Australian Government
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National Measurement Institute

CERTIFIED REFERENCE MATERIAL CERTIFICATE OF ANALYSIS

NMIA NA050 to NA055: SARS-CoV-2 Standard

Report ID: 210329 (supersedes ID 201221, revised expiry date) **Batch No.:** B200921

Certified concentration values of SARS-CoV-2 genome equivalents

NMIA Code	Concentration (copies/mL)	Expanded Uncertainty	Expanded Uncertainty (%)
NA050	116,000	28,000	24
NA051	49,000	12,000	25
NA052	12,400	3,100	25
NA053	3,700	1,200	33
NA054	1,290	530	41
NA055	420	280	66

The uncertainty has been calculated according to the Guide to the expression of uncertainty in measurement [1] and ISO Guide 35 [2] and is stated at the 95% level of confidence.

Measurand: The measurand is defined as the copy number concentration of the SARS-CoV-2 genome equivalents (GenBank Accession MT007544.1) in the solution supplied.

Expiry and storage: The property values are valid to 10 February 2022 for sealed vials stored at a maximum of -18 °C out of direct light.

Description: The Certified Reference Material (CRM) comprises a set of six vials, each vial contains 0.55 mL of diluted inactivated SARS-CoV-2 virus at the concentrations above in 1 mM citrate buffer pH 6.5 with yeast total RNA at 5 µg/mL.

Intended use: The CRM is intended to evaluate limit of detection for the entire measurement procedure including extraction and to calibrate qPCR measurement systems designed to detect and quantify the amount of viral genomes.

Instructions for use: Vials must be thawed and mixed thoroughly by vortexing prior to use. Vials are designed for single use and should not be frozen once thawed. Changes in the concentration of opened vials will depend on many factors and so the certified concentration does not apply after the first use. The recommended minimum sample size is 50 µL.

Metrological traceability: The concentration values were obtained by dividing reverse transcriptase - digital PCR measurements (RT-dPCR) of the CRM solutions by an RT-dPCR efficiency factor.

The certified copy number concentration for SARS-CoV-2 reference material in copies/mL is traceable to the International System of units (SI) via:

1. The copy number concentration method of RT-dPCR is traceable to the SI through the counting unit 1 when potential biases have been evaluated.
2. Reverse transcriptase efficiency was evaluated by comparison of RT-dPCR measured concentration to the Isotope Dilution Mass Spectrometry IDMS concentration of synthetic RNA templates. The IDMS concentration is traceable to the SI mole
3. RT-dPCR droplet volume was measured using a stage micrometer calibrated using a standard for length traceable to the SI metre
4. Gravimetric preparation of dPCR reaction mixtures using balances calibrated using a mass standard traceable to the SI kilogram.

Safety: The CRM contains inactivated SARS-CoV-2 virus that was confirmed non-viable. Use appropriate work practices when handling to avoid skin or eye contact, ingestion or inhalation of aerosols generated during handling.

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29 March, 2021

Terms and Conditions associated with the provision of this reference material can be found on the NMIA website.

CRM Characterisation Report

Characterisation approach: The certified reference material was characterised by direct RT-dPCR measurements of the diluted viral culture using two assays targeting each end of the viral genome. The raw RT-dPCR measurements were converted to viral genome concentration through an RT efficiency factor evaluated by dPCR and IDMS as described below; RT-dPCR partition volume was measured as published [3]. All measurements were performed in-house at the National Measurement Institute, Australia (NMIA).

Direct RT-dPCR Measurement method: The concentration of SARS-CoV-2 in a vial was measured by two hydrolysis probe RT-dPCR assays on the diluted inactivated virus solution without prior extraction. The assays targeted two regions, one region at each end of the genome; the 5′ end assay was developed at NMIA and the China CDC N gene assay was used for the 3′ end (Appendix 1). Assays were performed following the workflow recommended for the Bio-Rad QX200™ AutoDG™ Droplet Digital™ PCR System combined with Bio-Rad One-Step RT-ddPCR Advanced Kit for Probes; primer concentration was 900 nM and probe was 250 nM. Prior to thermal cycling reaction mixtures were incubated at 45 °C for 60 min for the reverse transcriptase reaction, followed by 10 minute activation at 95 °C then 50 cycles of a two-step thermal profile of 30 seconds denaturation at 95 °C, 60 seconds at 57.5 °C for combined annealing – extension and a final 10 minute inactivation step at 98 °C. A QX100 droplet reader with QuantaSoft v1.7.4 was used for emulsion analysis. Wells with less than 10,000 total accepted droplets were excluded from subsequent analysis.

