



# Tailored molecular tools for Asia

Update from WRLFMD

*Andrew Shaw*

**Acknowledgements:** Don King, Britta Wood, Valerie Mioulet, Nick Knowles, Anna Ludi, Ginette Wilsden, Jozhel Baguisi, Harry Bull, Andrew Shaw, Antonello Di Nardo, Jemma Wadsworth, Hayley Hicks



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# Tailored molecular tools for Asia

## Lineage-specific PCRs



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# Pool 1: Status in 2023

## Characterisation of different FMD virus lineages



Based on data from WRLFMD, RRLSEA, the OIE/FAO Lab Network and reported @SEACFMD

Country	O					A		Asia-1
	ME-SA/Ind-2001e	SEA / Mya-98	CATHAY	ME-SA / PanAsia	ME-SA/ PanAsia-2	ASIA / Sea-97	ASIA/Ind	
Cambodia	2019	2016		2019		2016		
Laos	2020	2017		2018		2018		
Malaysia	2022	2016	2005	2020	2009	2022*		
Myanmar	2021	2021				2021	2010	2017
Thailand	2021	2018	2012	2019		2022*		
Vietnam	2022	2019	2018	2018		2017		2006
PR China	2021	2020	2022	2019		2019		2009
Indonesia	2022							
Mongolia	2022	2018		2017		2016		

Is this the true picture of FMD in SEACFMD countries?

\* Not sequenced @ WRLFMD

..... or does under-sampling bias our understanding of the epidemiology?

[www.pirbright.ac.uk](http://www.pirbright.ac.uk)

# Lineage-specific real-time RT-PCRs for Asia

- Assays cover 8 FMDV lineages in South and Southeast Asia

Serotype	Lineage	Oligo name	Sequence (5'-3')	Reference
O	ME-SA/PanAsia & PanAsia2	FWD O-PA-FP1(sense)	TGGACCTGATGCARACCCC	Unpublished
		REV O-PA-RP1(antisense)	TCGTGTTTCACTGCCACYTC	
		Probe O-PA-P2 (sense)	FAM-CTCCGCACACCTAC-TAMRA	
	ME-SA/Ind-2001	FWD IND-2001d FPv3 (sense)	GAAGAGGGCCGARACATAC	Unpublished
		REV IND-2001d RPv2(antisense)	GCCACAATCTTYTGYTTGTG	
		Probe IND-2001d Pv2 (sense)	FAM-CTGCTSGCCATTACCCG-TAMRA	
	SEA/Mya-98	FWD O-Mya98-FP2 (sense)	CTGGGTGCCAAATGGAGCA	Unpublished
		REV O-Mya98-RP3(antisense)	CACGGTGTGGTGMCGTGT	
		Probe O-Mya98-P6 (sense)	FAM-ACCACCAACCAACGGCRTAC-TAMRA	
CATHAY	FWD O-CathFP3(sense)	GATGCARATCCCTGCYCAC	Unpublished	
	REV O-CathRP3(antisense)	ACCCARGTGAGRTC GCC		
	Probe O-CathP2 (sense)	FAM-CTGCGGACGGCCACTACTT-TAMRA		
A	ASIA/Iran-05	FWD A-JB-F (sense)	GCCACGACCATCCACGAGCT	Jamal & Belsham, 2015
		REV A-JB-R (antisense)	GTCCTGYGACRACACTCCAC	
		Probe A-JB-F-P (sense)	FAM-CTCGTGCGYATGAAACGTGCGYAGCT-TAMRA	
	ASIA/G-VII	FWD G-VII_FP (sense)	TGCTCAACTCCCTGCCTC	Saduakassova et al., 2018
		REV G-VII_RP (antisense)	GAGTTCGGCACGCTTCAT	
	ASIA/Sea-97	Probe G-VII_P (sense)	FAM-CCACYACCATCCACGAGCTG-BHQ1	Unpublished
FWD A-Sea-97_FP2(sense)		GGACAGGTTYGTGCARATC		
REV A-Sea-97_RP2(antisense)		CACCACAATCTCAAGATCAGA		
Asia1	-	Probe A-Sea-97_P2(sense)	FAM-GCGCGCGGCYACCTACTACTTT-TAMRA	Reid et al., 2014
		FWD Asia1forward3 (sense)	GCAGTWAAGGCYAGASCATYAC	
		REV Asia1reverse2 (antisense)	GCARAGGCCTAGGGCAGTATG	
		Probe Asia1probe4 (sense)	FAM-AGCTGTTGATCCGCATGAAACGYGCG-TAMRA	

- “molecular toolbox” combines previously published assays and unpublished content
- Primer and probe sets previously circulated (see table)
- Now in Press (Frontiers in Vet Sci.)*

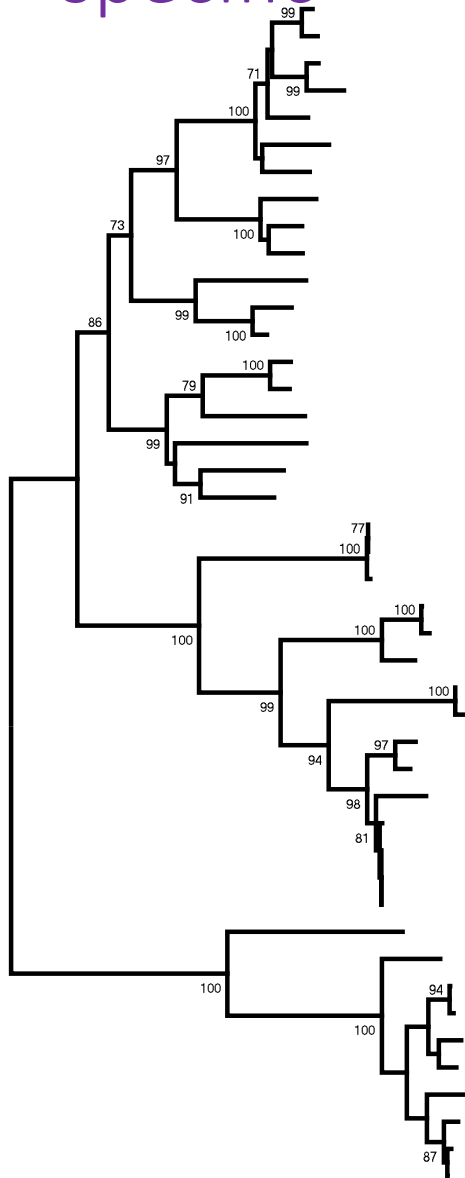
# Homologous and cross-reactive results

- Assays evaluated using FMDV isolates (n=85)
  - Samples representing the different lineages
  - 3D rRT-PCR used to benchmark the results

