WaterRA ColoSSoS Project

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

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Bioanalysis Section, Australian National Measurement Institute

14th June 2021
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*

Analysed straight away = **Day 0**

- **S1**
- **S2**
- **S3**
- **S4**

RNA samples analyzed in duplicate and at two concentrations

100 µL V1 = 740,000 *cp*

100 µL V2 = 66,000 *cp*

 Stored at 4°C for 2 days prior to analysis = **Day 2**

- **S5**
- **S6**
- **S7**
- **S8**
- **S9** (NEC1)
- **S10** (NEC2)

RNA samples analyzed in duplicate and at two concentrations

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

*Copies of SARS-CoV-2 genome equivalents*
Please fill in all the cells coloured yellow

<table>
<thead>
<tr>
<th>Concentration Factor</th>
<th>Vol. before processing (mL)</th>
<th>CF (Dilution 1)</th>
<th>Vol. after virus recovery (mL)</th>
<th>CF (Dilution 2)</th>
<th>Vol. of eluted RNA (mL)</th>
<th>RNA Dilution Factor 1</th>
<th>RNA Dilution Factor 2</th>
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<tbody>
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Laboratory Name:  
Analyst Name:  
Analysis date (first day):  
V1 Spike aliquot number:  
V2 Spike aliquot number:  

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Replicate Number</th>
<th>Cq Value</th>
<th>Estimated Copy Number/ 81 qPCR</th>
<th>Mean Estimated Copy Number/ 81 qPCR</th>
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<tbody>
<tr>
<td>Sample 1</td>
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<td>Sample 4</td>
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Negative Controls  
Sample:  
Sample 9 (NEC)  
NTC  

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<tr>
<th>Sample Number</th>
<th>Replicate Number</th>
<th>Cq Value</th>
<th>Calculated log value</th>
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Calibrant  
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Slope (m)  
Intercept  
Correlation coefficient (r²)  
Efficiency  
Number of points on calibration curve  

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<tr>
<th>0.00</th>
<th>0.50</th>
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<tr>
<td>Cq</td>
<td>Log 10 concentration</td>
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Analysis of calibrant data

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

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

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 $\rightarrow$ copies per $\mu$L of extracted RNA

Concentration Factor (CF) $\rightarrow$ copies per mL of wastewater
Concentration Factor (CF)

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

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

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

<table>
<thead>
<tr>
<th>Virus Recovery</th>
<th>RNA Extraction</th>
<th>RT-PCR</th>
</tr>
</thead>
</table>
| Eight labs used electronegative membranes with range of pretreatments: | Seven labs used Qiagen kits:  
- PowerSoil  
- PowerWater  
- PowerMicrobiome  
- PowerViral | Five labs used PerkinElmer kit including China CDC assays |
| - centrifugation,  
- acidification,  
- addition of MgCl₂ | Three labs used ThermoFisher kits:  
- MagMax Viral Pathogen  
- MagMax Microbiome Ultra | Two labs used Promega kit including US CDC assays |
| One lab used 20 % PEG | Other kits used:  
- Macherey-Nagel RNA stool kit  
- Roche High Pure Viral Nucleic Acid  
- Vazyme fast pure viral RNA/DNA | Other kits used:  
- ThermoFisher Combo kit,  
- Vazyme kit, |
| Two lab used ultrafiltration:  
- centrifugal filters  
- hollow-fibre concentrating pipette | | Three labs bought primers, probes, enzymes and buffers separately, including one using 2-step RT-PCR. |
Recoveries

\[
\text{Percentage of recoveries of ‘V1’ spiked samples, assuming 740,000 SARS-CoV-2 genome equivalents were spiked into the 50 mL of wastewater (14,800 copies per mL of wastewater).}
\]

\[
\text{Error bars are expanded relative uncertainties (95%) capturing participant measurement variation and V1 measurement uncertainty.}
\]
## Detailed comparison of protocols with highest recoveries

<table>
<thead>
<tr>
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<th>Lab 7</th>
<th>Lab 13</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-treatment</strong></td>
<td>Centrifugation @3,270 g spin at 4 °C for 30 min (solids removal)</td>
<td>Centrifugation @10,000 g for 30 min with extract from solids added back to cleared supernatant</td>
</tr>
<tr>
<td><strong>pH Adjustment</strong></td>
<td>pH 3.5</td>
<td>pH 7.0-7.2</td>
</tr>
<tr>
<td><strong>Virus recovery</strong></td>
<td>0.45 µm electronegative membrane, vacuum pump</td>
<td>20 % PEG precipitation, 2 hr incubation at 4 °C, 10,000 g spin for 30 min</td>
</tr>
<tr>
<td><strong>RNA extraction</strong></td>
<td>RNeasy PowerMicrobiome® Kit (Qiagen) Shield™ reagent and phenol added during bead beating</td>
<td>High Pure Viral Nucleic Acid kit (Roche)</td>
</tr>
<tr>
<td><strong>Elution/diluent</strong></td>
<td>Nuclease-free water</td>
<td>Nuclease-free water</td>
</tr>
<tr>
<td><strong>Style of RT-PCR</strong></td>
<td>1-step</td>
<td>2-steps</td>
</tr>
<tr>
<td><strong>Assays</strong></td>
<td>China CDC assays: N gene (FAM) and ORF1ab (Texas Red)</td>
<td>China CDC assay: N gene, FAM-labelled probe</td>
</tr>
<tr>
<td><strong>Temp. during plate set-up</strong></td>
<td>25 °C</td>
<td>4 °C</td>
</tr>
<tr>
<td><strong>Enzymes/mastermixes</strong></td>
<td>SARS-COV-2 Real Time 1-step RT-PCR assay kit (PerkinElmer)</td>
<td>SuperScript III® Reverse Transcriptase (Thermofisher) PerfeCta® qPCR ToughMix (Quanta Biosciences)</td>
</tr>
</tbody>
</table>
| **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
Acknowledgements

• WaterRA for supporting and co-ordinating this study:
  • Dr Kelly Hill and Marlene Hsu

• Victorian Infections Diseases Reference Laboratory for providing gamma-irradiated SARS-CoV-2:
  • Prof. Bruce Thorley and Dr. Julian Druce

• Task Leader for the ColoSSoS Task Group 2 and ColoSSoS Project Manager:
  • Dr Dan Deere

• All of the study participants

• Sydney Water for providing the domestic wastewater sample:
  • Kate McLennan and Anna Flack

• Pathwest and Sydney Water Laboratories for unwittingly assisting with the validation of the V1 and V2 material:
  • Jake Gazeley and Sudhi Payyappat

• The NMI Bioanalysis Team:
  • Dr D.G. Burke, (Manager); Dr K.R. Emslie (NMI Honorary Fellow); Dr A. Baoutina; Dr S. Bhat; Dr M. Forbes-Smith; Ms F. Hall; Dr D. Lynch; Mr J. McLaughlin; Ms L. Partis; Dr L.B. Pinheiro
Contact us for inquiries and calibrant CRM sales

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