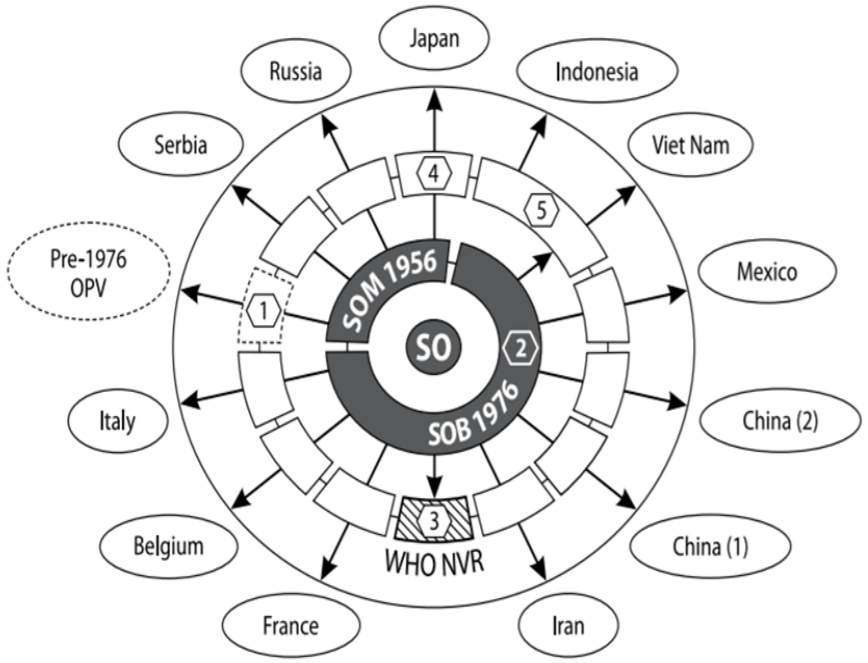
Pearson correlation coefficient

Analysis	Lab								
	1	2	3	4	5	6	7	8	Overall
In-House	0.997	0.979	0.994	0.994	0.990	0.995	0.995	0.992	0.996
NIBSC	0.996	0.984	0.995	0.993	0.996	0.996	0.991	0.993	0.996

Whole-genome NGS analysis of OPV strains

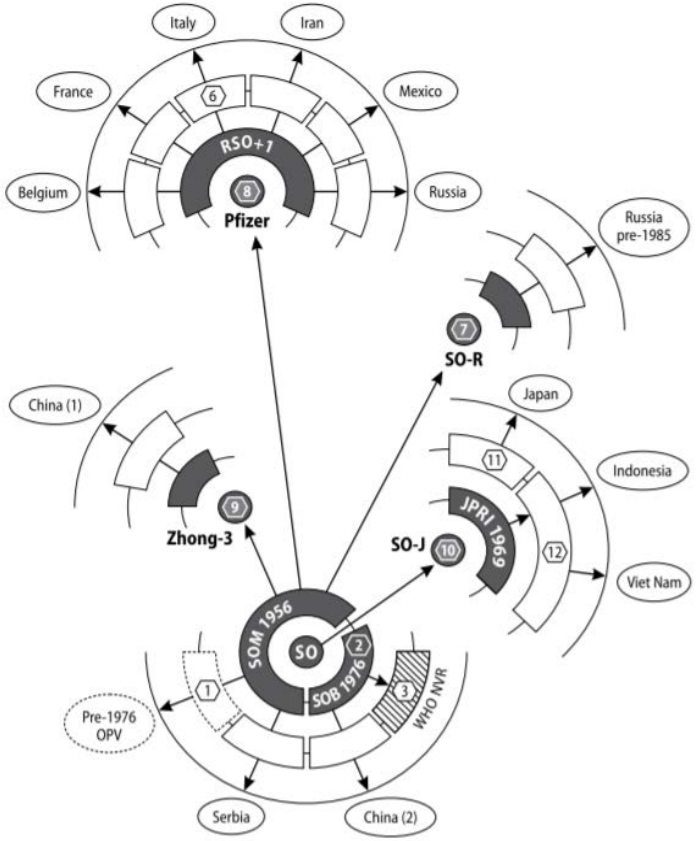
Passage history of OPV seeds

Figure 2.1
History of seed virus and reference materials used to produce type 1 and type 2 OPV from Sabin 1 and Sabin 2

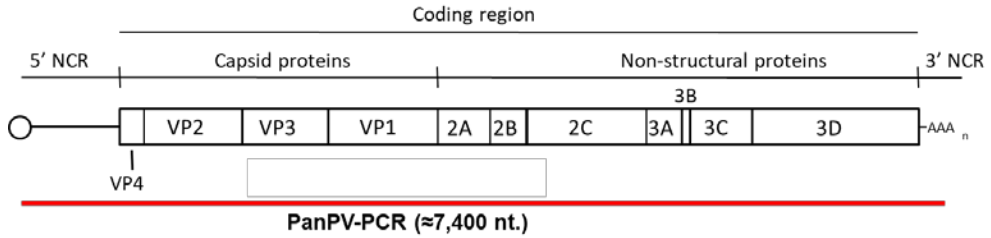


WHO Technical Report Series No. 980, 2014

Figure 2.2
History of seed virus and reference materials used to produce type 3 OPV from Sabin 3



NGS analysis of OPV strains – SNP analysis



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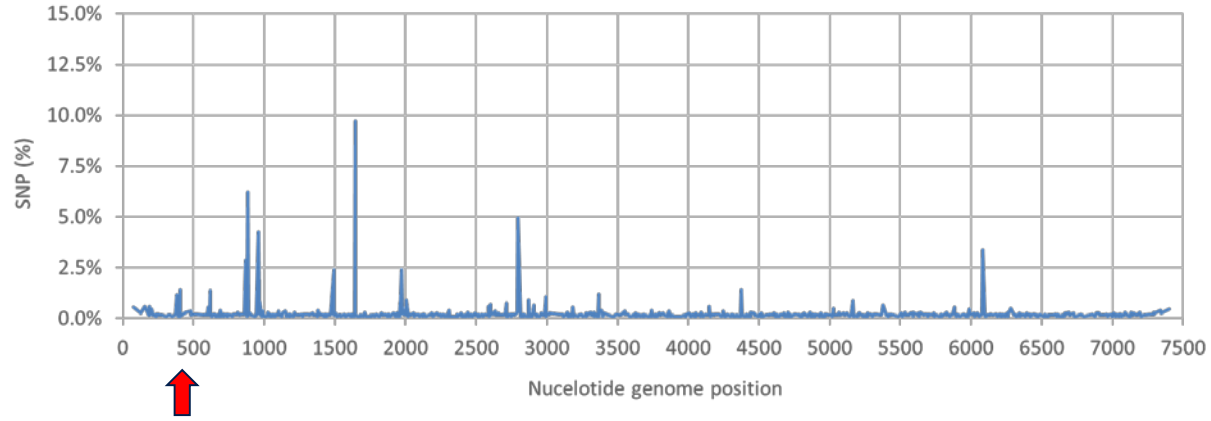
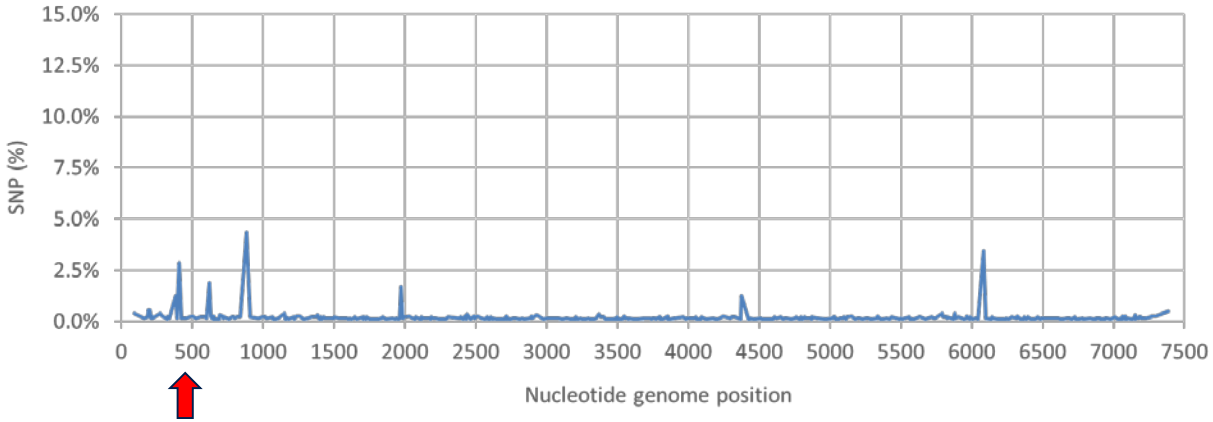
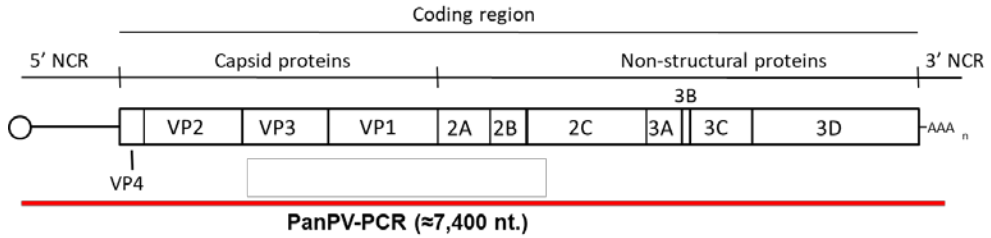