		Avg Ct per rRT-PCR assay (# samples)								
		O/PanAsia & PanAsia2	O/Ind-2001	O/Mya-98	O/CATHAY	A/G-VII	A/Iran-05	A/Sea-97	Asia1	3D
Sample lineage	O/PanAsia & PanAsia2	<b>18.16</b> (8)	29.23 (3)	<b>26.90*</b> (1)						18.80 (9)
	O/Ind-2001	18.30 (4)	<b>20.69</b> (10)							22.11 (10)
	O/Mya-98	19.30 (1)	30.78 (1)	<b>25.38</b> (15)						23.30 (15)
	O/CATHAY				<b>20.13</b> (10)					23.09 (10)
	A/G-VII					<b>20.04</b> (10)	34.71 (4)			21.03 (10)
	A/Iran-05						<b>20.94</b> (8)			21.18 (8)
	A/Sea-97					<b>27.48*</b> (1)		<b>16.17</b> (12)		17.77 (13)
	Asia1								<b>19.07</b> (10)	18.36 (10)

- Assays correctly detected 83/85 of the isolates
- Critical point: heterologous signal, whilst generally weaker, is evident for 1.9% of the tests (13/680 assays); assays must be compared with 3D.

# O - specific



- O/MOG/19/2018
- O/MOG/15/2017
- O/MAY/2/2018
- O/SKR/5/2019
- O/SRL/5/2017
- O/BHU/22/2017
- O/SAU/9/2018
- O/SRL/2/2018
- O/SRL/4/2016
- O/SRL/11/2014 (MF947482)
- O/VIT/17/2018
- O/VIT/22/2012
- O/MOG/12/2017
- O/IRN/11/2010 (MT443278)
- O/IRN/2/2010 (MT443197)
- O/AFG/34/2017 (MT443044)
- O/IRN/6/2015 (MN276044)
- O/TUR/27/2014 (MT443880)
- O/IRN/7/2015 (MT443385)
- O/MAY/10/2016
- O/MAY/9/2016
- O/MAY/12/2016
- O/MOG/7/2018
- O/MOG/25/2018
- O/MYA/1/2015
- O/VIT/47/2018
- O/VIT/31/2019
- O/HKN/6/2010 (JQ070302)
- O/HKN/20/2010 (HM229661)
- O/HKN/1/2011 (JQ070306)
- O/HKN/10/2010
- O/HKN/13/2010
- O/HKN/14/2010
- O/HKN/7/2010 (JQ070303)
- O/HKN/24/2010 (KM243162)
- O/HKN/5/2011 (KM243167)
- O/HKN/10/2015
- O/HKN/8/2015
- O/HKN/8/2014
- O/HKN/1/2015
- O/HKN/6/2018
- O/HKN/2/2016
- O/HKN/3/2016
- O/HKN/4/2016

O/PanAsia & PanAsia-2	O/Ind-2001	O/Mya-98	O/CATHAY	A/G-VII	A/Iran-05	A/Sea-97	Asia1	3D
UD	26.65	UD	UD	UD	UD	UD	UD	29.29
17.87	15.26	UD	UD	UD	UD	UD	UD	17.18
23.31	22.12	UD	UD	UD	UD	UD	UD	20.7
14.75	12.66	UD	UD	UD	UD	UD	UD	15.1
UD	23.27	UD	UD	UD	UD	UD	UD	22.33
UD	21.86	UD	UD	UD	UD	UD	UD	21.46
17.26	18.04	UD	UD	UD	UD	UD	UD	13.73
UD	18.68	UD	UD	UD	UD	UD	UD	21.43
UD	21.41	UD	UD	UD	UD	UD	UD	26.48
UD	26.97	UD	UD	UD	UD	UD	UD	33.73
UD	UD	26.9	UD	UD	UD	UD	UD	22.19
17.13	UD	UD	UD	UD	UD	UD	UD	17.51
19.49	UD	UD	UD	UD	UD	UD	UD	15.42
18.18	UD	UD	UD	UD	UD	UD	UD	20.39
13.58	24.74	UD	UD	UD	UD	UD	UD	13.73
18.99	28.46	UD	UD	UD	UD	UD	UD	20.49
17.08	UD	UD	UD	UD	UD	UD	UD	19.52
23.14	UD	UD	UD	UD	UD	UD	UD	19.83
17.67	34.5	UD	UD	UD	UD	UD	UD	20.07
UD	UD	26.63	UD	UD	UD	UD	UD	25.55
UD	UD	26.53	UD	UD	UD	UD	UD	26.04
UD	UD	24.01	UD	UD	UD	UD	UD	22.78
UD	UD	24.05	UD	UD	UD	UD	UD	26.97
UD	30.78	28.28	UD	UD	UD	UD	UD	24.42
19.3	UD	17.45	UD	UD	UD	UD	UD	15.24
UD	UD	25.14	UD	UD	UD	UD	UD	14.87
UD	UD	34.02	UD	UD	UD	UD	UD	21.94
UD	UD	28.53	UD	UD	UD	UD	UD	23.64
UD	UD	24.23	UD	UD	UD	UD	UD	22.91
UD	UD	27.03	UD	UD	UD	UD	UD	27.75
UD	UD	25.64	UD	UD	UD	UD	UD	25.22
UD	UD	25.89	UD	UD	UD	UD	UD	23.51
UD	UD	29.19	UD	UD	UD	UD	UD	29.01
UD	UD	20.81	UD	UD	UD	UD	UD	19.68
UD	UD	UD	21.88	UD	UD	UD	UD	20.95
UD	UD	UD	20.21	UD	UD	UD	UD	23.33
UD	UD	UD	17.35	UD	UD	UD	UD	19.73
UD	UD	UD	18.43	UD	UD	UD	UD	22.82
UD	UD	UD	22.71	UD	UD	UD	UD	24.11
UD	UD	UD	17.6	UD	UD	UD	UD	22.34
UD	UD	UD	23.78	UD	UD	UD	UD	28.26
UD	UD	UD	22.41	UD	UD	UD	UD	22.28
UD	UD	UD	17.64	UD	UD	UD	UD	20.43
UD	UD	UD	19.33	UD	UD	UD	UD	26.6