The measurement equation used for calculating RT-dPCR copy number concentration is:

$$C_{RT\text{-}dPCR,assay} = -\ln\left(1 - \frac{N_P}{N_T}\right) \times \frac{10^6}{V_P} \times \frac{(m_{dPCR\ premix} + m)}{m} \times \frac{\rho}{\rho_{dPCR\ mix}} \quad (1)$$

where $C_{RT\text{-}dPCR,assay}$ is copy number concentration (copies/mL) in the test solution measured by RT-dPCR using the specific assay oligonucleotides (Appendix 1), N_p and N_T are the number of positive droplets and number of total accepted droplets, respectively, V_P is droplet volume (nL), m and $m_{dPCR\ premix}$ are the mass (g) of reference material solution and assay premix obtained by adding assay specific primers and probe and Bio-Rad One-Step RT-ddPCR Advanced Kit for Probes, respectively, and ρ and $\rho_{dPCR\ mix}$ are the density (g/cm^3) of the reference material solution and the dPCR reaction mix, respectively.

The density of the dPCR reaction mix was 1032.63 kg/m^3 measured at NMIA using an Anton Paar density measuring cell model DMA 5000 at controlled temperature of 20 °C. The relative expanded uncertainty of the density measurement was 0.0048% (Report Number 201152)

Conversion to SARS-CoV-2 Genome equivalents: Since RT-dPCR values are obtained from dPCR measurement of complimentary DNA produced in the RT step, the original RNA concentration can be calculated when the efficiency of conversion to cDNA is evaluated. RT efficiency was evaluated for the assays cited in this report as the ratio of RT-dPCR value divided by the IDMS concentration of short synthetic templates. The RT-dPCR values were divided by the corresponding reverse transcriptase efficiency factor (E_{RT}) described below to give SARS-CoV-2 genome equivalents.

The average of the two target region RNA concentrations was used to give the viral genome concentration using Equation 2.

$$C_{SARS\text{-}CoV\text{-}2} = \frac{\frac{C_{RT\text{-}dPCR,NMIA}}{E_{RT,NMIA}} + \frac{C_{RT\text{-}dPCR,ChinaCDC2}}{E_{RT,China\ CDC\ 2}}}{2} \quad (2)$$

Reverse Transcriptase and dPCR efficiency: Efficiency of reverse transcriptase was evaluated by measuring the concentration of HPLC purified short synthetic RNA templates corresponding to the assay target region by dPCR and by Isotope Dilution Mass Spectrometry (IDMS); efficiency was defined as the ratio of RT-dPCR to IDMS measured concentration.

Table 1 RT-dPCR efficiency for the assays used to assign the certified value

Assay	Assay Start Position	Target Gene	Efficiency
NMIA	445	ORF 1ab	0.758
China CDC 2	28882	N	0.726

Isotope dilution mass spectrometry method: Synthetic templates were manufactured by a commercial supplier and purified at NMIA by HPLC fractionation; purity of HPLC fractions was evaluated by HPLC prior to concentration measurement by IDMS [4]. The HPLC fraction was enzymatically digested to nucleotide monophosphates (NMPs) and the concentrations of NMPs in the digest were evaluated with calibration solutions that were measured by QNMR or by spectrophotometry using a molar absorption coefficient determined from the QNMR measured NMP solutions providing traceability to the SI mole.

Source of inactivated virus: The inactivated virus solution was produced at Victorian Infectious Diseases Reference Laboratory and has a batch number of SARS-CoV-2/Victoria/1/2020; the cell line and media used to culture the virus was Vero and MEM with 2% FBS. The original source of the virus was from a patient returning to Australia from Wuhan. The isolate has been sequenced (GenBank Accession MT007544.1, GISAID EPI_ISL_406844)[5]. The solution as received was diluted with a proprietary buffer to give solutions with the target concentrations.