NGS analysis of OPV strains – SNP analysis



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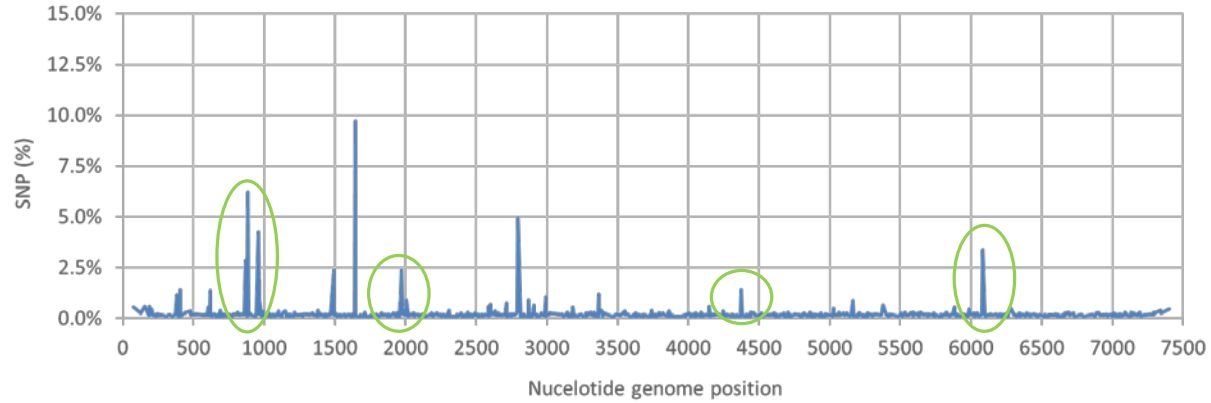

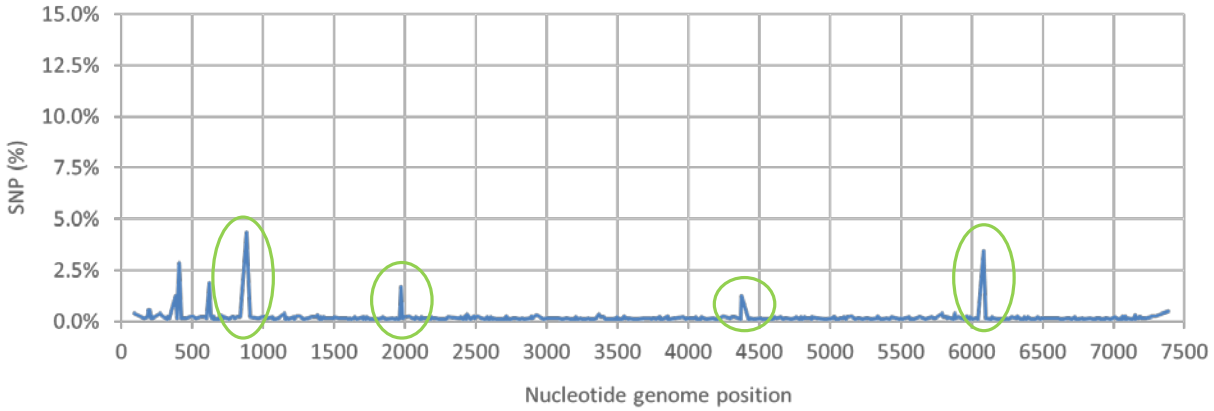

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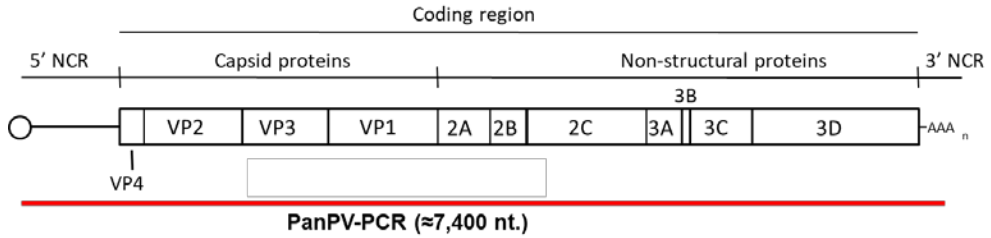

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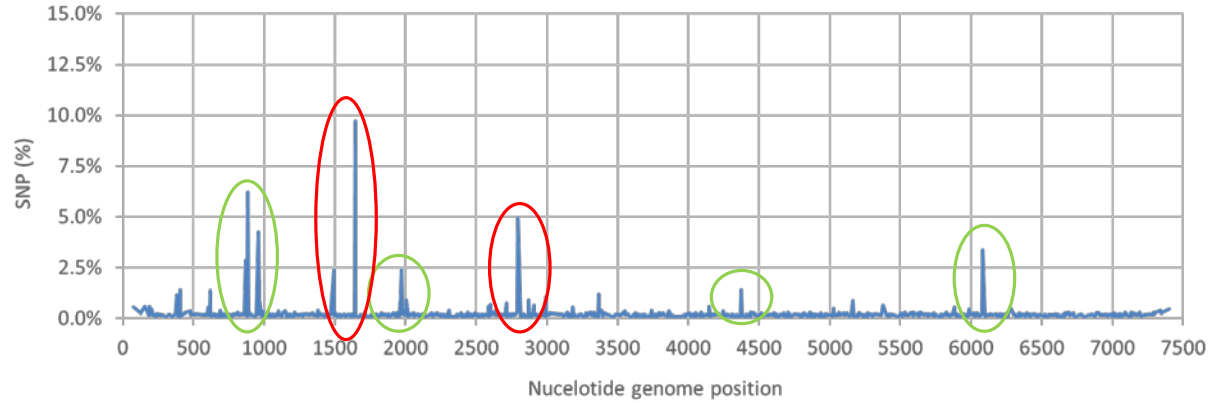
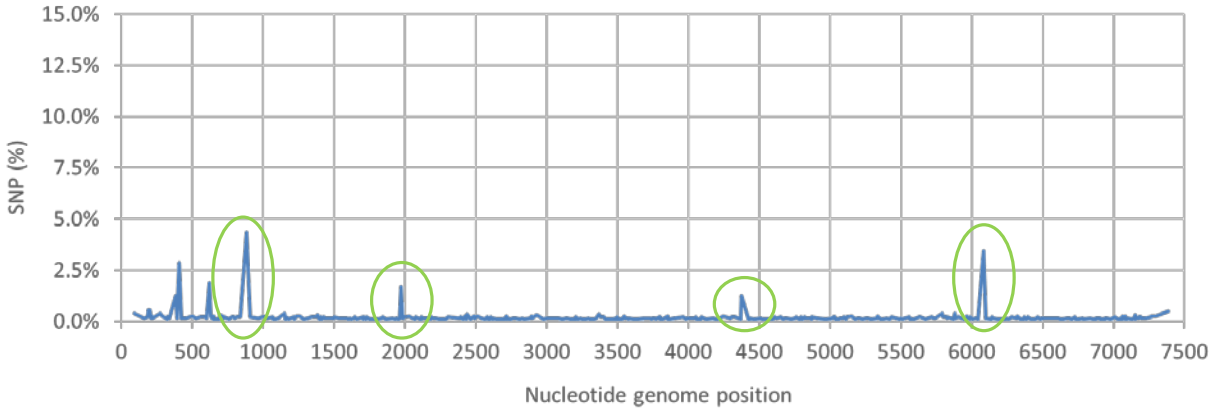
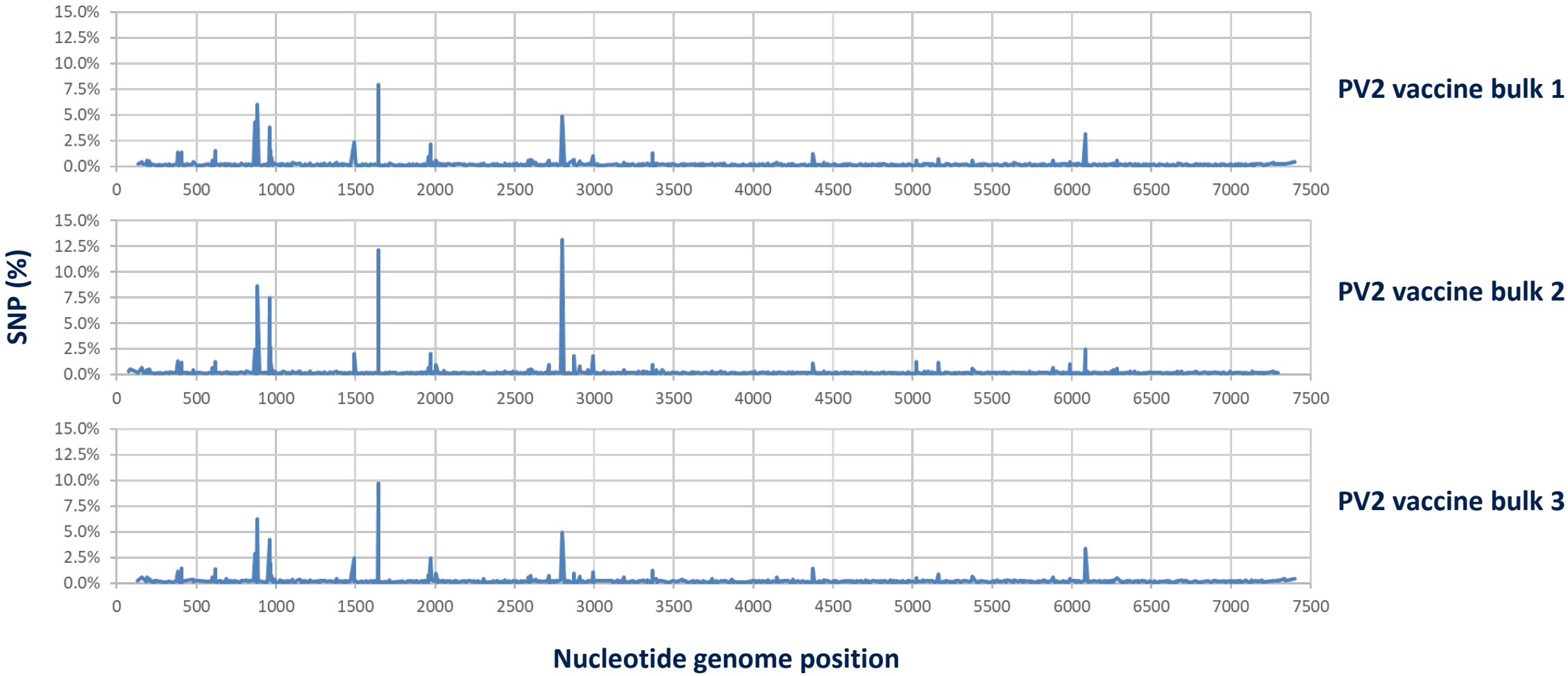




