Australian Government
Department of Industry, Science, Energy and Resources

National Measurement Institute

Water Research AUSTRALIA

ColoSSoS
Collaboration on Sewage Surveillance of SARS-CoV-2

WaterRA ColoSSoS Project

Inter-laboratory Study: SARS-CoV-2 in Wastewater

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Wastewater surveillance for SARS-CoV-2

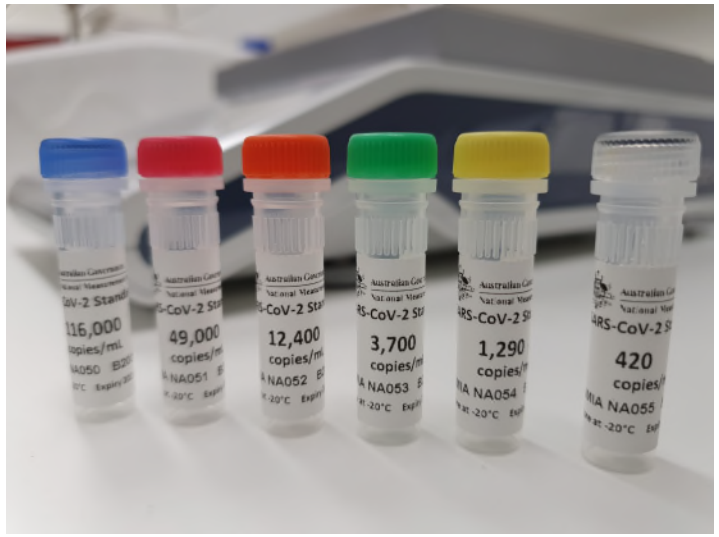
- Phase 1: Method development
  - Multiple labs developed methods independently, using a range of materials for validation and calibration
    - Recovery methods: electronegative membranes, ultrafiltration, PEG, ultracentrifugation
    - RNA extraction methods: range of kits available
    - RT-qPCR kits, mastermixes, 1-step or 2-step and assays
    - Choice of calibrant / reporting units

- Phase 2: Compare method performance between different service providers
  - Limit of detection (LOD) / yield
  - Reproducibility
  - Quantitative accuracy

- Participants:
  - 11 in Australia and 1 in New Zealand
  - 2 water utilities, 3 Government labs, 2 commercial labs, 5 University labs;

Plan for the ColoSSoS inter-laboratory study

- Study materials provided:
  - 10 x 50 mL aliquots of domestic wastewater (grab sample, untreated)
  - 2 x frozen aliquots of inactivated virus ('V1' and 'V2') for spiking
  - 3 x sets of calibrant CRM (6 dilutions prepared from inactivated virus)

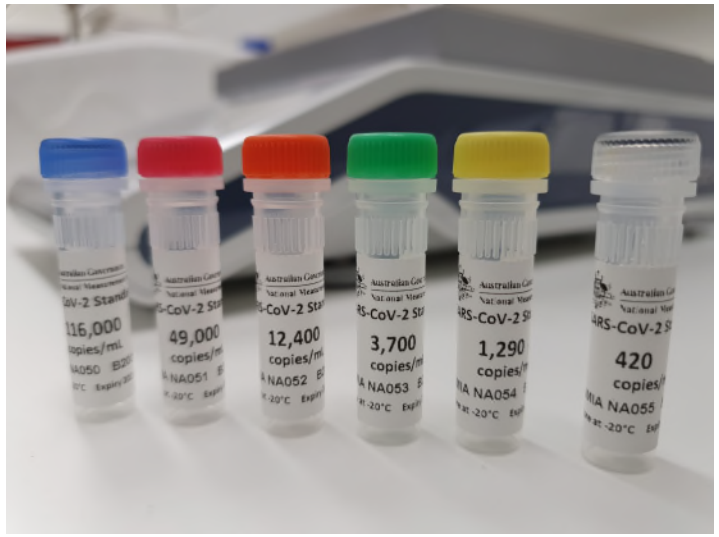


We were *very fortunate* to have domestic wastewater expected to be free of the virus and gamma-irradiated SARS-CoV-2 provided by the Victorian Infectious Diseases Reference Laboratory (VIDRL) – both game changers.