O/Ind-2001 & PanAsia-2

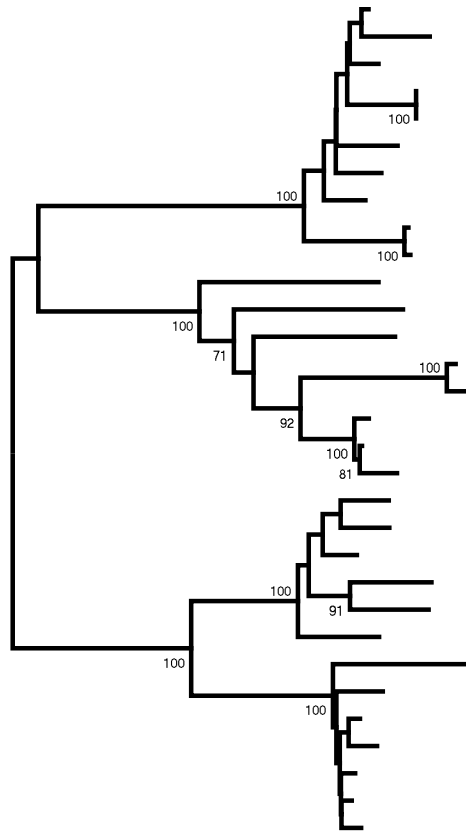
O/PanAsia

O/Mya-98

O/CATHAY

0.02

# A - specific



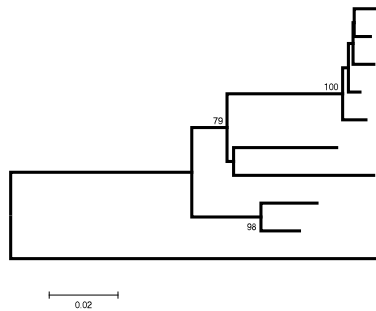
A/IRN/20/2016 (KY982296)  
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 A/SAU/20/2016 (KY982313)  
 A/IRN/25/2018  
 A/IRN/39/2016  
 A/TUR/12/2017  
 A/BHU/27/2017  
 A/BHU/28/2017  
 A/IRN/7/2017 (MT442308)  
 A/IRN/6/2016 (MT442304)  
 A/AFG/74/2011 (MT442114)  
 A/AFG/50/2017 (MT442138)  
 A/IRN/2/2018 (MT442312)  
 A/IRN/3/2018 (MT442313)  
 A/IRN/2/2017 (MT442306)  
 A/IRN/10/2018 (MT442317)  
 A/VIT/25/2017  
 A/LAO/2/2014  
 A/MAY/15/2014  
 A/TAI/9/2019  
 A/TAI/11/2019  
 A/VIT/26/2016  
 A/SKR/4/2018  
 A/MOG/2/2016  
 A/CAM/5/2016  
 A/VIT/22/2016  
 A/MOG/13/2013  
 A/LAO/1/2015  
 A/MOG/4/2013

0.02

O/PanAsia & PanAsia-2	O/Ind-2001	O/Mya-98	O/CATHAY	A/G-VII	A/Iran-05	A/Sea-97	Asia1	3D
UD	UD	UD	UD	21.86	UD	UD	UD	21.72
UD	UD	UD	UD	15.22	30.36	UD	UD	13.95
UD	UD	UD	UD	12.99	25.95	UD	UD	15.29
UD	UD	UD	UD	22.05	41.78	UD	UD	21.88
UD	UD	UD	UD	20.3	36.63	UD	UD	20.53
UD	UD	UD	UD	16.01	UD	UD	UD	17.28
UD	UD	UD	UD	22.05	UD	UD	UD	23.97
UD	UD	UD	UD	30.45	UD	UD	UD	20.28
UD	UD	UD	UD	19.59	UD	UD	UD	28.59
UD	UD	UD	UD	19.85	UD	UD	UD	26.85
UD	UD	UD	UD	UD	16.08	UD	UD	14.49
UD	UD	UD	UD	UD	22.1	UD	UD	22.74
UD	UD	UD	UD	UD	22.53	UD	UD	29.58
UD	UD	UD	UD	UD	26.4	UD	UD	25.77
UD	UD	UD	UD	UD	17.68	UD	UD	15.52
UD	UD	UD	UD	UD	20.97	UD	UD	22.24
UD	UD	UD	UD	UD	20.27	UD	UD	18.75
UD	UD	UD	UD	UD	21.46	UD	UD	20.34
UD	UD	UD	UD	UD	UD	14.62	UD	14.83
UD	UD	UD	UD	UD	UD	15.05	UD	21.52
UD	UD	UD	UD	UD	UD	15.14	UD	21.4
UD	UD	UD	UD	UD	UD	21.09	UD	16.13
UD	UD	UD	UD	UD	UD	14.32	UD	15.04
UD	UD	UD	UD	UD	UD	14.22	UD	13.59
UD	UD	UD	UD	UD	UD	15.28	UD	17.96
UD	UD	UD	UD	UD	UD	19.95	UD	18.35
UD	UD	UD	UD	UD	UD	20.44	UD	21.8
UD	UD	UD	UD	UD	UD	13.92	UD	14.28
UD	UD	UD	UD	27.48	UD	UD	UD	16.46
UD	UD	UD	UD	UD	UD	13.73	UD	14.1
UD	UD	UD	UD	UD	UD	16.34	UD	25.6