Whole-genome NGS analysis of OPV strains – PV2



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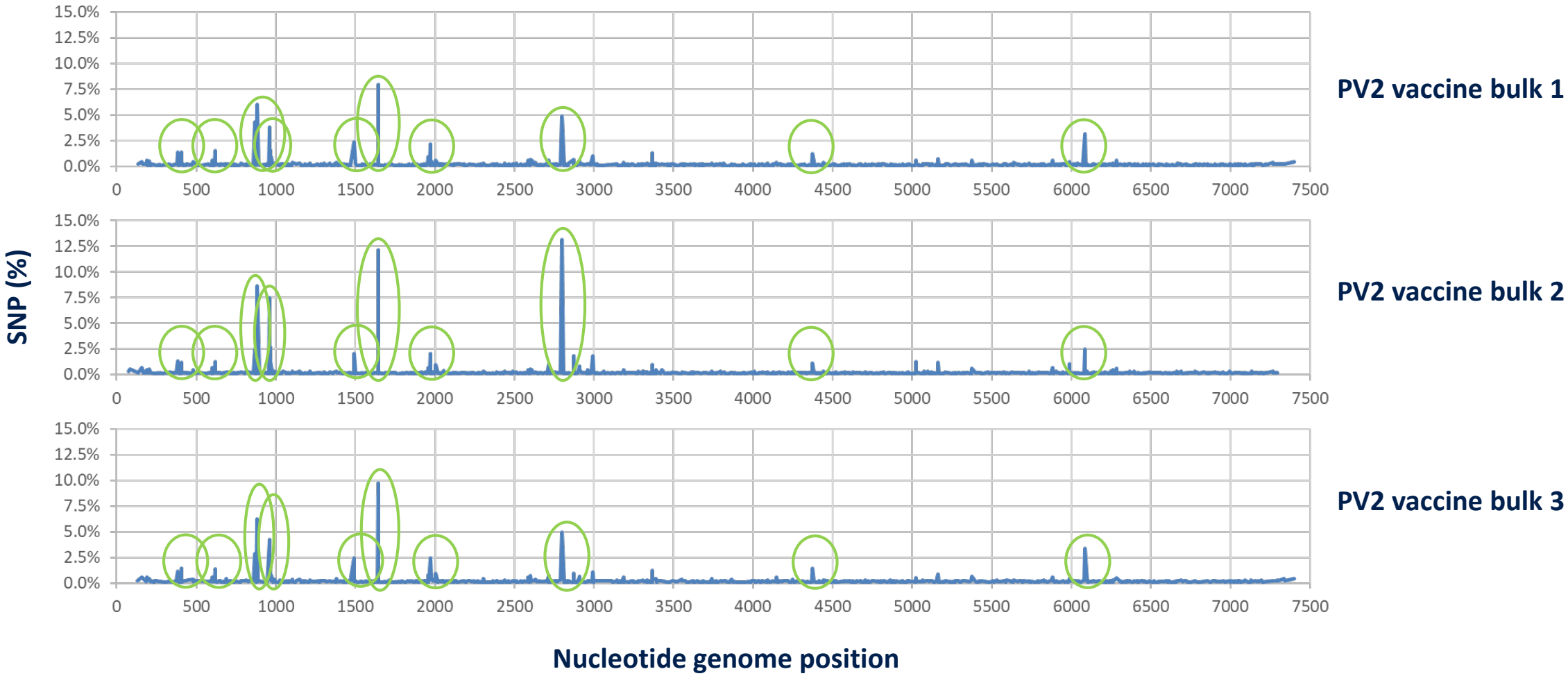
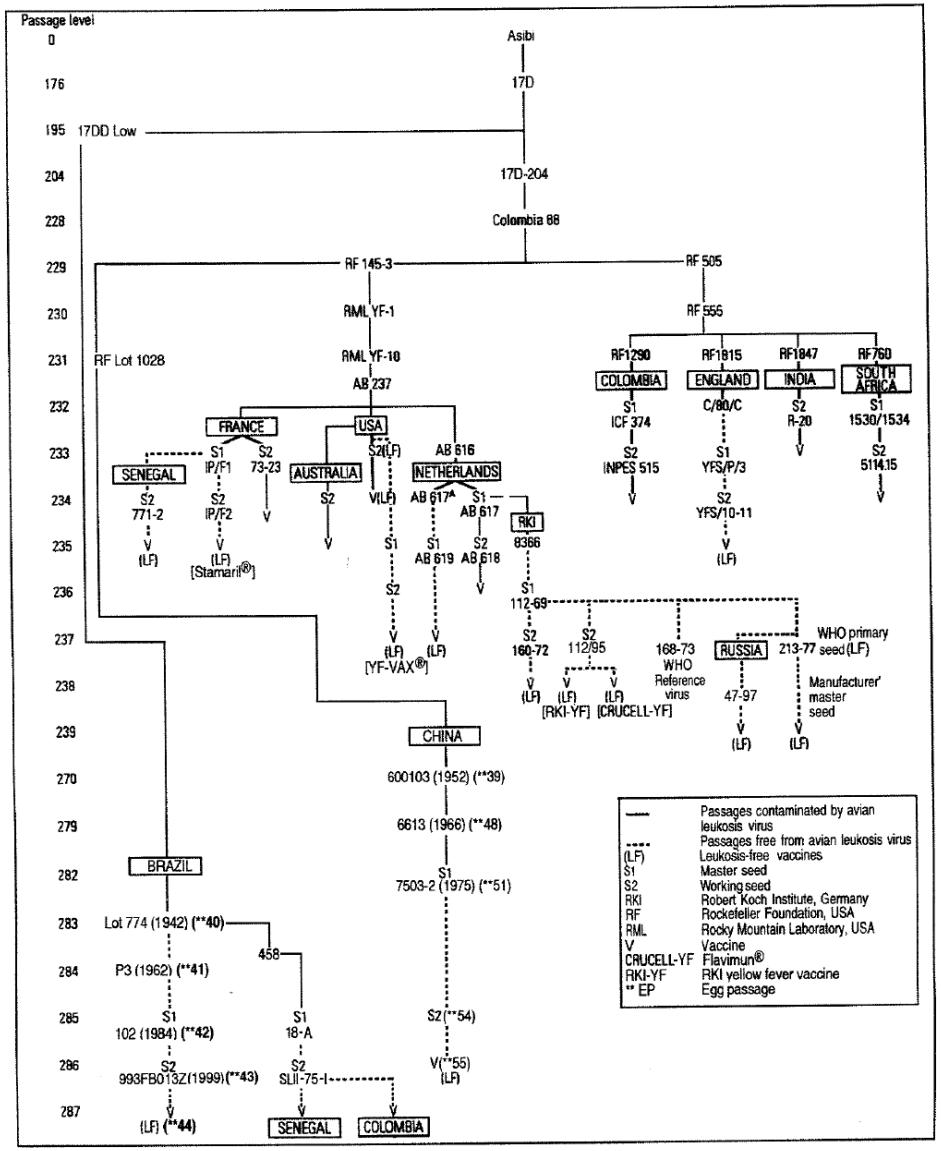