NMI Calibrant CRM

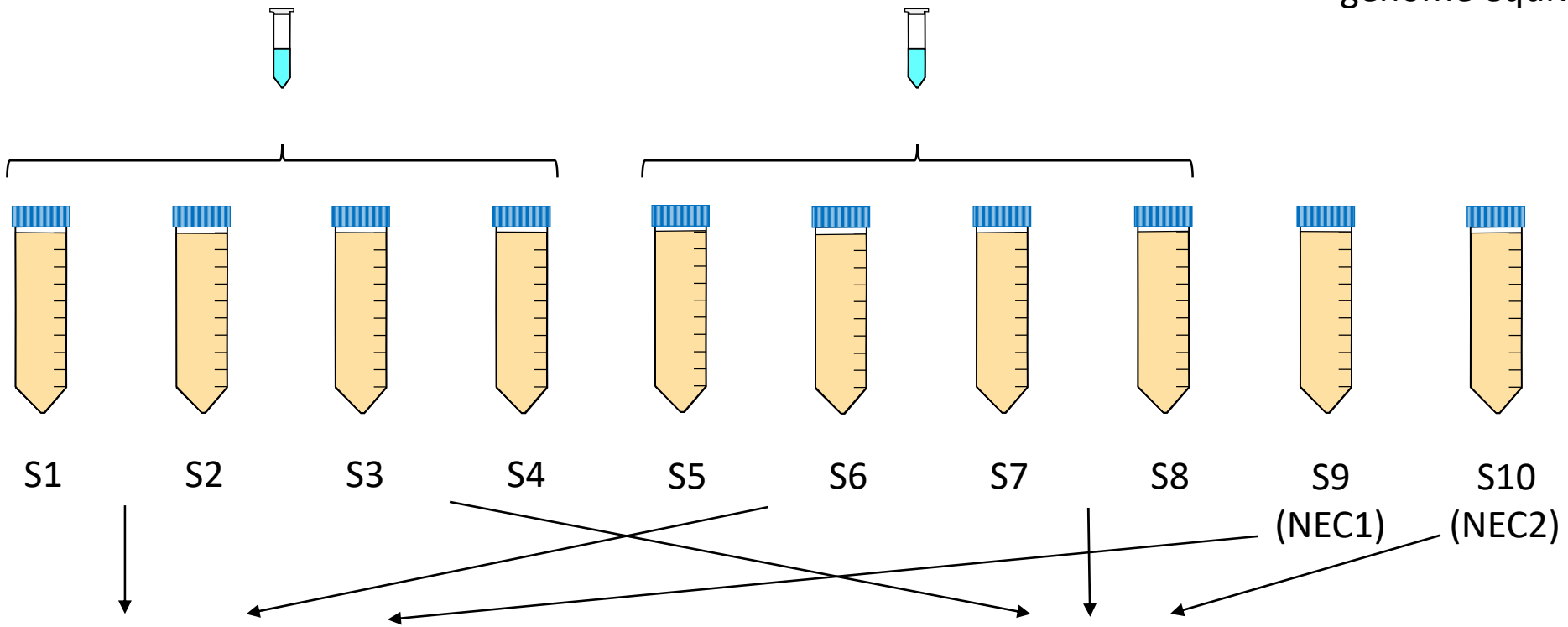
- The NMI calibrant:
  - Prepared from gamma-irradiated SARS-CoV-2 virus supplied by VIDRL
  - 6-point dilution series: 560, 245, 62, 18.5, 6.5 and 2.1 copies per 5 µL
  - Quantified in "**copy number concentration of SARS-CoV-2 genome equivalents**"
  - Quantified using RT-dPCR
  - Measurement uncertainty includes reverse transcription efficiency for conversion of RNA to DNA, using isotope dilution mass spectrometry (IDMS) data
  - Provides traceability to the International System of Units (SI mole)

- 5 µL of each dilution to be added directly into the RT-PCR well



Study Instructions

*copies of SARS-CoV-2 genome equivalents



Analysed straight away = **Day 0**
RNA samples analyzed in duplicate and at two concentrations

Stored at **4°C for 2 days** prior to analysis = **Day 2**
RNA samples analyzed in duplicate and at two concentrations

Calibrant (6 dilutions) analyzed in duplicate, plus NECs and NTCs

Results spreadsheet

Please fill in all the cells coloured yellow

Laboratory Name:
Analyst Name:
Analysis date (first day)
V1 Spike aliquot number:
V2 Spike aliquot number:

Concentration Factor

Vol. before processing (mL)	50
Vol. after virus recovery (mL)	
Vol. used in RNA extraction (mL)	
Vol. of eluted RNA (mL)	
RNA Dilution Factor 1	
RNA Dilution Factor 2	

CF (Dilution 1)
CF (Dilution 2)

First Day, Assay 1

	Calibrant Number	Replicate Number	Copy Number per RT-qPCR	Log (Copy Number)	Cq Value
Calibrant	NA050	C1.1	580	2.76	
		C1.2	580	2.76	
	NA051	C2.1	245	2.39	
		C2.2	245	2.39	
	NA052	C3.1	62.0	1.79	
		C3.2	62.0	1.79	
	NA053	C4.1	18.5	1.27	
		C4.2	18.5	1.27	
	NA054	C5.1	6.5	0.81	
		C5.2	6.5	0.81	
	NA055	C6.1	2.1	0.32	
		C6.2	2.1	0.32	

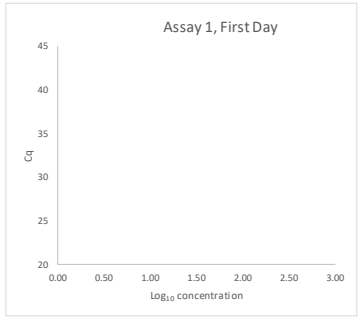
First Day, Assay 2

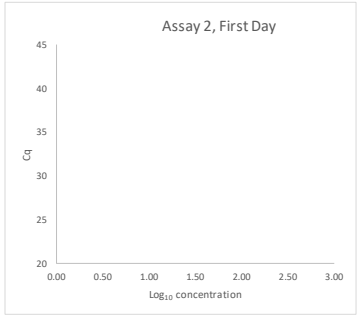
	Calibrant Number	Replicate Number	Copy Number per RT-qPCR	Log (Copy Number)	Cq Value
Calibrant	NA050	C1.1	580	2.76	
		C1.2	580	2.76	
	NA051	C2.1	245	2.39	
		C2.2	245	2.39	
	NA052	C3.1	62.0	1.79	
		C3.2	62.0	1.79	
	NA053	C4.1	18.5	1.27	
		C4.2	18.5	1.27	
	NA054	C5.1	6.5	0.81	
		C5.2	6.5	0.81	
	NA055	C6.1	2.1	0.32	
		C6.2	2.1	0.32	

	Sample Number	Replicate Number	Cq Value	Calculated log value	Estimated Copy Number/ RT-qPCR	Mean Estimated Copy Number/ RT-qPCR
Samples	Sample 1	S1.1				
		S1.2				
		S1.3				
		S1.4				
	Sample 2	S2.1				
		S2.2				
		S2.3				
		S2.4				
	Sample 5	S5.1				
		S5.2				
		S5.3				
		S5.4				
	Sample 6	S6.1				
		S6.2				
		S6.3				
		S6.4				
Negative Controls	Sample 9 (NEC)	S9.1				
		S9.2				
	NTC	NTC1				
		NTC2				

	Sample Number	Replicate Number	Cq Value	Calculated log value	Estimated Copy Number/ RT-qPCR	Mean Estimated Copy Number/ RT-qPCR
Samples	Sample 1	S1.1				
		S1.2				
		S1.3				
		S1.4				
	Sample 2	S2.1				
		S2.2				
		S2.3				
		S2.4				
	Sample 5	S5.1				
		S5.2				
		S5.3				
		S5.4				
	Sample 6	S6.1				
		S6.2				
		S6.3				
		S6.4				
Negative Controls	Sample 9 (NEC)	S9.1				
		S9.2				
	NTC	NTC1				
		NTC2				

	Assay 1	Assay 2
Slope (m)		
Intercept		
Correlation coefficient (r^2)		
Efficiency		
Number of points on calibration curve		