A/G-VII  
 A/Iran-05  
 A/SEA-97

# Asia 1 - specific

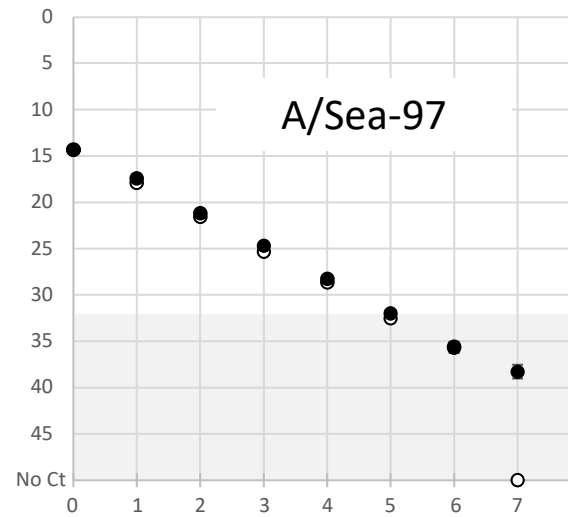
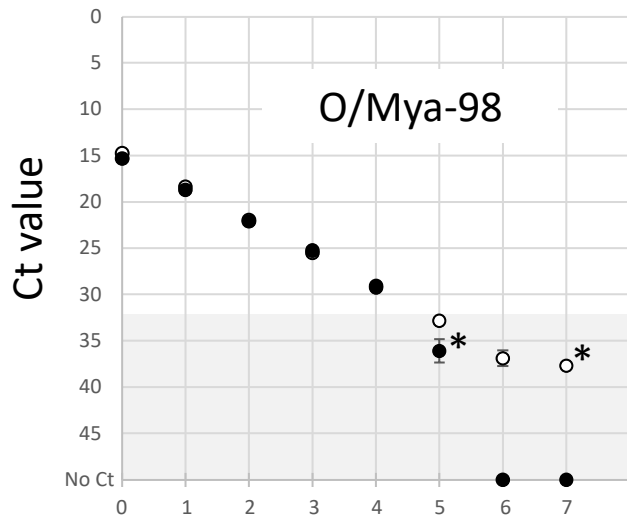
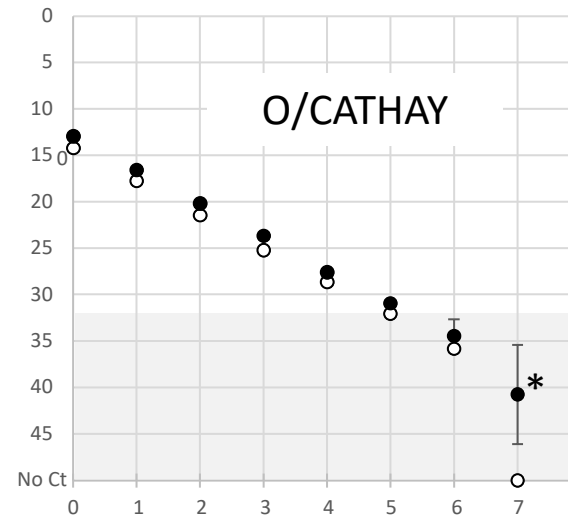
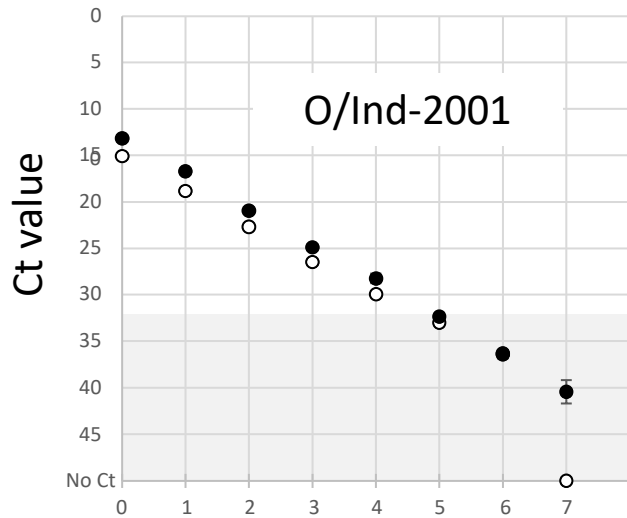


Asia1/PAK/43/2015 (MT442764)  
 Asia1/IRN/26/2016 (MT442662)  
 Asia1/TUR/18/2015 (MT442813)  
 Asia1/IRN/1/2015 (MT442659)  
 Asia1/IRN/20/2015 (MT442661)  
 Asia1/IRN/19/2018 (MT442666)  
 Asia1/PAK/10/2019  
 Asia1/IRN/1/2020  
 Asia1/PAK/69/2019  
 Asia1/NEP/38/2017