Assessment of Fit for Purpose: Suitability of this certified reference material for quantitative RT-PCR analysis was demonstrated by direct analysis of the CRM using the in-house developed assay targeting the ORF1ab gene and shown in Figure 1. The amount of viral RNA measured by users may vary due to many factors including variability in extraction recovery, reverse transcriptase efficiency and choice of assay.

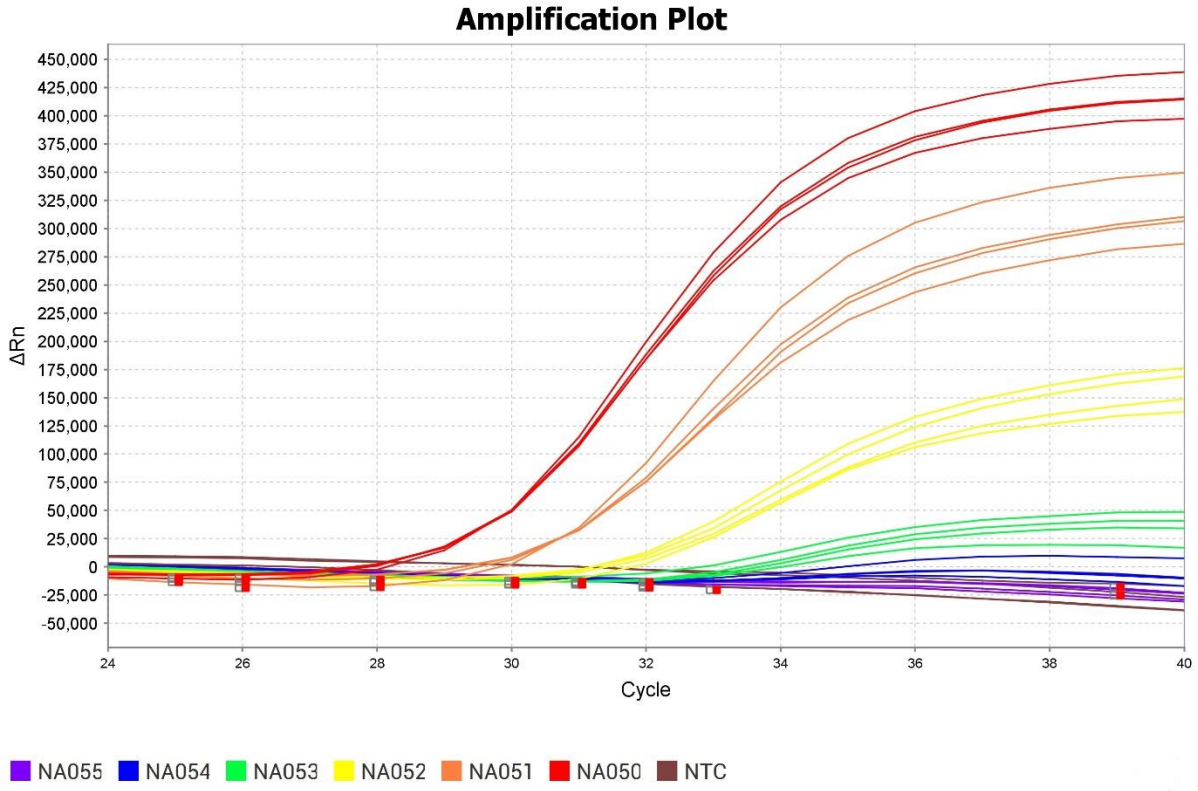

Figure 1 Amplification plots of SARS-CoV-2 Standard using the NMIA assay targeting the ORF1ab gene

Quantitative PCR: As an aid to evaluating RT-qPCR assay efficiency, Table 2 shows differences in theoretical cycle threshold quantitation values (ΔCq) calculated using an ideal slope of -3.3 for 100% and intercept Cq of 36 cycles as given in Equation 3.

$$C_q = -3.3 \times log_{10} C + 36 \quad (3)$$

Table 2. Theoretical Cq differences between CRM solutions

NMIA Code	Certified concentration (copies/mL)	ΔCq
NA050	116,000	NA
NA051	49,000	1.2
NA052	12,400	2.0
NA053	3,700	1.7
NA054	1,290	1.5
NA055	420	1.6

Homogeneity: Assessment of the inter-vial homogeneity of SARS-CoV-2 standard was conducted in accordance with ISO Guide 35 [2]. Ten vials were selected from the batch using a randomised sampling plan and two sub-samples from each vial (one from the top and one from the bottom) were analysed by RT-dPCR. The within-vial and between-vial variances were calculated using the ANOVA method and was included in the combined measurement uncertainty. Negative control assays run in parallel with the lowest concentration solution had a SARS-CoV-2 genome equivalent of 20 copies/mL.