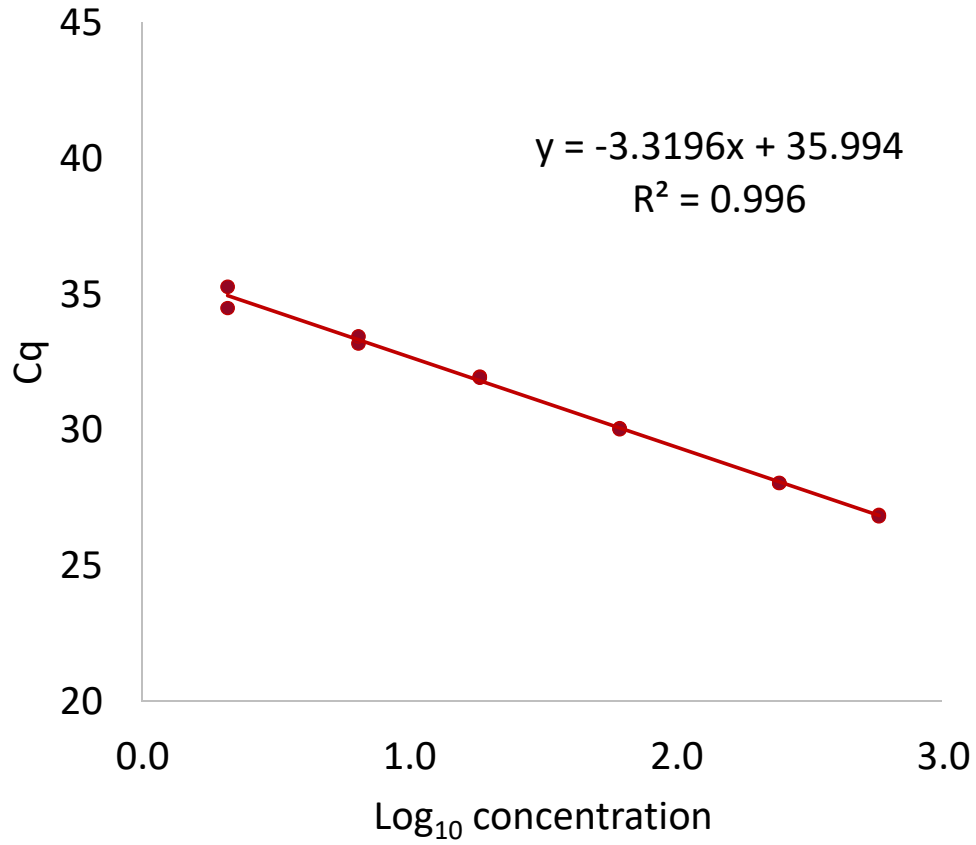




Analysis of calibrant data

$$\log_{10} c_i = \frac{C_q - Intercept}{Slope}$$

	Calibrant Number	Replicate Number	Copy Number per RT-qPCR	Log (Copy Number)	Cq Value
Calibrant	**NA050**	C1.3	580	2.76	26.84
		C1.4	580	2.76	26.79
	NA051	C2.3	245	2.39	28.02
		C2.4	245	2.39	28.03
	NA052	C3.3	62.0	1.79	30.04
		C3.4	62.0	1.79	30.00
	NA053	C4.3	18.5	1.27	31.94
		C4.4	18.5	1.27	31.91
	NA054	C5.3	6.5	0.81	33.16
		C5.4	6.5	0.81	33.42
	NA055	C6.3	2.1	0.32	34.47
		C6.4	2.1	0.32	35.25

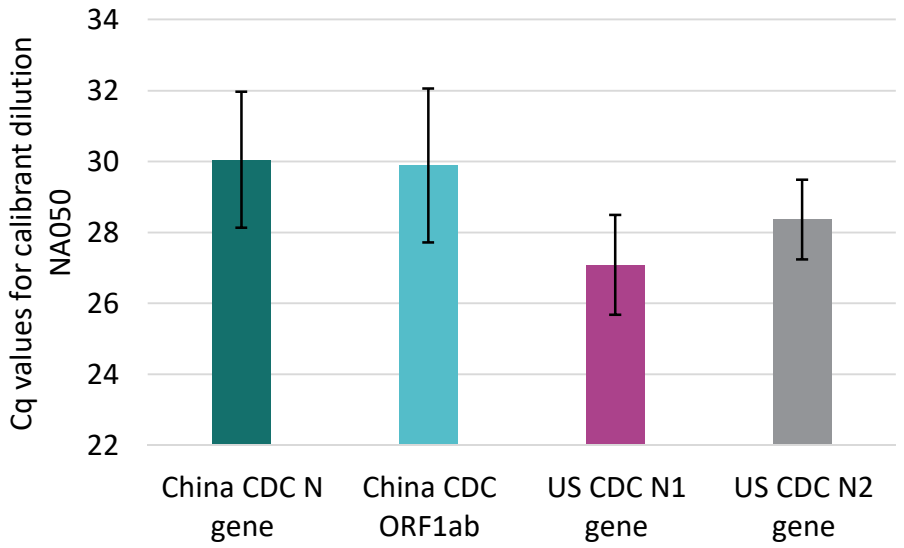


Lab 6, US CDC N1 Assay
Slope of -3.32 = 100 % RT-qPCR efficiency

Reasons for using a calibrant

1. Measure reaction efficiencies
2. Report in appropriate units:

Copy number concentration of SARS-CoV-2 genome equivalents



Average Cq values produced by all participants using most concentrated calibrant dilution (NA050) @ 580 copies/5 µL ±20 %.