NGS analysis of live-attenuated yellow fever vaccines



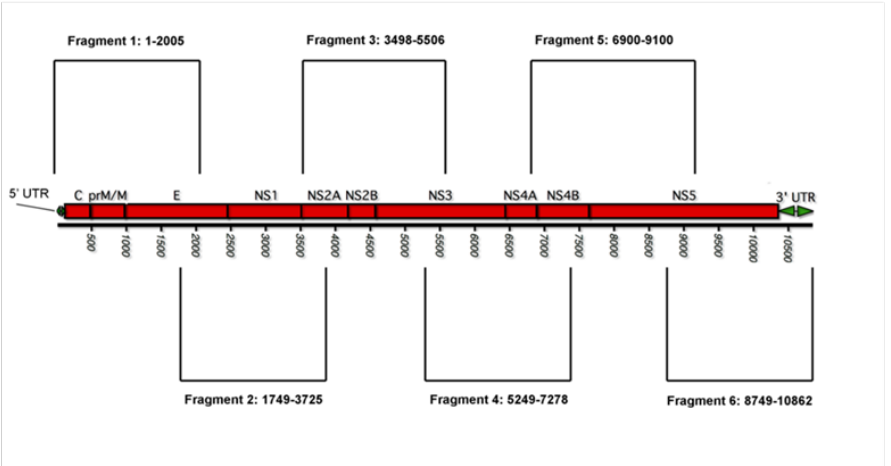
YF-17D is one of the most effective vaccines ever made. In the 73 years that have elapsed since its development, the vaccine has been administered to over 540 million people globally

MAJOR ARTICLE

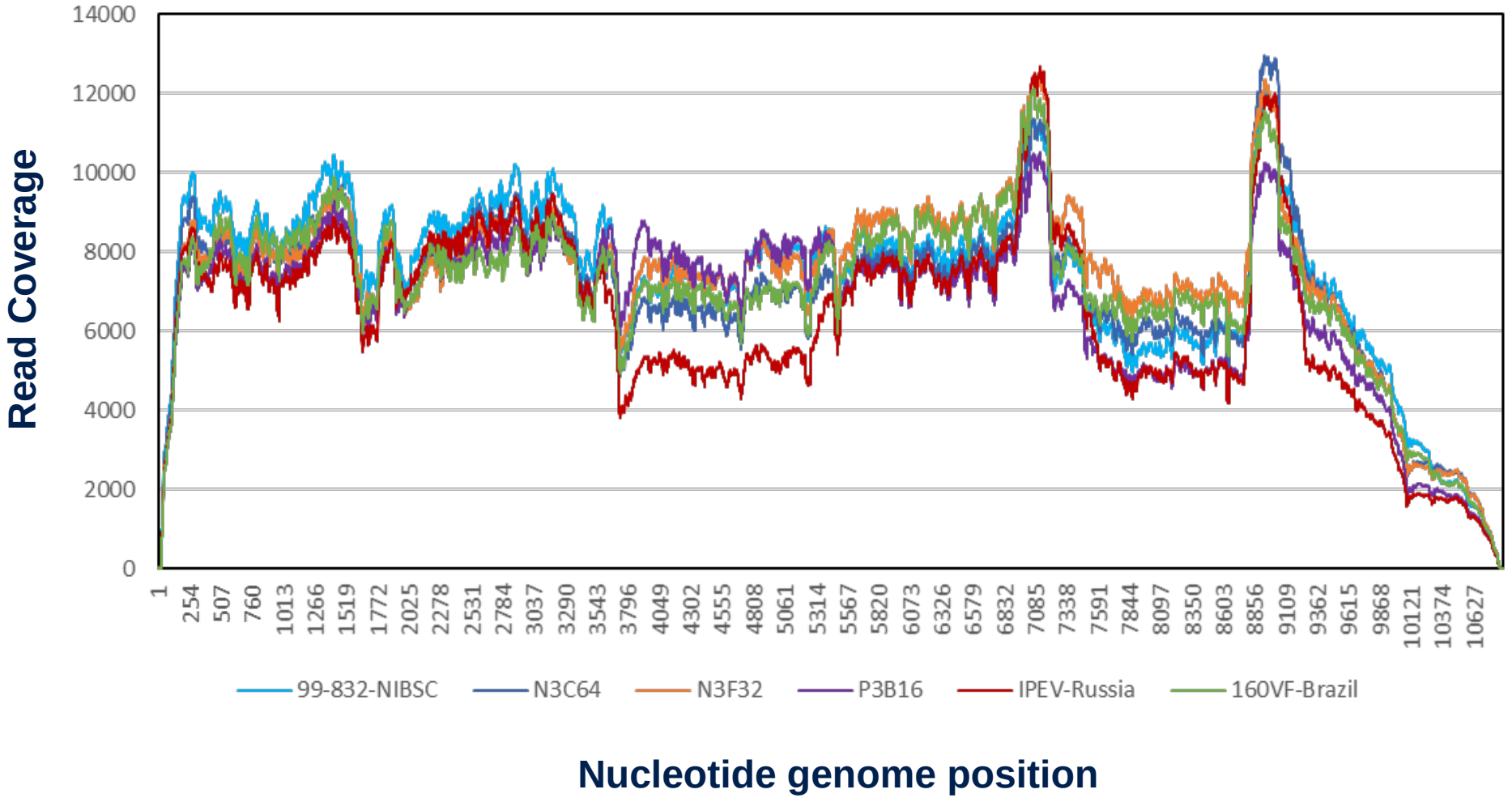
Comparison of the Live Attenuated Yellow Fever Vaccine 17D-204 Strain to Its Virulent Parental Strain Asibi by Deep Sequencing

Andrew Beck,[1,2] Robert B. Tesh,[1,2] Thomas G. Wood,[3] Steven G. Widen,[3] Kate D. Ryman,[4] and Alan D. T. Barrett[1,2]