O/PanAsia & PanAsia-2	O/Ind-2001	O/Mya-98	O/CATHAY	A/G-VII	A/Iran-05	A/Sea-97	Asia1	3D
UD	UD	UD	UD	UD	UD	UD	23.22	24.11
UD	UD	UD	UD	UD	UD	UD	15.26	14.96
UD	UD	UD	UD	UD	UD	UD	16.37	14.94
UD	UD	UD	UD	UD	UD	UD	17.91	18.62
UD	UD	UD	UD	UD	UD	UD	20.13	17.92
UD	UD	UD	UD	UD	UD	UD	16.36	16.64
UD	UD	UD	UD	UD	UD	UD	23.11	22.61
UD	UD	UD	UD	UD	UD	UD	18.08	15.73
UD	UD	UD	UD	UD	UD	UD	17.75	17.33
UD	UD	UD	UD	UD	UD	UD	22.47	20.8



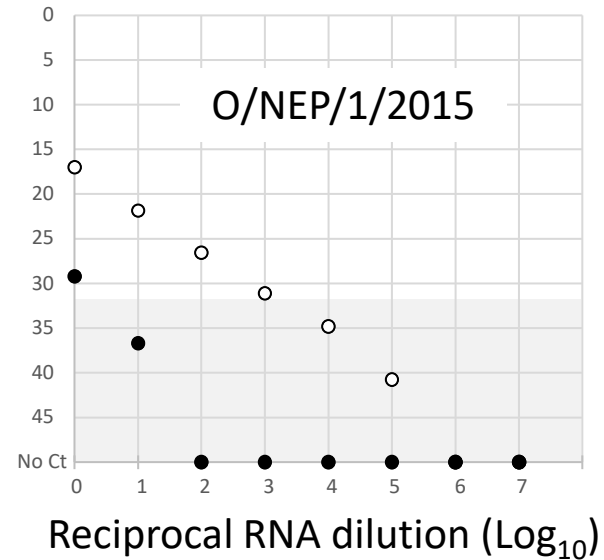
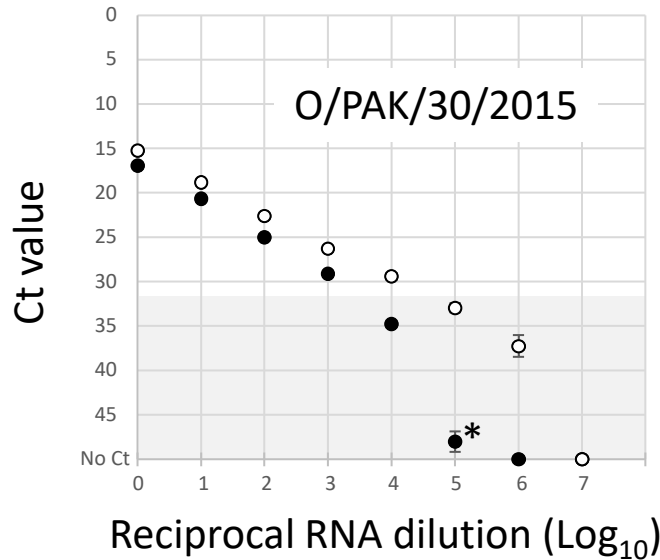
# Equivalent sensitivity to Callahan 3D



Reciprocal RNA dilution (Log<sub>10</sub>)

Reciprocal RNA dilution (Log<sub>10</sub>)

# The challenge of FMDV sequence diversity



	Forward: O-PA-FP1	Probe: O-PA-P2	Reverse (antisense): O-PA-RP1
	TGGACCTGATGCARACCCC	CTCCGCACYGCCACCTAC	GARGTGGCAGTGAAACACGA
PAK/30/2015	TGGACCTGATGCAAACCCC	CTCCGCACCGCCACCTAC	GAGGTAAGCAGTGAAACACGA
NEP/1/2015	TGGACCTGATGCAAGCCCC	CTCCGCACAGGCCACCTAC	GAGGTGGCTGTGAAACACGA

# SAT2/XIV: laboratory tools



- New lineage-specific rRT-PCR for SAT/XIV developed by KVI, Israel and WRLFMD
  - Initial results indicate that a ver2 primer/probe set detect SAT2/XIV viruses but not serotype O, A, Asia 1 or SAT2/VII viruses
  - Further information: <https://www.foot-and-mouth.org/science/lineage-specific-pcr>
- Encouraging data from FMD (ŞAP) Institute, Türkiye and WRLFMD indicate that the SAT 2 specific of the antigen-ELISA produced by IZSLER can detect this viral lineage
- SP-ELISAs can be used to detect responses, but be aware that cross-reactivity with antibodies from infection/vaccination with other FMDV serotypes may complicate sero-diagnosis

# The way forward?

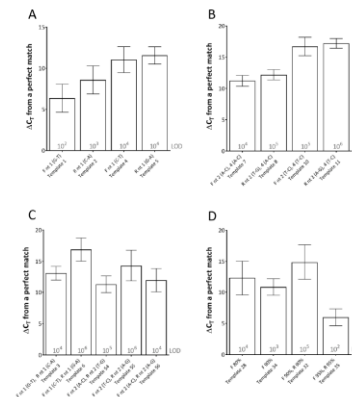
- Lineage-specific assays target capsid encoding sequences
- The performance of these lineage-specific assays is constrained by sequence diversity of the FMDV genome.
  - This is true, even when mismatches are accounted for by degenerate bases in the oligonucleotide sequences
  - Intra-lineage sensitivity
  - Inter-lineage specificity
- Based on feedback, full genome sequences have been shared with WOAHA
- Assays may only have a relatively short life-span before field isolates evolve!
- In silico tool to predict assays performance?



