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(Article begins on next page)

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CCQM-K181

SARS-CoV-2 RNA copy number quantification

Final Report

December 2024

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SUMMARY

Nucleic acid amplification tests for SARS-CoV-2, the virus responsible for the COVID-19 pandemic, primarily target RNA as the analyte. These tests detect the presence of SARS-CoV-2 specific RNA sequences, confirming infection through *in vitro* diagnostic methods. However, the lack of a standardized reference measurement system has led to varied units and unclear traceability in reporting RNA content quantities, complicating comparisons between different tests [1-5]. To address this challenge, a pilot study CCQM-P199b was initiated during the pandemic, to establish traceability to SARS-CoV-2 RNA quantification. Subsequently the present comparison study (CCQM-K181), coordinated by NIM, LGC, NIBSC and NIST was conducted as a follow up to CCQM-P199b. The key comparison CCQM-K181 aims to support participants in establishing calibration measurement capability (CMC) claim of SARS-CoV-2 RNA quantification.

Sixteen NMIs/DIs laboratories participated in the CCQM-K181 "SARS-CoV-2 RNA copy number quantification". Participants were requested to evaluate the copy number concentration, expressed in μL^{-1}, of the RNA molecule containing the SARS-CoV-2 open reading frame 1ab (*ORF1ab;* partial region) coding region (NC_045512.2: 13201-15600), the nucleocapsid (N) coding region (NC_045512.2: 28274-29533) and envelope (E) coding region (NC_045512.2: 26245-26472). Materials were provided at two concentration levels: high concentration Study Material 1 (S1, at a nominal concentration of 10^5 μL^{-1}) and low concentration Study Material 2 (S2, at a nominal concentration of 10^1 μL^{-1}). An additional Study Material (S0, at a nominal concentration of 10^8 μL^{-1} in aqueous solution without any RNA background) was supplied upon request to be quantified by an orthogonal method, isotope dilution mass spectrometry (IDMS). S1 and S2 were gravimetrically diluted from S0 in an aqueous buffer solution containing yeast total RNA background.

Fifteen laboratories reported results for S1 and 14 laboratories submitted results for S2. One laboratory applied 2-step RT-dPCR and the remainder of laboratories used one-step RT-dPCR. Three laboratories also performed IDMS measurement on S0, and two of them corrected their RT-PCR data with correction factors assuming an incomplete reverse transcription of the RNA molecules. Three laboratories with four independent measurements measured mass concentration in the high concentration Study Material S0 by IDMS, with two of the laboratories converting their results to copy number concentration values (three values in total). Consensus reference values and their uncertainties for the diluted S1 and S2 materials were calculated based on the IDMS results obtained for S0 and the gravimetric dilution factors applied. The KCRVs with their expanded uncertainties for S1 and S2 were determined to be $(8.27\pm0.58)\times10^5$ μL^{-1} and $(6.4\pm0.6)\times10^1$ μL^{-1}, respectively.

Successful participation in CCQM-K181 demonstrates CMC for determining RNA copy number concentration range from 10^1 μL^{-1} to 10^6 μL^{-1} of defined SARS-CoV-2 target sequences in a

non-target RNA matrix or as a single template in aqueous solution. This may include measurement capabilities such as: (1) value assignment of primary reference materials; (2) value assignment of calibration solutions; (3) measurement of RNA sequence copy number concentration using RT-dPCR.

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ACRONYMS

CCQM	Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology
CMC	Calibration and Measurement Capability
CRM	Certified Reference Material
CV	Coefficient of variation, expressed in %: $CV = 100 \cdot s/\bar{x}$
DI	Designated Institute
DNA	Deoxyribonucleic acid
DoE	Degrees of equivalence
dPCR	Digital PCR
HPLC	High performance liquid chromatography
IDMS	Isotope dilution-mass spectrometry
JCTLM	Joint Committee for Traceability in Laboratory Medicine
KC	Key Comparison
KCRV	Key Comparison Reference Value
LC	Liquid chromatography
MAD	Median absolute deviation from the median (MAD)
MADe	$1.4826 \cdot MAD$, where $MAD = median(\lvert x_i - median(x_i) \rvert)$
NA	Nucleic Acid
NMI	National metrology institute
NAWG	Working Group on Nucleic Acid Analysis
PCR	Polymerase chain reaction
qPCR	Real-time quantitative PCR
RMP	Reference Measurement Procedure
RNA	Ribonucleic acid
RT-dPCR	Reverse transcription dPCR
RT-qPCR	Reverse transcription qPCR
RV	Reference value
SEC	Size exclusion chromatography
CMP	Cytidine monophosphate
UMP	Uridine monophosphate
AMP	Adenosine monophosphate
GMP	Guanosine monophosphate
NMP	Nucleoside monophosphate
ssRNA	Single strand RNA