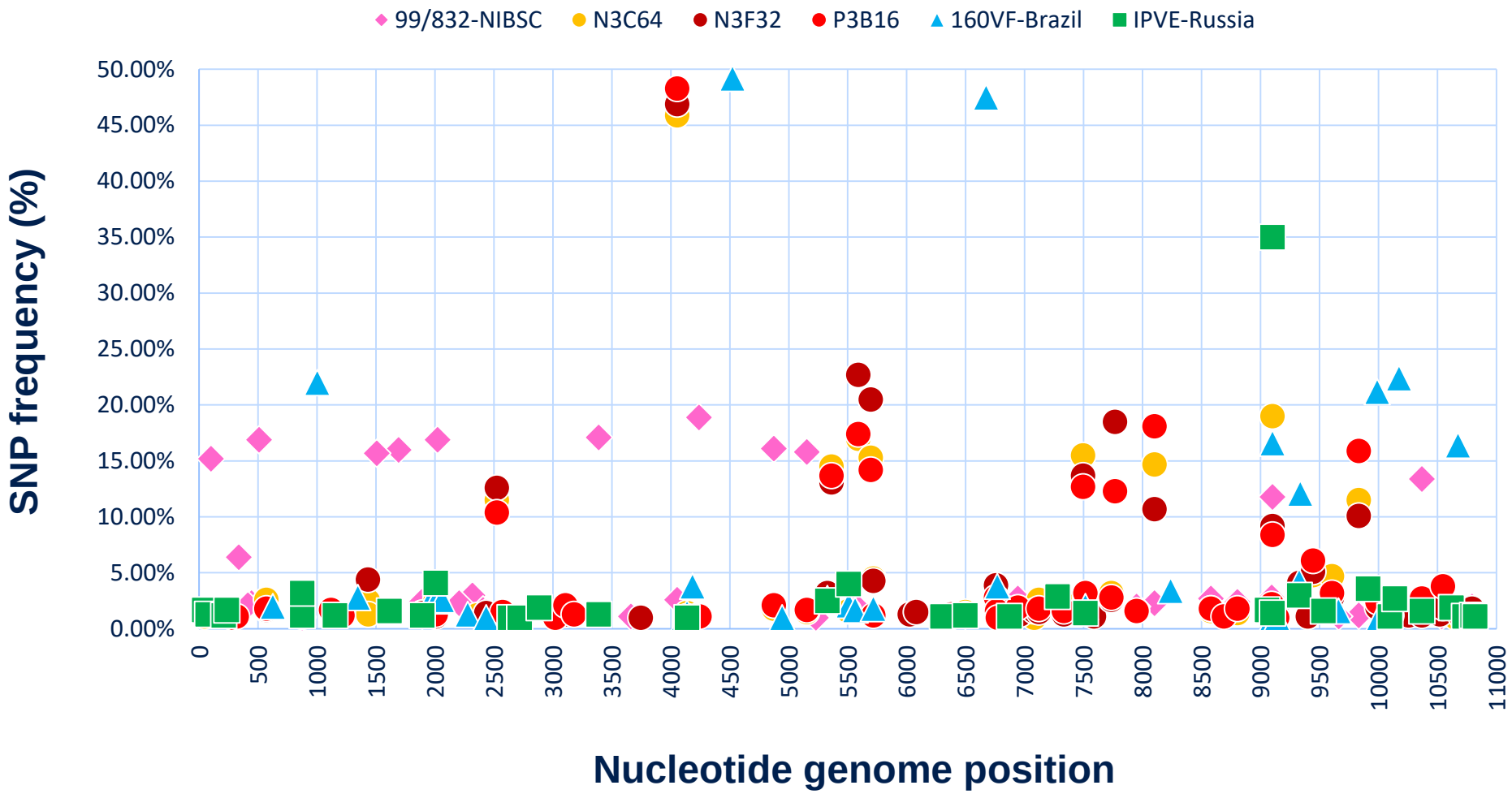
[1]Department of Pathology, [2]Sealy Center for Vaccine Development, and [3]Molecular Genomics Core Facility, University of Texas Medical Branch, Galveston; and [4]Center for Vaccine Research, University of Pittsburgh, Pennsylvania



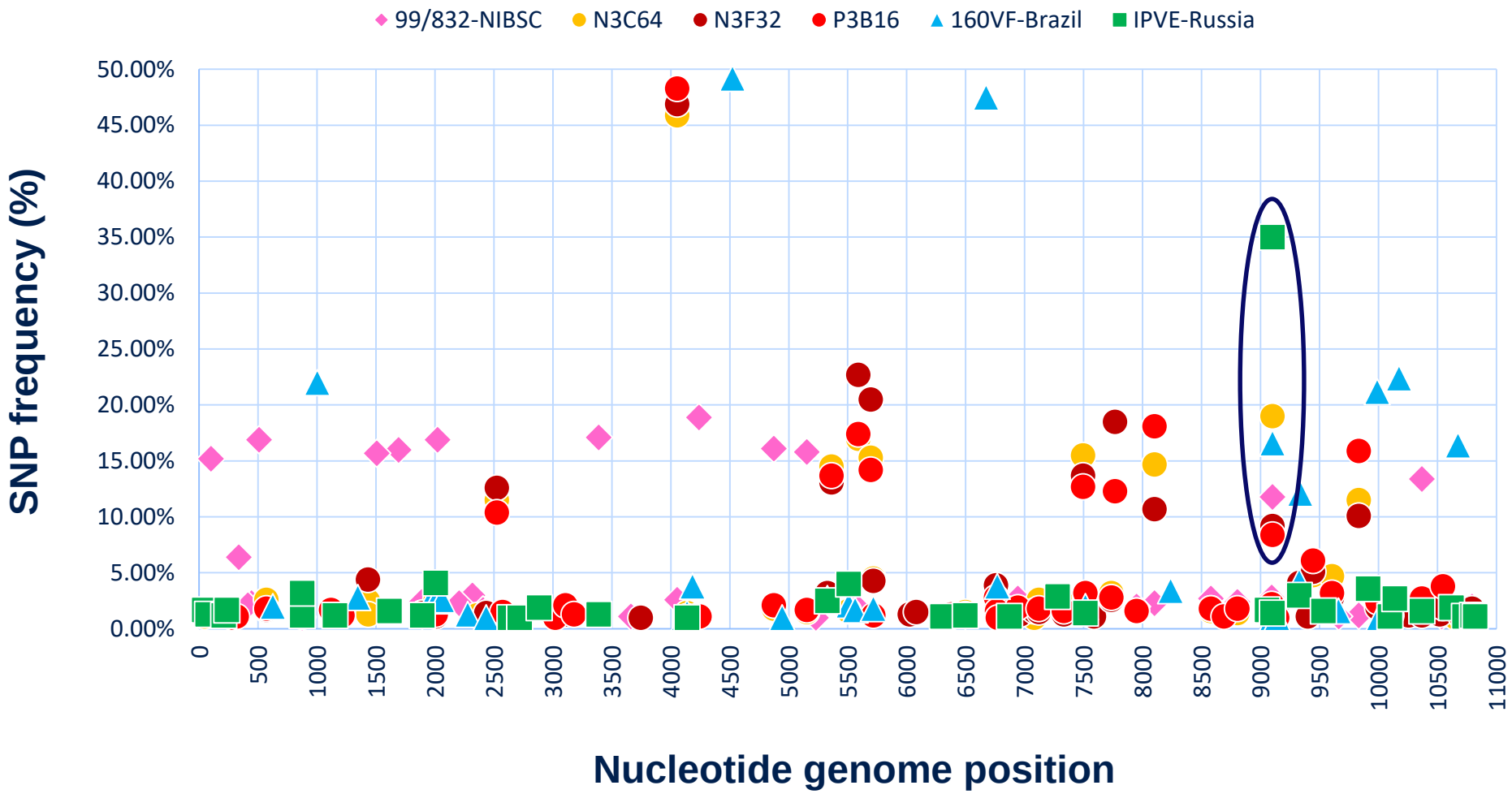
NGS Read Coverage - YFV



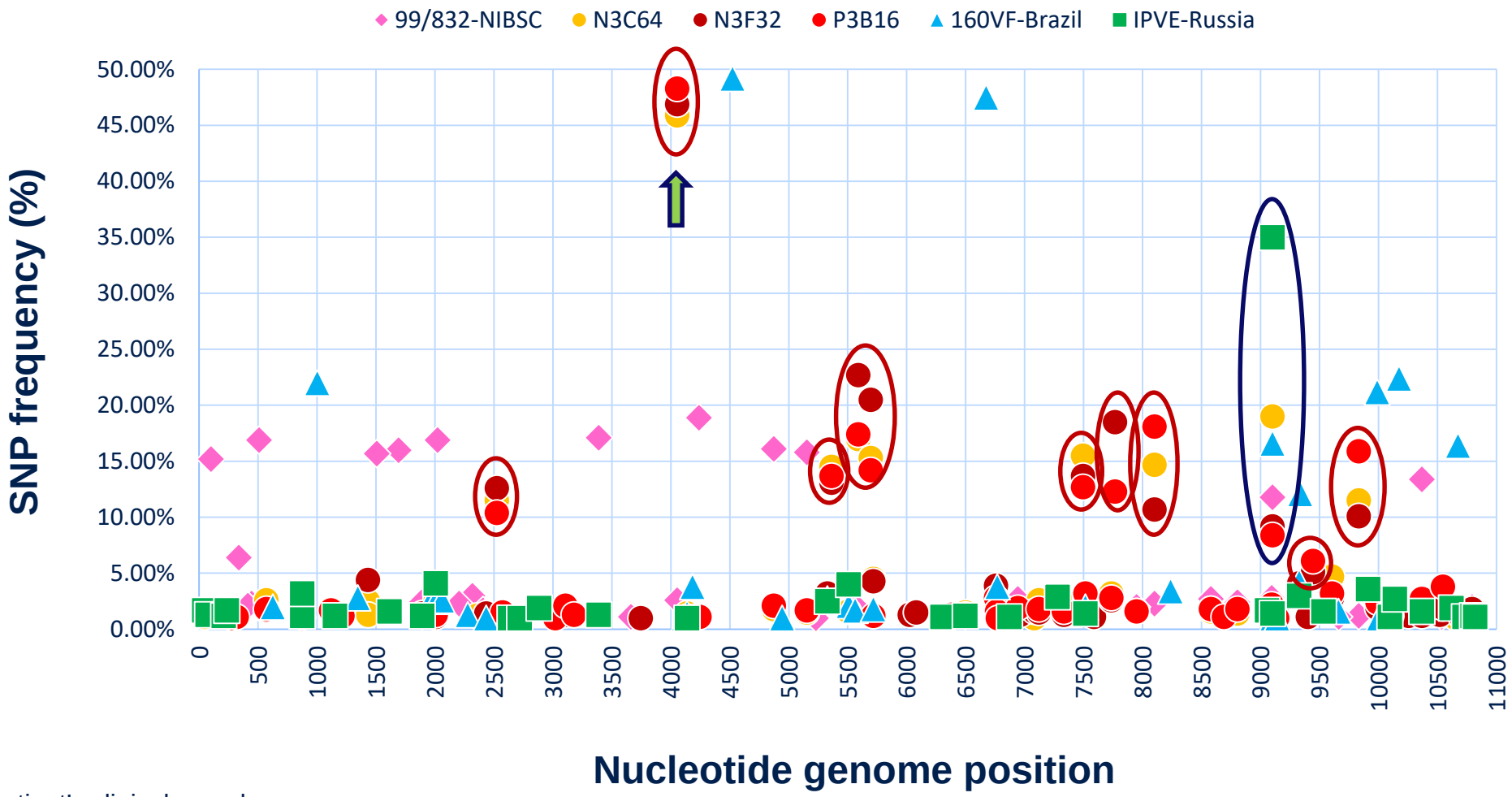
Single Nucleotide Polymorphism (SNP) analysis of YFV