Stability: An accelerated stability study was conducted at 22 °C for a minimum of 26 days and a small reduction in concentration was observed. The time period was extrapolated to storage at -20 °C and the uncertainty of the slope including the degradation component was included in the uncertainty budget. Stability at the recommended storage temperature -20 °C will be monitored.

Genome coverage and target gene bias: The viral genome was analysed using five representative assays defined in Appendix), each targeting a unique region, and concentration results are given in Table 3. There was no significant difference (ANOVA, 95% confidence level) between the five concentration values after conversion to SARS-CoV-2 genome equivalents. The standard deviation between the five concentration values was used in the uncertainty budget as the assay bias factor. Since there was no significant difference between assays, two assays (NMIA and China CDC 2) were selected for homogeneity measurements to give representative coverage of the entire genome and assignment of the certified value. RT-dPCR values for these five target regions before conversion to genome concentration using the reverse transcriptase efficiency factors were significantly different (ANOVA 95% confidence level).

Table 3. Indicative copy number concentrations of the 12,400 copy/mL certified solution (NA052) for a range of targets along the genome.

Assay	NMIA	Charite 2	US CDC 1	China CDC 2	US CDC 2
Gene	ORF1ab	E	N1	N	N2
Assay start position (nucleotides from sequence start)	445	26269	28287	28882	29164
RT-dPCR copies/mL	9,000	8,200	11,500	9,900	11,400
RT efficiency	0.758	0.724	0.918	0.726	0.865
NA052 SARS-CoV-2 genome equivalents copies/mL	11,900	11,300	12,500	13,700	13,200

Commutability: This material contains gamma irradiated SARS-CoV-2 virus. It is suitable for analysis involving RNA extraction and RNA analysis by methods such as RT- PCR for assays targeting any region on the SARS-CoV-2 genome. A formal commutability study has not yet been undertaken.

Droplet volume: Droplet volume measurements were conducted over a five month period using optical microscopy as previously described [3]. A total of 46,549 droplets from 54 independent RT-dPCR reaction mixtures were used for droplet volume measurement; the average droplet volume was 0.763 nL with a standard deviation of 0.016 nL which was within the current control limit. The current control chart mean droplet volume of 0.760 nL was used for calculating copy number concentration in the reference material solution.

Viral RNA recovery: The certified concentration values were obtained by direct RT-dPCR analysis of the standard solutions; the solutions were not extracted prior to RT-dPCR analysis.

Measurement uncertainty: Measurement uncertainty was estimated according to international standards [1, 2] using the standard operating procedures of the National Measurement Institute, Australia. All factors that could reasonably be expected to affect the measurement result were identified and the standard uncertainty of each was estimated from experimental data. The relative standard uncertainties of the various components were combined as described in the Guide to the Expression of Uncertainty in Measurement [1]. The combined standard uncertainty was expanded to provide a level of confidence of 95% using a coverage factor (k) calculated from the combined degrees of freedom evaluated using the Welch-Satterthwaite equation.

Factors contributing to the measurement uncertainty were batch homogeneity (between vial variance), method precision (within vial variance), stability during long term storage at -20 °C (estimated from accelerated stability study), the RT-dPCR efficiency, the volume of droplets in the RT-dPCR assay (Type B component reflecting the uncertainty of measuring a single droplet), and assay bias. The observed precision of the analytical method is reflected in the within vial variance and encompasses both the Poisson model precision and the precision of droplet volume between wells. The density of the RT-dPCR mix and the gravimetric mass measurements did not contribute significantly to the combined measurement uncertainty. Factors such as the goodness of the elliptical fit, operator bias, microscope calibration, and effect of focus were all considered in the estimation of the Type B uncertainty for droplet volume.