SYMBOLS

d_i	degree of equivalence: x_i - KCRV
$\%d_i$	percent relative degree of equivalence: $100 \cdot d_i/\text{KCRV}$
k	coverage factor: $U(x) = k \cdot u(x)$
n	number of quantity values in a series of quantity values
s	standard deviation of a series of quantity values: $s = \sqrt{\sum_{i=1}^{n} (x_i - \bar{x})^2 / (n-1)}$
t_s	Student's t-distribution expansion factor
$u(x_i)$	standard uncertainty of quantity value x_i
$\bar{u}(x)$	pooled uncertainty: $\bar{u}(x) = \sqrt{\sum_{i=1}^{n} u^2(x_i) / n}$
$U(x)$	expanded uncertainty
$U_{95}(x)$	expanded uncertainty defined such that $x \pm U_{95}(x)$ is asserted to include the true value of the quantity with an approximate 95 % level of confidence
$U_{k=2}(x)$	expanded uncertainty defined as $U_{k=2}(x) = 2 \cdot u(x)$
x	a quantity value
x_i	the i^{th} member of a series of quantity values
$\bar{x}$	mean of a series of quantity values: $\bar{x} = \sum_{i=1}^{n} x_i / n$

INTRODUCTION

Quantitative viral genome copy measurements are important in many pathogens detection where viral loads are used to guide treatment, such as HIV and Hepatitis C. The diagnostic response to the COVID-19 pandemic also highlighted the importance of quantitative measurements in informing the performance of tests. This study will provide NMIs with a route to demonstrate the core competencies to deliver measurement services of RNA copy number concentration to respond to national needs in the global response to the SARS-CoV-2 pandemic. It is proposed to apply the aims and approach of the "CCQM-P154 Absolute Quantification of DNA" study to 'absolute' quantification of RNA and will follow on CCQM-P199 and P199b studies.

The aim of CCQM-P154 was to assess the quantification of low-levels of DNA in an absolute manner without the aid of calibration using enumeration-based techniques (digital PCR and direct counting). The results reported for the low-level material by enumeration-based methods were consistent with values reported by laboratories using orthogonal methods (IDMS, UV-CE) for the approximately 100,000 times more concentrated high-level material from which the low-level material was prepared [6]. The close agreement between the mean results of the four alternative approaches tested (CV 1.8%) strongly supports the accuracy of more recently developed enumeration-based techniques.

Previous CCQM pilot studies have demonstrated NMI capabilities to perform accurate measurements of viral RNA sequence copy number concentration (HIV-1 in P199 and SARS-CoV-2 in CCQM-P199b). Reverse transcription-digital PCR (RT-dPCR) was used by most laboratories in these studies. Reported values for RNA copy number concentration were within ± 40% (CCQM-P199b). A good agreement between RT-dPCR and the orthogonal methods measurement results was observed in CCQM-P199b. This provides evidence for the overall trueness in the RT-dPCR results however, between-laboratory variability (as reflected in reproducibility metrics of ~20% CV) suggests that sources of bias such as partition volume, RT efficiency or assay performance can add uncertainty to RT-dPCR-based measurement results. In this key comparison, an orthogonal method (IDMS) was employed to assign RNA copy number concentration values to two gravimetrically diluted samples. This assignment was based on the concentration measured by IDMS in the undiluted RNA preparation.

The objective of the key comparison is to demonstrate participating laboratories' proficiency in quantifying target gene copy number concentration. It also serves to substantiate their Calibration Measurement Capability (CMC) claims when measuring purified RNA template within a mixed RNA solution containing non-target background (yeast total RNA).

The following sections of this report document the timeline of CCQM-K181, the measurands, study material, participants, results, and the measurement capability claims that participation in CCQM-K181 can support. The Appendices reproduce the official communication materials and summaries of information about the results provided by the participants.