Single Nucleotide Polymorphism (SNP) analysis of YFV



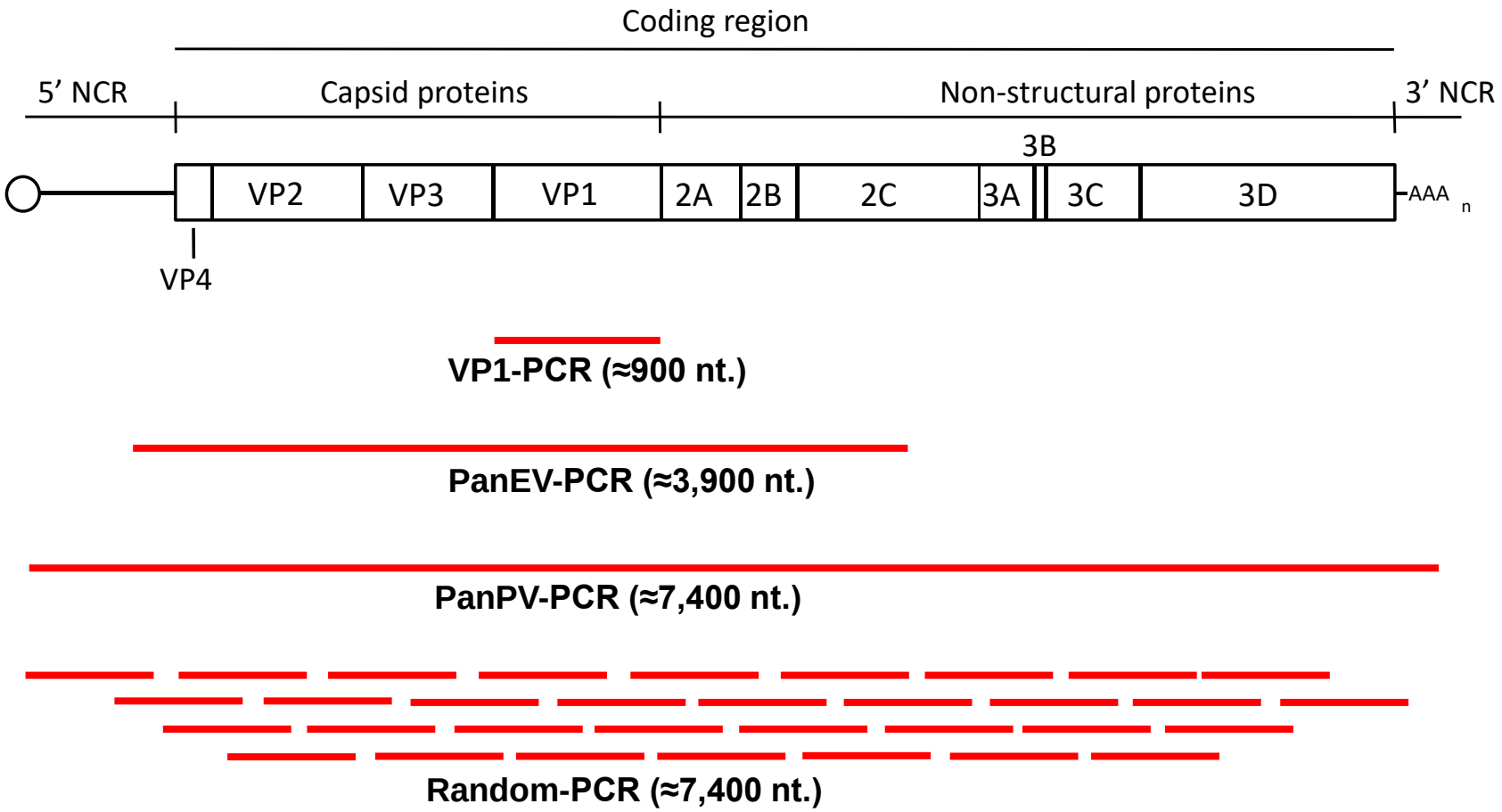
Single Nucleotide Polymorphism (SNP) analysis of YFV



Mutation in patient's clinical sample

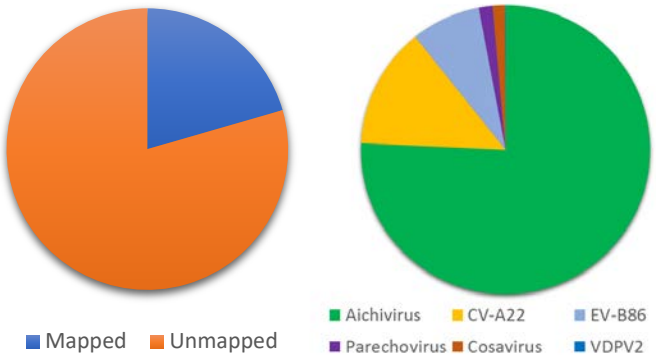
Detection by NGS of human enteroviruses in sewage and clinical samples