Article

## GoPrime: Development of an In Silico Framework to Predict the Performance of Real-Time PCR Primers and Probes Using Foot-and-Mouth Disease Virus as a Model

Emma L A Howson <sup>1,2</sup>, Richard J Orton <sup>2,3</sup>, Valerie Mioulet <sup>1</sup>, Tiziana Lembo <sup>2</sup>, Donald P King <sup>1,\*</sup> and Veronica L Fowler <sup>1</sup>





# Tailored molecular tools for Asia

improved methods for sequencing  
FMDV



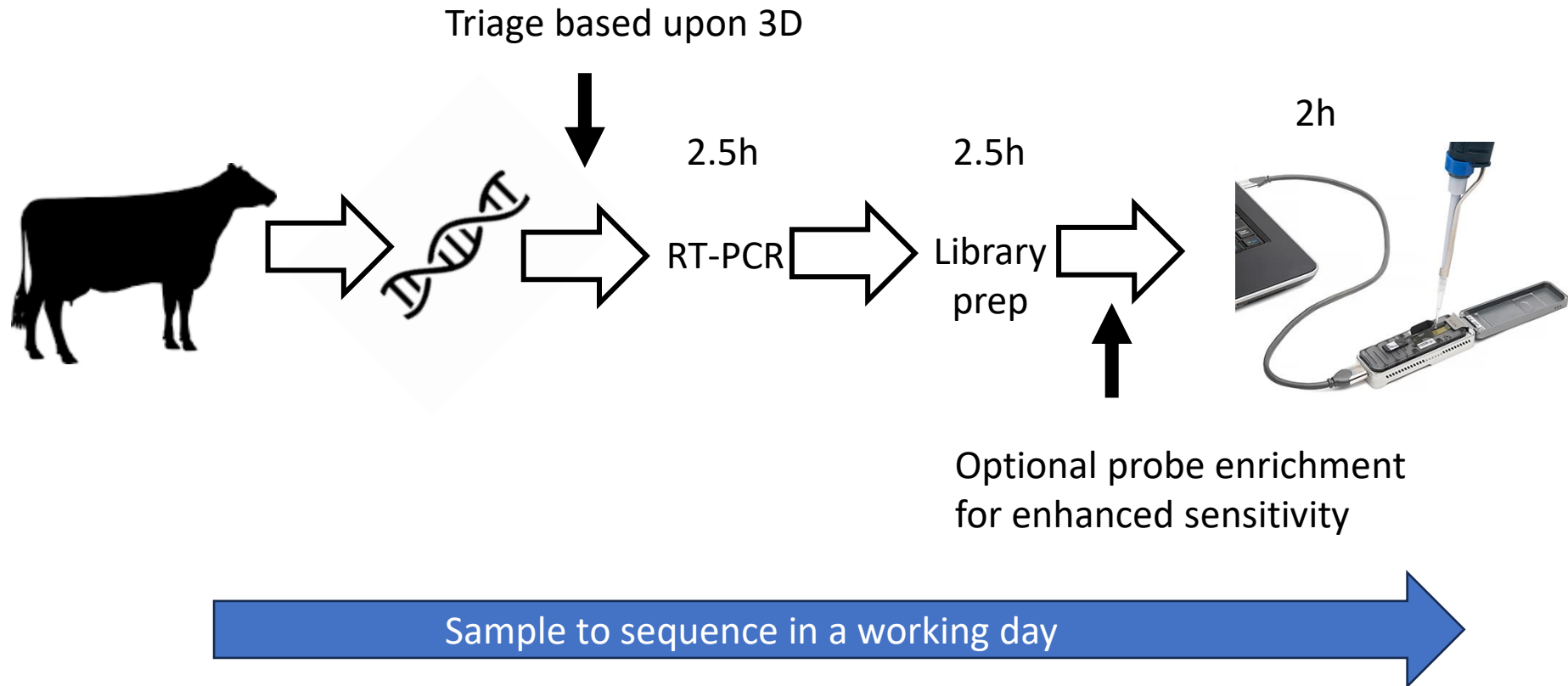
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# Enhanced sequencing capacity using MinION?

- Rapid and portable sequencing method for full genome sequencing
- Sample-to-sequence <24 hrs
- Equipment requirements: PCR machine, Qubit, MinION,
- pan-FMDV pools of primers have been designed to amplify overlapping amplicons
- Preliminary data indicates that the protocols can be applied for the O/ME-SA/Ind-2001e lineage
- WRLFMD can provide assistance to set up these methods and analyse data

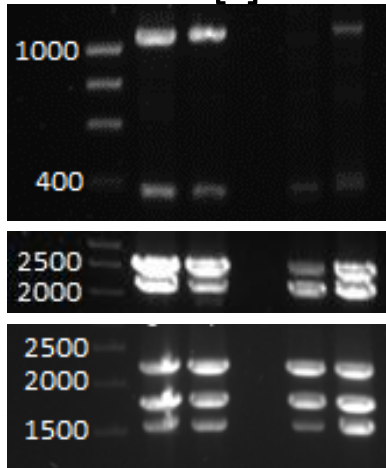


# The nanopore sequencing process

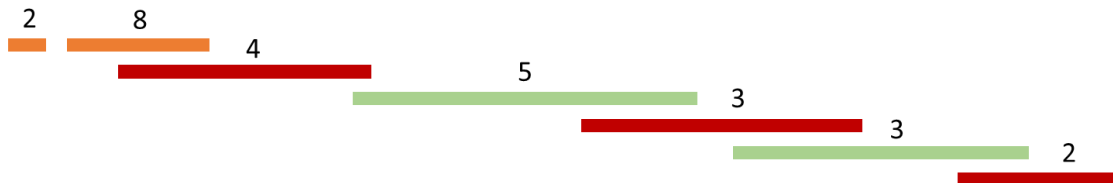
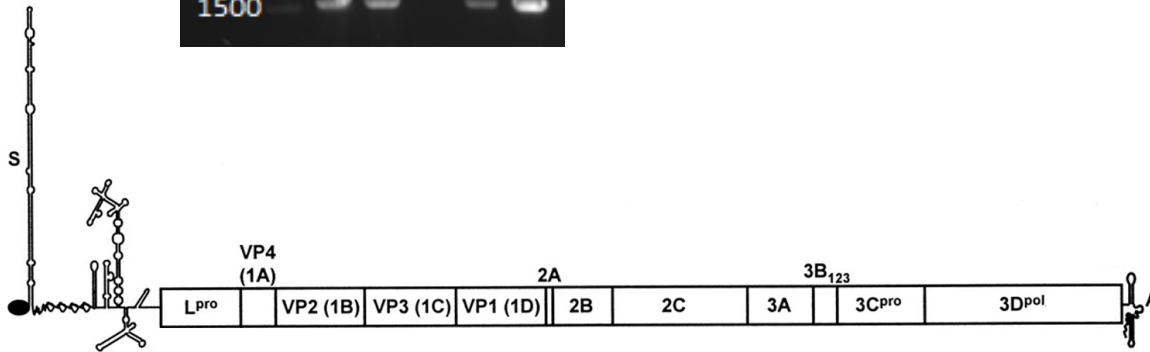