Reported Cq values for the highest calibrant dilution range from 25.31 to 33.77

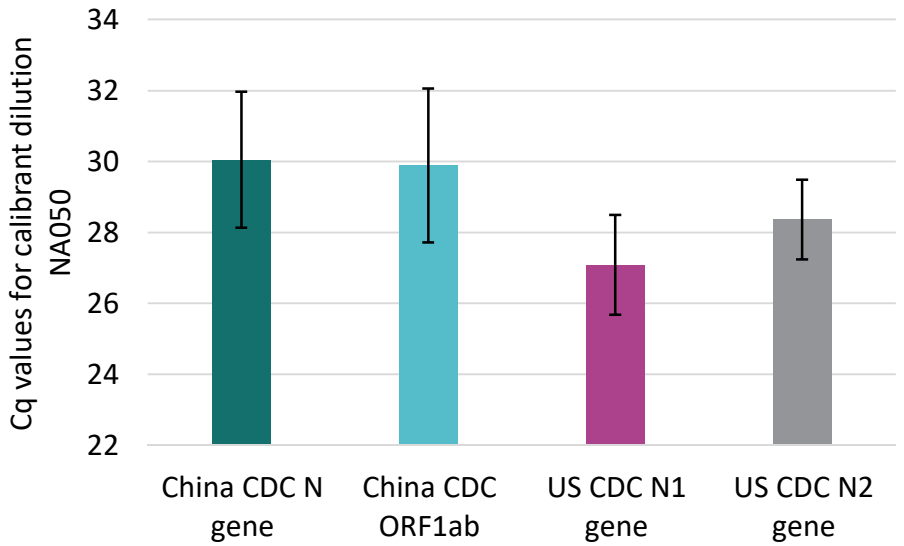
Reporting in Cq values, a ΔCq of 8.43 represents **345x difference in copy number concentration**.
In reality this is the **same material** measured by 12 different labs using 4 different RT-PCR assays

Reasons for using a calibrant

1. Measure reaction efficiencies
2. Report in appropriate units
3. Reduced assay-specific bias

Assay biases:

- amplicon length
- secondary structures
- fluorescence chemistries
- threshold position