Generation of RT-PCR products for NGS analysis

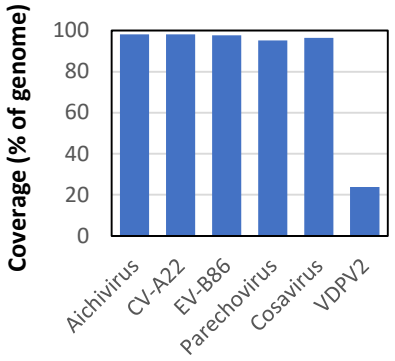


Direct detection and identification of PV by NGS – Stool

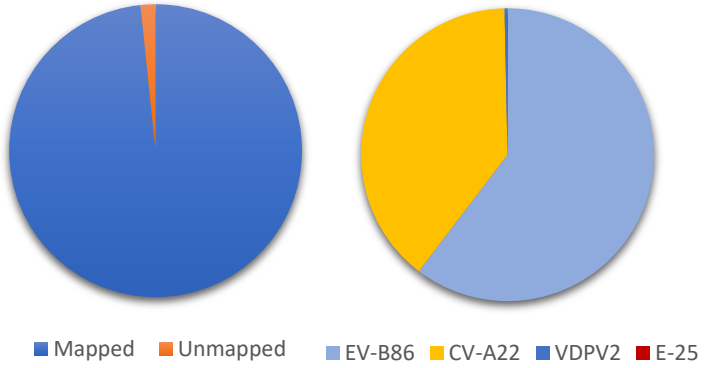
Random



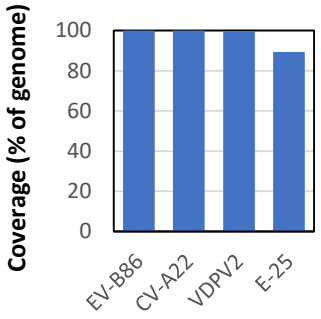
VDPV2-0.06%



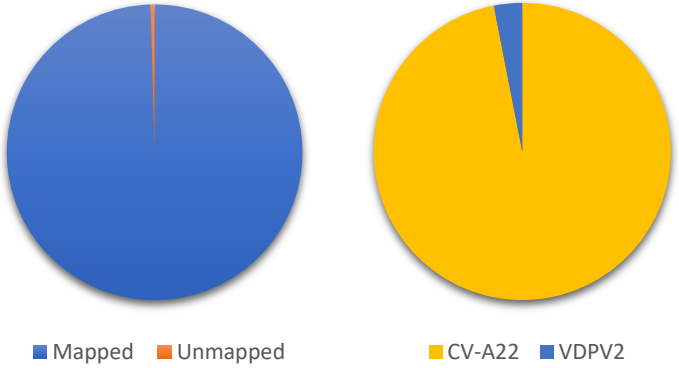
PanEV-ECRA



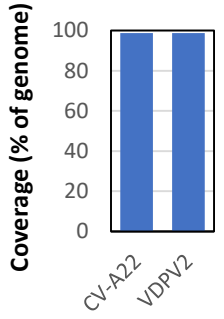
VDPV2-0.28%



PV-WG

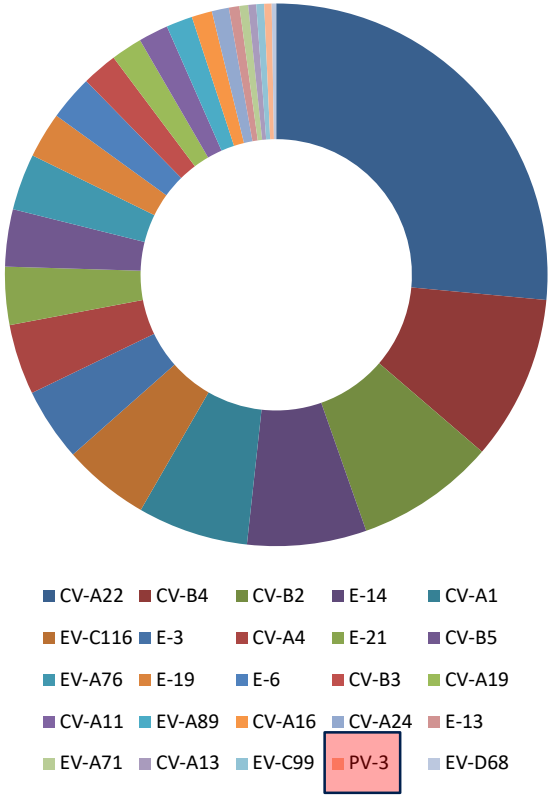


VDPV2-3.07%

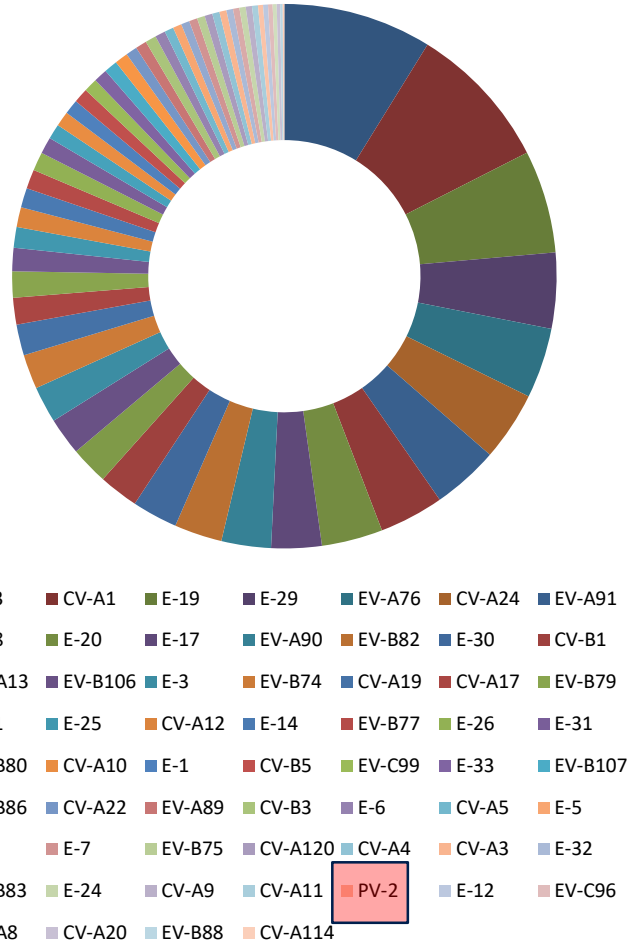


Direct detection of PV by NGS - sewage

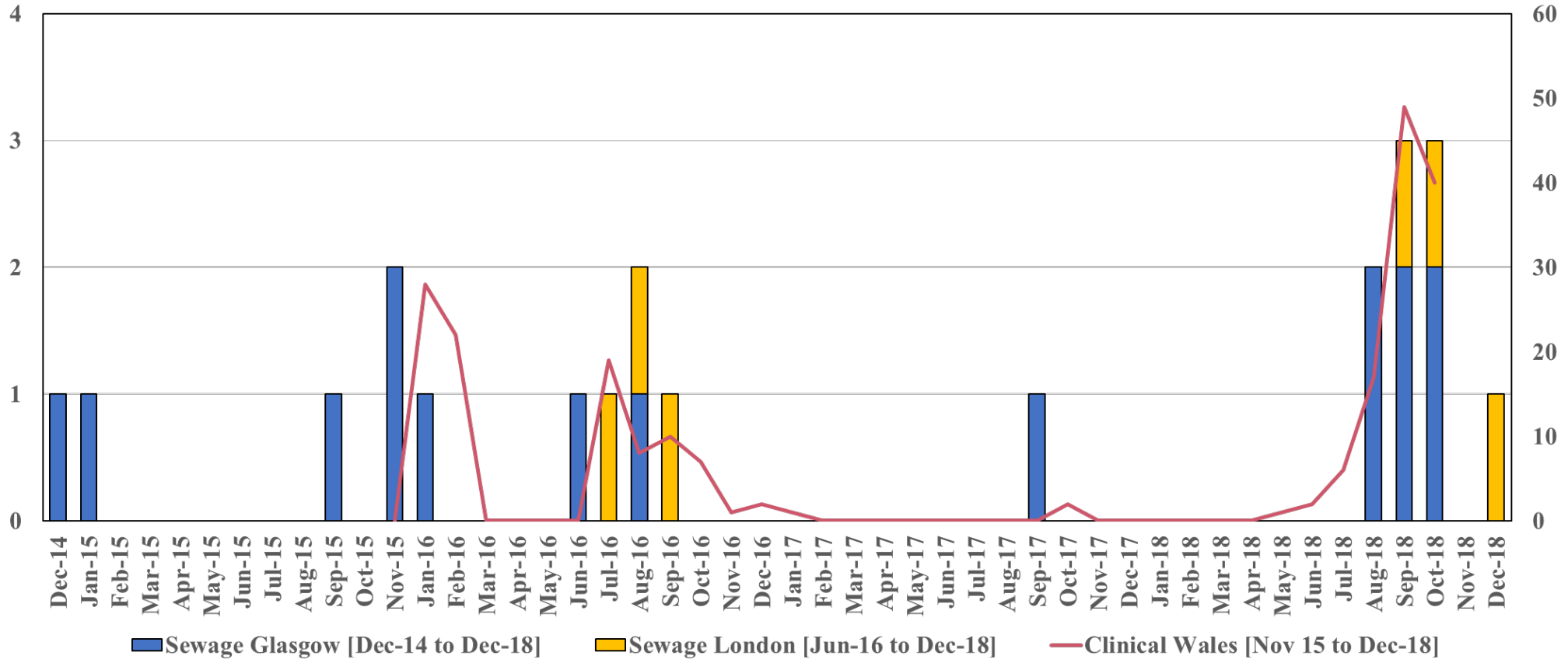
London (UK), September 2016



Karachi (Pakistan), January 2015



Circulation of EV-D68 in the UK between 2015-2018 vs clinical cases



Medicines & Healthcare products Regulatory Agency

NIBSC
Confidence in Biological Medicines

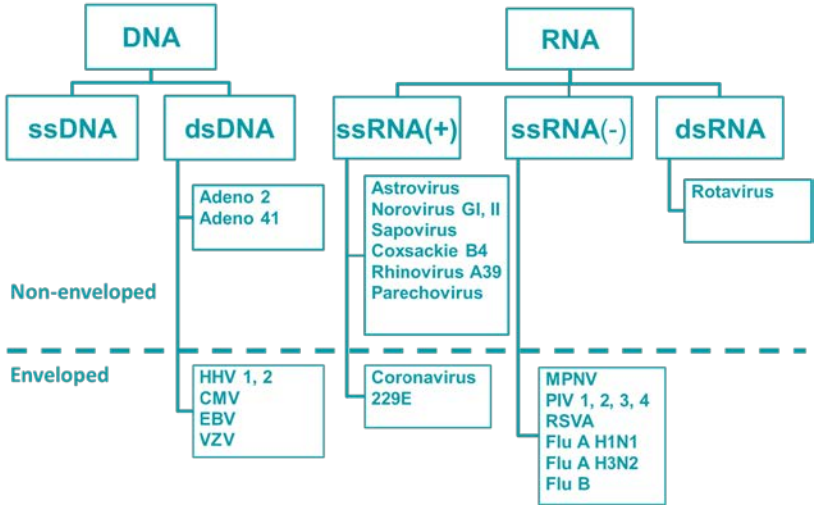
Reference Materials for Adventitious Virus Detection by Metagenomics