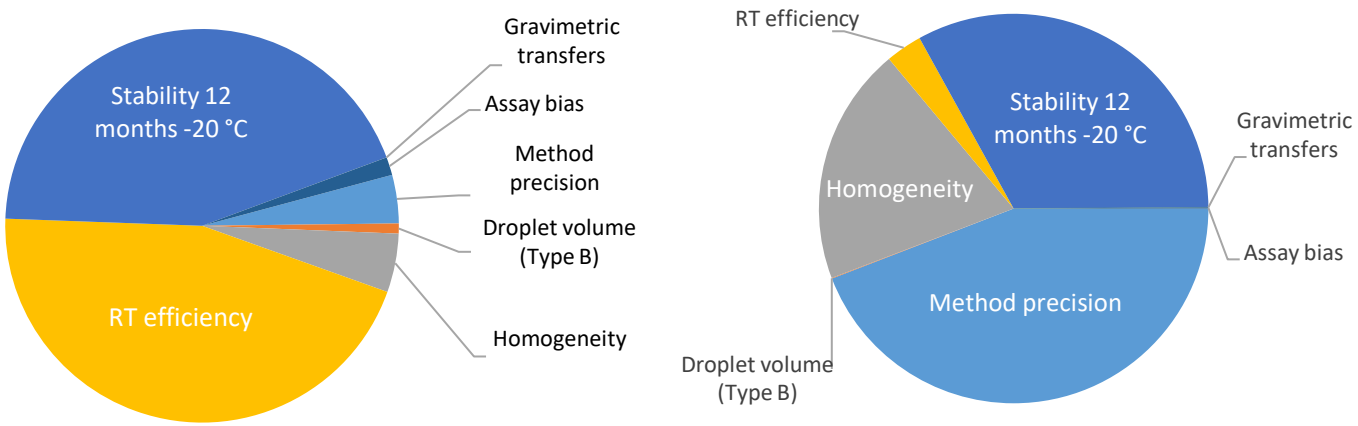

Figure 2. Relative proportions of the standard uncertainties of measuring system components for NA050 (left) and NA 055 (right) using NMIA assay as an example (China CDC 2 assay was similar).

References:

1. *Evaluation of measurement data — Guide to the expression of uncertainty in measurement*. Vol. JCGM 100:2008. 2008: Joint Committee for Guides in Metrology.
2. *Reference materials-Guidance for characterization and assessment of homogeneity and stability*, in *ISO Guide 35:2017(E)*. 2017, International Organisation for Standardisation: Switzerland.
3. Emslie, K.R., et al., *Droplet Volume Variability and Impact on Digital PCR Copy Number Concentration Measurements.* Analytical Chemistry, 2019. **91**(6): p. 4124-4131.
4. Thüring, K., et al., *Analysis of RNA modifications by liquid chromatography–tandem mass spectrometry.* Methods, 2016. **107**: p. 48-56.
5. Caly, L., et al., *Isolation and rapid sharing of the 2019 novel coronavirus (SARS-CoV-2) from the first patient diagnosed with COVID-19 in Australia.* Medical Journal of Australia, 2020. **212**(10): p. 459-462.

Appendix 1

Oligonucleotide sequences (**5'-3'**) for PCR assays of *SARS-CoV-2* CRM.

Assay	Gene	Forward primer sequence	Reverse primer sequence	Probe sequence, fluorophore and quencher	Length bp
Charite2	E	ACAGGTACGTTAATAGTTAATAGCGT	ATATTGCAGCAGTACGCACACA	HEX-ACACTAGCCATCCTTACTGCGCTTCG-BHQ	113
USCDC1	N1	GACCCCAAAATCAGCGAAAT	TCTGGTTACTGCCAGTTGAATCTG	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1	72
USCDC2	N2	TTACAAACATTGGCCGCAAA	GCGCGACATTCCGAAGAA	FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ1	67
ChinaCDC 2	N	GGGGAACTTCTCCTGCTAGAAT	CAGACATTTTGCTCTCAAGCTG	HEX-TTGCTGCTGCTTGACAGATT-BHQ1	99
NMIA	ORF1ab	TTTGCCTCAACTTGAACAGC	TTCTGCTACCAGCTCAACCA	FAM-TCGGATGCTCGAACTGCACCTC-BHQ1	94