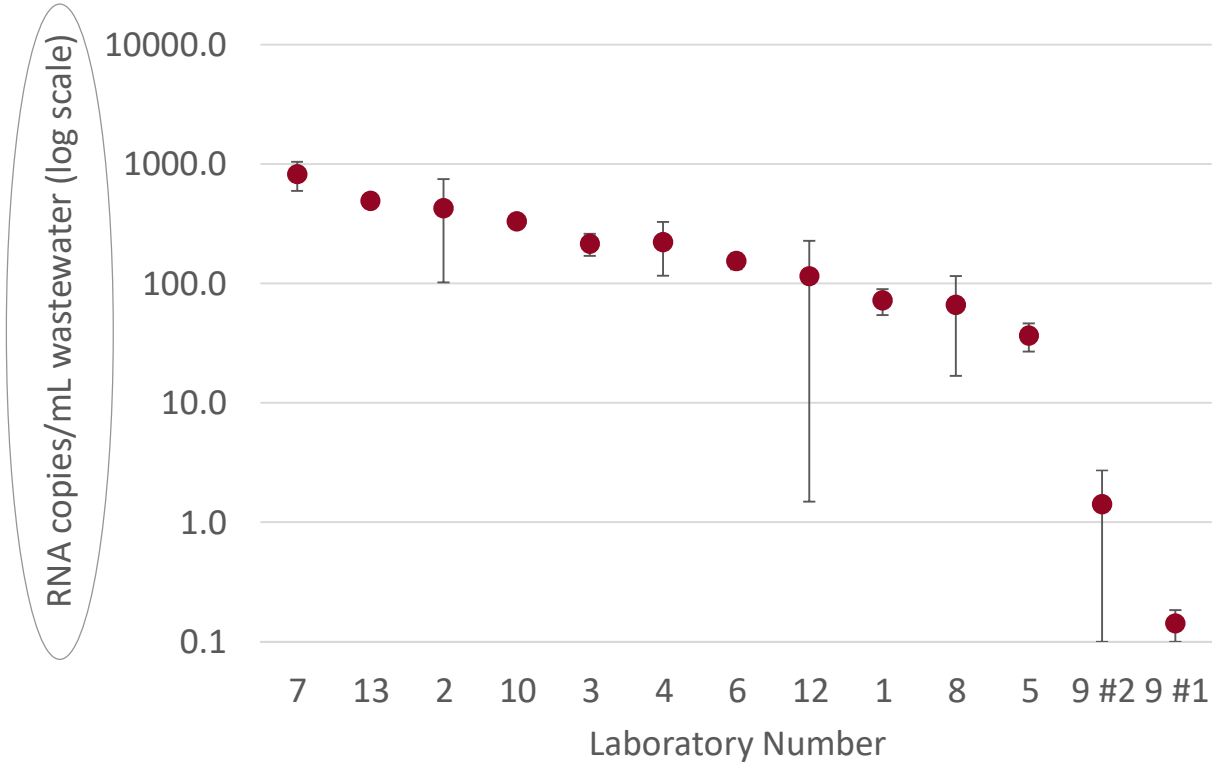


Average Cq values produced by all participants using most concentrated calibrant dilution (NA050) @ 580 copies/5 µL ±20 %.

It's preferable to report in traceable units rather than Cq values

Reasons for using a calibrant

1. Measure reaction efficiencies
2. Report in appropriate units
3. Reduced assay-specific biases
4. Provides traceability, allowing direct comparison of data between different laboratories



Calibrant → copies per µL of extracted RNA

Concentration Factor (CF) → copies per mL of wastewater

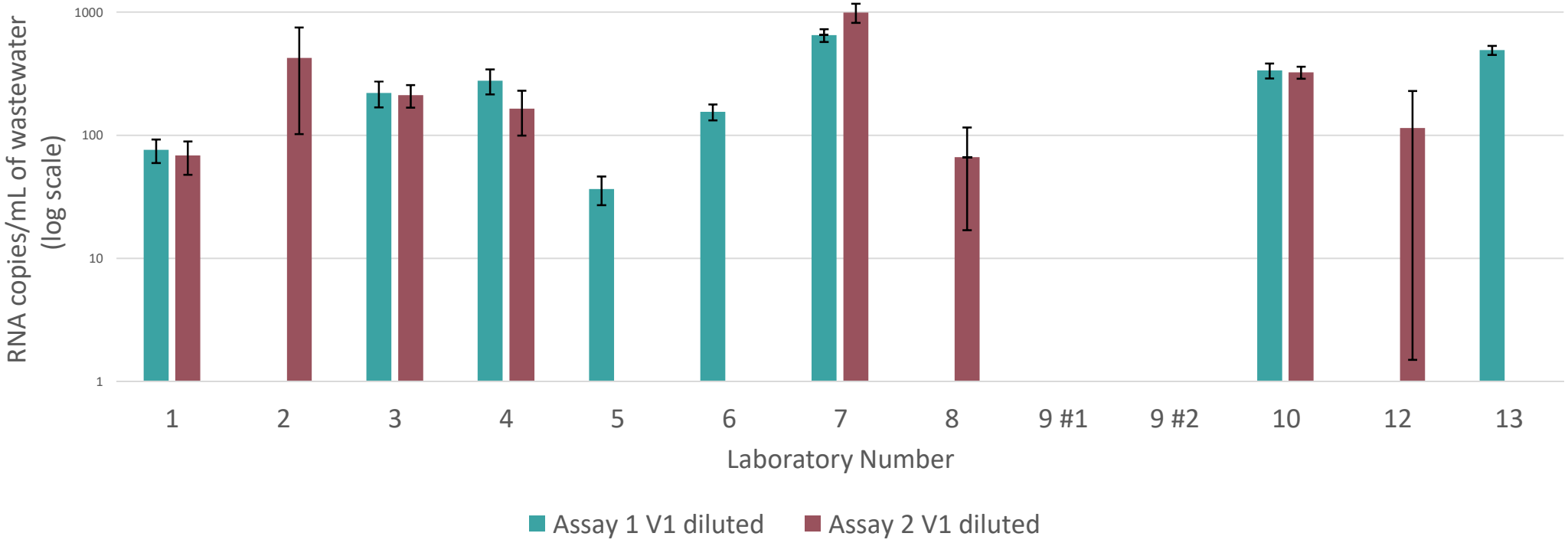
Concentration Factor (CF)