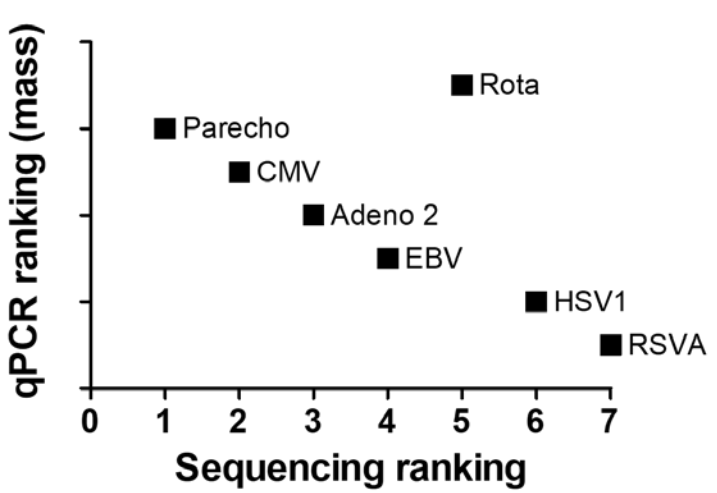
Edward.Mee@nibsc.org

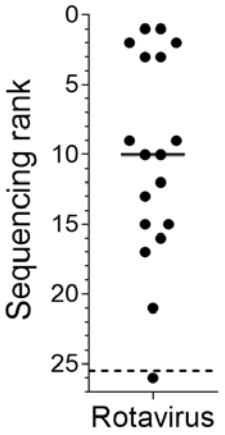
nibsc.org

1st generation reference material

- Candidate reference material containing 25 viruses representing diverse genome types and sizes
- 16 laboratories processed the reagent using various deep-sequencing virus detection assays
- Large variation between-labs in detection efficiency/sequencing rank
- 6 viruses were detected by all laboratories. Remaining viruses were detected by 4-14 participants
- Viruses detected least frequently corresponded to those not detected by qPCR
- 6 non-target viruses were reported by three or more participants (serum origin?)







Vaccine 34 (2016) 2035–2043

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

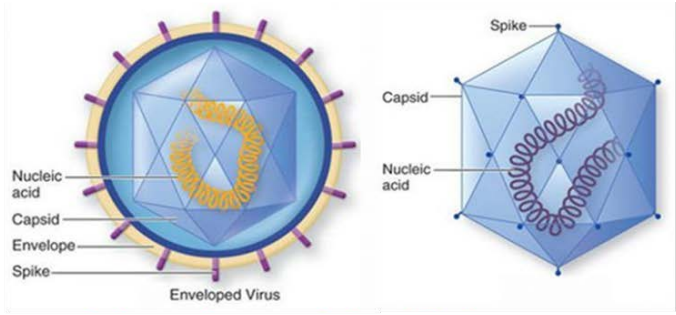
Development of a candidate reference material for adventitious virus detection in vaccine and biologicals manufacturing by deep sequencing

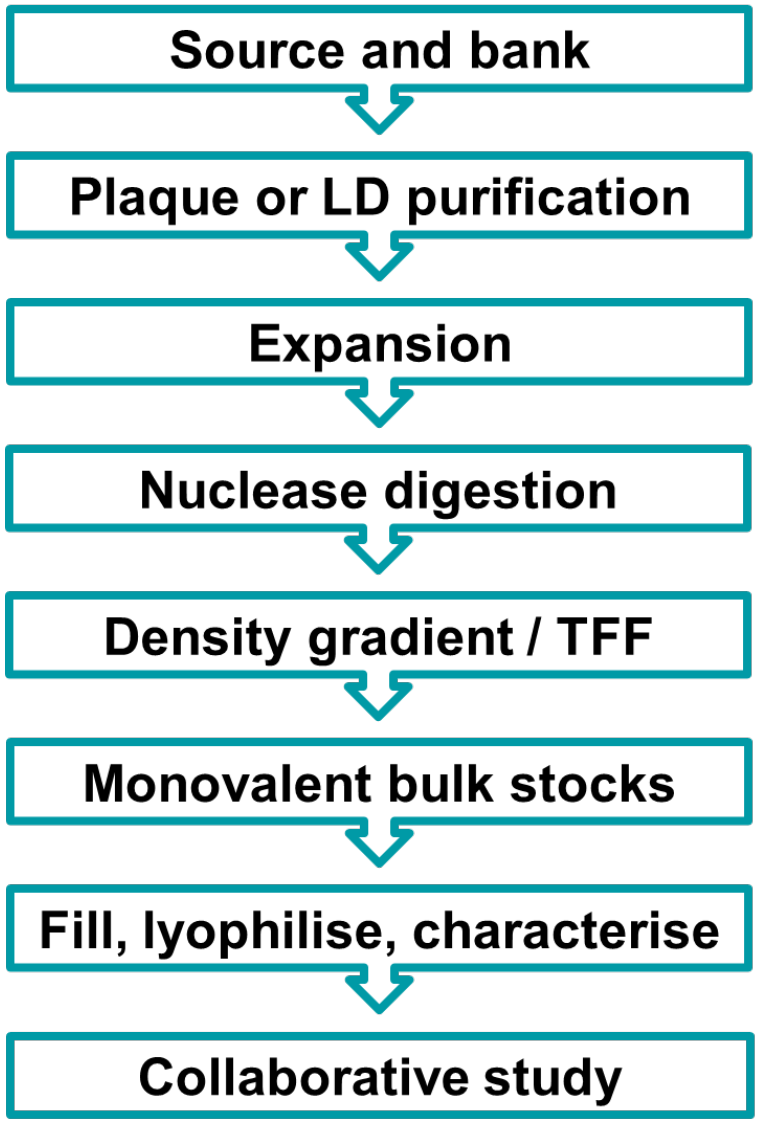
Edward T. Mee[a,*], Mark D. Preston[b], CS533 Study Participants[1], Philip D. Minor[a], Silke Schepelmann[a]

- Reagent available from NIBSC catalogue
  - www.nibsc.org/products ref: 11/242-001
  - £103 per vial (1ml)
  - ~ 400 sold ~800 vials remaining

2nd generation reference material

- Fewer viruses - representing major virus structures
- High titre, defined background
- Lyophilised
- Practical considerations
  - BSL2
  - Grow to high titre in cells / eggs
  - Distinct from vaccine viruses where possible
- Intended uses:
  - Development / comparison of AV methods
  - Define LOD by spiking into relevant matrix





MTA with donor
Characterise and bank virus

Minimise diversity of target
Exclude non-target viruses

Reduce host and non-encapsidated NA

Enrich complete viral particles
Further reduce background NA

Stabilise, ensure consistency
Facilitate shipping

Determine fitness for purpose
Snapshot of current methods

Acknowledgements

- Kostya Chumakov (FDA)
- Majid Laassri (FDA)
- Bethany Charlton
- Thomas Wilton
- Laura Crawt
- Edward Mee
- Dimitra Klapsa
- Manasi Majumdar
- Begona Valdazo-Gonzalez
- Gill Cooper
- Mark Preston
- Martin Fritzsche
- Jason Hockley
- Peter Rigsby
- Nadine Holmes
- Collaborative study participants

NIBSC
Elaine Pegg
Mark Preston
Stacey Efstathiou
Silke Schepelmann
Philip Minor

Material donors
Michael Nicoll
Rob Anderson
Cristina Margaretto
Sheila Govind

Margaret Hosie – U. Glasgow
Falko Steinbach – APHA, UK
Sarah McDonald – Wake Forest U
Colin Parrish – Cornell

PDA/FDA AVDTIG members

edward.mee@nibsc.org

Test validity and pass-fail criteria

Validity criteria

- Include the following vaccines and controls in your test:
  - cDNA-derived vaccine with 0% 472C.
  - Low MAPREC reference or validated reference with similar 472C content.
  - High MAPREC reference or validated reference with similar 472C content in duplicate.
  - Test vaccines.
  - Negative RT-PCR control if applicable.
- Perform 5 independent NGS determinations of 472C content for each reference and test sample.
- Standard deviation 5 independent NGS determinations to be <0.3
- Ratio of NGS values for 472C between duplicate determinations of High MAPREC reference between 0.85 and 1.15.
- Ratio of NGS values for 472C between Low MAPREC reference and High MAPREC reference <1.0.

Pass/fail decision:

1. If ratio of NGS values for 472C between test vaccine and NGS reference* is <1.0, vaccine passes.
2. If ratio of NGS values for 472C between test vaccine and NGS reference is ≥1.0, vaccine fails.
3. If vaccine fails, the test can be repeated once and combined results used for pass/fail decision. If ratio of NGS values for 472C between test vaccine (average value between the two tests) and NGS reference is <1.0, vaccine passes. If it is ≥1.0, vaccine fails.

* Based on similar 472C content with WHO IS for MAPREC, both Sample 3H (vaccine 96/650) and 3B/I (96/578, High MAPREC reference are suitable NGS references for this purpose.