# Genome tiling strategy

1 2 [-] 3 4



1: CAM/5/2015 (A/ASIA/Sea-97)  
 2: TAI/8/2019 (A/ASIA/Sea-97)  
 3: TAI/34/2016 (O/SEA/Mya-98)  
 4: VIT/1/2018 (O/ME-SA/PanAsia)

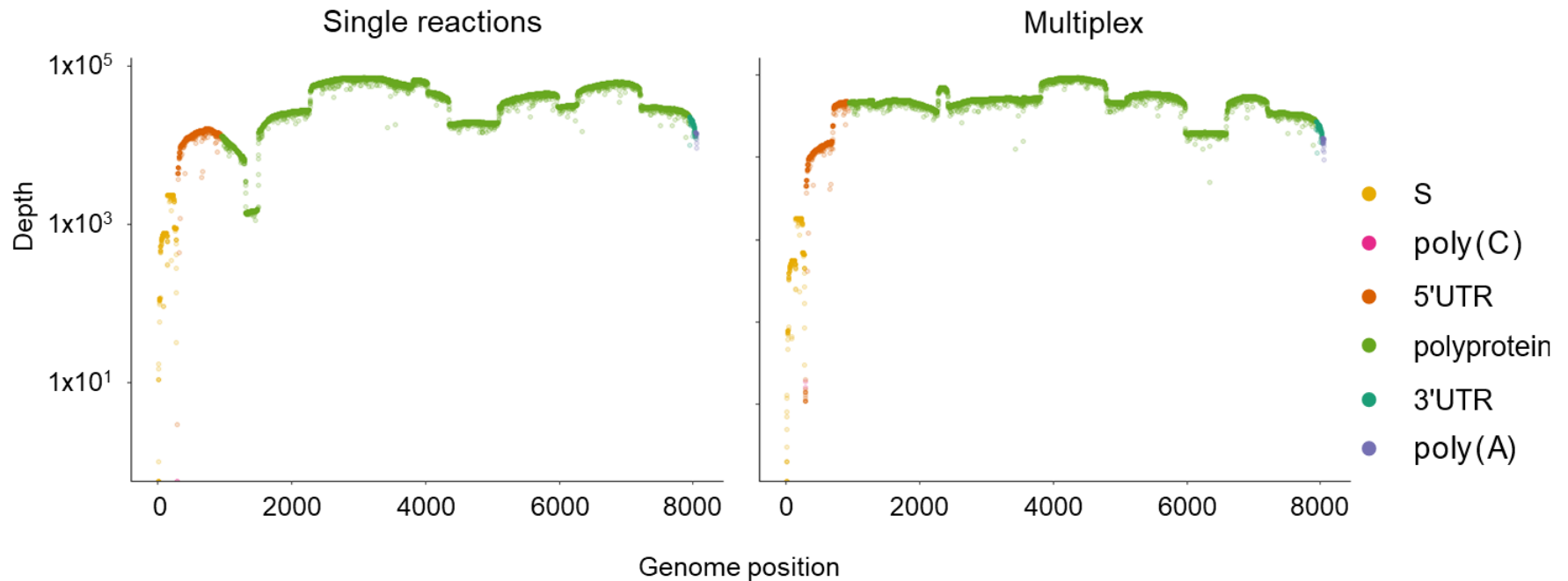




# Simple methods of genome amplification for sequencing

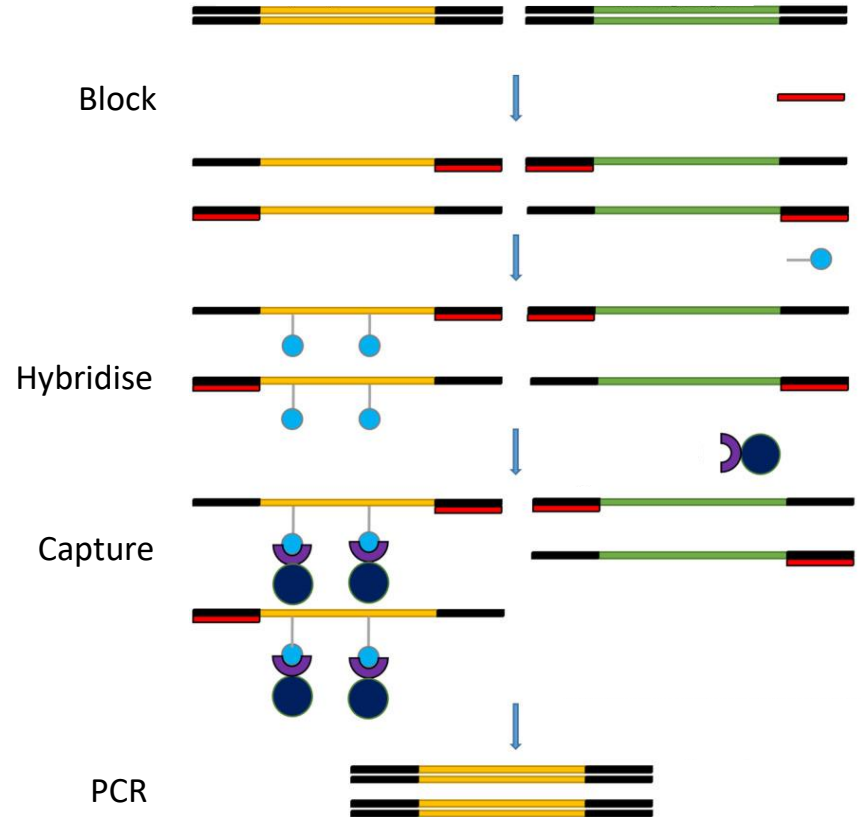
- Performing amplification in a multiplex (multiple amplicons per PCR reaction) rather than single-plex (one PCR reaction per amplicon) fashion has no effect upon sequencing efficacy.