$$CF = \frac{V_{sample\ before\ processing}}{V_{after\ recovery}} \times \frac{V_{concentrate\ used\ for\ RNA\ extraction}}{V_{after\ RNA\ extraction}} \times \frac{1}{DF}$$

Allows conversion of copies **per µL of extracted RNA** into copies **per mL of wastewater**

Reference: Pecson, B. M. *et al*. (2021) "Reproducibility and sensitivity of 36 methods to quantify the SARS-CoV-2 genetic signal in raw wastewater: findings from an interlaboratory methods evaluation in the US". Environmental Science Water Research and Technology 7: 504-520.

Wastewater samples S1 and S2: spiked with ‘V1’, analyzed on Day 0



Labs 5, 6 and 13 only used one assay; labs 2, 8 and 12 had data excluded for one assay

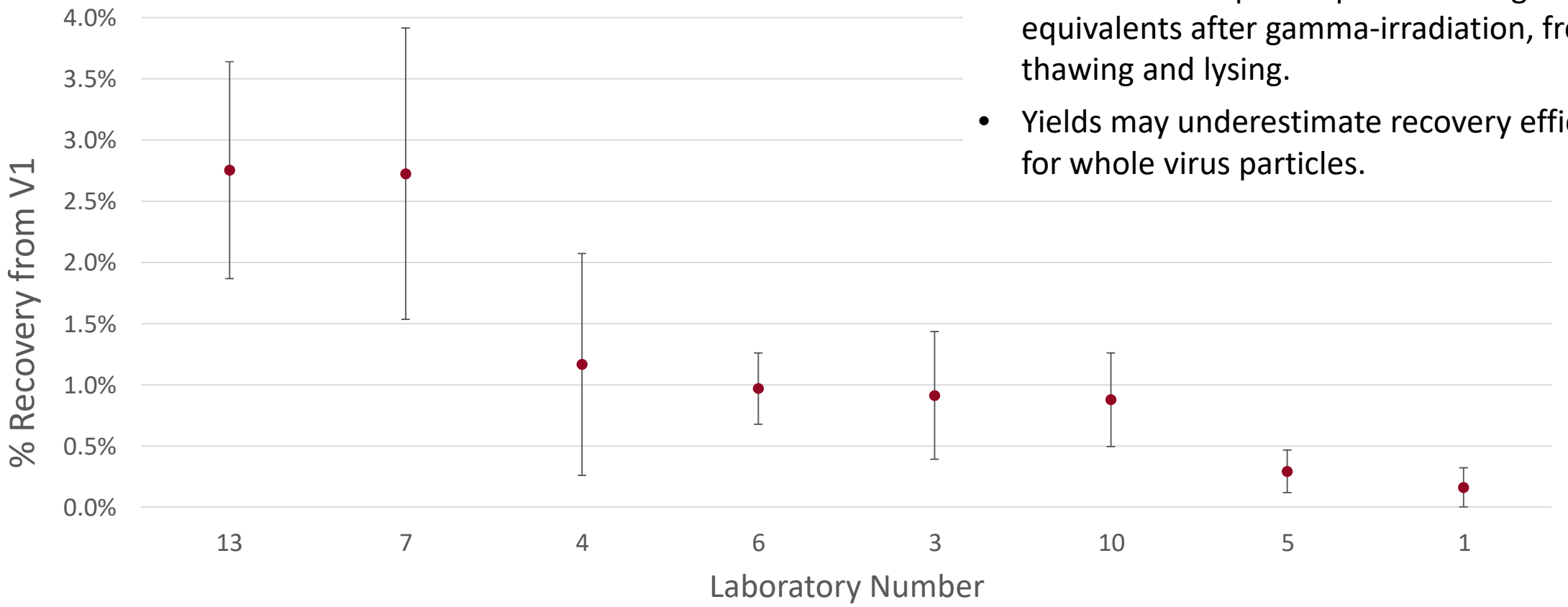
Summary of protocols used by participants

Virus Recovery	RNA Extraction	RT-PCR
Eight labs used electronegative membranes with range of pre-treatments: • centrifugation, • acidification, • addition of $MgCl_2$ One lab used 20 % PEG Two lab used ultrafiltration: • centrifugal filters • hollow-fibre concentrating pipette	Seven labs used Qiagen kits: • PowerSoil • PowerWater • PowerMicrobiome • PowerViral Three labs used ThermoFisher kits: • MagMax Viral Pathogen • MagMax Microbiome Ultra Other kits used: • Macherey-Nagel RNA stool kit • Roche High Pure Viral Nucleic Acid • Vazyme fast pure viral RNA/DNA	Five labs used PerkinElmer kit including China CDC assays Two labs used Promega kit including US CDC assays Other kits used: • ThermoFisher Combo kit, • Vazyme kit, Three labs bought primers, probes, enzymes and buffers separately, including one using 2-step RT-PCR.

Recoveries

$$= \frac{Total\ viral\ RNA\ genome\ equivalents\ recovered}{Total\ viral\ RNA\ genome\ equivalents\ added}\ x\ 100$$

- Based on ‘V1’ spike = quantified in genome equivalents after gamma-irradiation, freeze-thawing and lysing.
- Yields may underestimate recovery efficiency for whole virus particles.



Percentage of recoveries of ‘V1’ spiked samples, assuming 740,000 SARS-CoV-2 genome equivalents were spiked into the 50 mL of wastewater (14,800 copies per mL of wastewater).
Error bars are expanded relative uncertainties (95%) capturing participant measurement variation and V1 measurement uncertainty.

Detailed comparison of protocols with highest recoveries

	Lab 7	Lab 13
Pre-treatment	Centrifugation @3,270 g spin at 4 °C for 30 min (solids removal)	Centrifugation @10,000 g for 30 min with extract from solids added back to cleared supernatant
pH Adjustment	pH 3.5	pH 7.0-7.2
Virus recovery	0.45 µm electronegative membrane, vacuum pump	20 % PEG precipitation, 2 hr incubation at 4 °C, 10,000 g spin for 30 min
RNA extraction	RNeasy PowerMicrobiome® Kit (Qiagen) Shield™ reagent and phenol added during bead beating	High Pure Viral Nucleic Acid kit (Roche)
Elution/diluent	Nuclease-free water	Nuclease-free water
Style of RT-PCR	1-step	2-steps
Assays	China CDC assays: N gene (FAM) and ORF1ab (Texas Red)	China CDC assay: N gene, FAM-labelled probe
Temp. during plate set-up	25 °C	4 °C
Enzymes/mastermixes	SARS-COV-2 Real Time 1-step RT-PCR assay kit (PerkinElmer)	SuperScript III® Reverse Transcriptase (Thermofisher) PerfeCta® qPCR ToughMix (Quanta Biosciences)
Cycling parameters	RT: 37°C, 2 min, 50°C, 5 min, 42°C, 35 min Enzyme Activation: 94°C, 10 min Denaturation: 94°C, 10 sec Annealing: 55°C, 15 sec, Extension: 65°C, 45 sec Number of Cycles: 45	RT: 50°C, 30 min Enzyme Activation: 95°C, 180 sec Denaturation: 95°C, 15 sec Annealing/Extension: 60°C, 30 sec Number of Cycles: 45
Dilution(s) for RNA	Neat and 1-in-10 dilution	Neat and 1-in-4 dilution
Concentration Factors	CF1: 323, CF2: 32	CF1: 200, CF2: 50

Conclusions

- All participants successfully detected RNA from the inactivated V1 virus spiked into wastewater
- Highest recoveries, great reproducibility and clean RNA using:
  - electronegative membranes with centrifugation and acidification as pretreatments
  - 20 % PEG
    - Major implications for lower income regions and ColoSSoS DFAT program
    - PEG method widely used for environmental surveillance of poliovirus – familiar and low cost*
- SARS-CoV-2 calibrant and Concentration Factor for comparable reporting units:

"RNA copy number concentration of SARS-CoV-2 genome equivalents per mL of wastewater"

*World Health Organization (2003), Guidelines for environmental surveillance of poliovirus circulation, WHO/V&B/03.03

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  - Dr Dan Deere
- All of the study participants
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Contact us for inquiries and calibrant CRM sales

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