## Coverage following nanopore sequencing (A/TAI/8/2019)



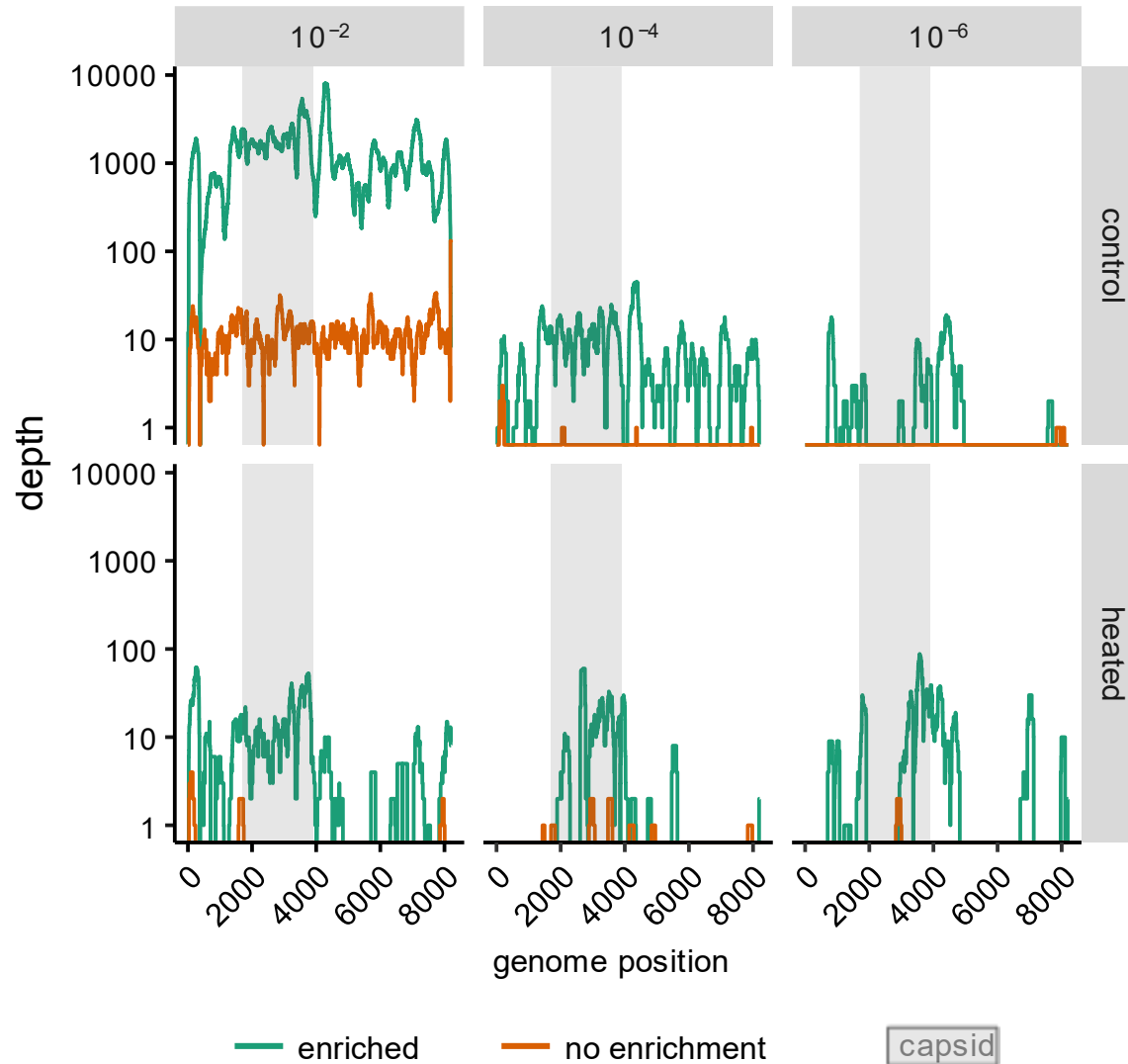
# Probe based enrichment

- Biotinylated probes can be used to pull out defined target sequences
- Widely used to enhance sensitivity of NGS
- Designed a panel of 26,275 FMDV-specific oligos



Adapted from Munyuza et al, 2022

# Probe enrichment enhances FMDV genome coverage



# Nanopore sequencing: summary

- Nanopore technology could be deployed into FMDV laboratories in Southeast Asia to rapidly characterise FMDVs.
- Simple protocols.
- High sensitivity obtained via PCR of overlapping tiles.
- Further increase in sensitivity theoretically possible using probe-based enrichment.
- Requires hardware and computational input (support possible via WRLFMD if necessary).
- Consumables cost can be as low as ~\$25/sample.
- Email [Andrew.shaw@pirbright.ac.uk](mailto:Andrew.shaw@pirbright.ac.uk) for protocols/advice.

# Acknowledgements

- Support for the WRLFMD and research projects
- Collaborating FMD Reference Laboratories and field teams
- Partners within the WOA/FAO FMD Lab Network



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