MEASURANDS

Copy number concentration of *single stranded* RNA synthetic molecule (obtained by *in vitro* transcription) containing the SARS-CoV-2 *E, N* and partial *ORF1ab* coding regions * in a non-target RNA matrix or as a single template in aqueous solution.

Figure 1. Schematic drawing of the single-stranded RNA transcript showing the T7 promoter regions and the *Sal*I and *Not*I restriction sites used.

**Genome accession and coordinates provided in Appendix A*

STUDY MATERIALS

Background

Three Study Materials (S0, S1 and S2) were designed and prepared by NIM. All materials were synthetic ssRNA, non-infectious and required level 1 containment. RNA sequence information is provided in Appendix A. It was expected that all study participants analyze Study Material 1 and 2, whereas S0 was supplied only to laboratories which would perform orthogonal method (IDMS) analysis. Study participants were provided with four units of S1 and S2 and five additional units of S0 upon request.

Preparation of Study Materials

S0 was composed of a single *in vitro* transcribed SARS-CoV-2 RNA construct (**Appendix A**) containing the complete *E* and *N* coding regions and a segment of *ORF1ab* at an approximate concentration of 10^8 μL^{-1} in buffered solution (1 mM sodium citrate, pH 6.5 (RNA Storage Solution Thermo Fisher Scientific P/N AM7001). *In vitro* transcription was performed using MEGAscript™ T7 Transcription Kit (AM1334, Thermo Fisher Scientific, USA). RNA transcripts were purified with MEGAclear™ Kit (Thermo Fisher Scientific, USA). A total of 100 units were prepared, with each unit containing 100 µL.

S1 was prepared by gravimetric dilution of **S0** using a Mettler Toledo XP56 balance to 5 decimal places. It was diluted ~451 times at an approximate concentration of 10^5 μL^{-1} in a buffered solution (1 mM sodium citrate, pH 6.5 (RNA Storage Solution Thermo Fisher Scientific P/N AM7001) supplemented with ~5 ng μL^{-1} yeast total RNA (purchased from Sigma). A total of 200 units, each containing 50 µL, were prepared.

S2 was further prepared by gravimetric dilution of **S1** using a Mettler Toledo XP56 balance to 5 decimal places. It was further diluted ~10^5 times to obtain an approximate concentration of 10^1 μL^{-1} in a buffered solution (1 mM sodium citrate, pH 6.5 (RNA Storage Solution Thermo Fisher Scientific P/N AM7001) supplemented with ~5 ng μL^{-1} yeast total RNA (purchased from Sigma). A total of 200 units, each containing 50 µL, were prepared.

The standard uncertainty of weighing using for the Mettler Toledo XP56 balance was ± 0.002 mg (based on the calibration certificate). Further details can be found in Appendix B. Following cleaning of the balance, linearity was tested using a set of laboratory standard weights covering the range 0.1 g to 200 g.

Homogeneity Assessment of Study Materials

The homogeneity of S1 and S2 was evaluated by RT-dPCR targeting the *ORF1ab*, *E* and *N* coding regions. The detailed methods used are described in Appendix B. Twelve units for S1 and eight units for S2 were taken randomly and analysed on triplicate.

Analysis and results of homogeneity studies

One-way ANOVA with *F*-test in accordance with the requirements as stipulated in ISO Guide 35 was used to determine whether there were significant between-unit differences in copy number concentration of S1 and S2. For S1 and S2, the value of the relevant *F*-test is smaller than the *F* critical value at 0.05 confidence level, which indicates that the inhomogeneity levels of the study materials were insignificant. The respective relative u_{bb} using the different RT-PCR assays are shown in Table 1.

Table 1: Study Material homogeneity results of S1 and S2.

Study Material	**S1**			**S2**		
Coding region	***F*-value**	$F_{0.05(11,24)}$	***Relative* u_{bb}**	***F*-value**	$F_{0.05(7,16)}$	***Relative* u_{bb}**
N	1.60	2.25	1.1%	1.10	2.66	2.5%
E	1.70	2.25	1.2%	1.72	2.66	2.8%
ORF1ab	1.72	2.25	1.2%	1.55	2.66	2.3%

Stability Assessment of Study Material

Design of short-term stability studies

For this key comparison, the short-term stability (STS) was performed as for comparable RNA materials in P199b, at 4 ºC and dry ice for 0, 3, and 7 days. This indicated that the materials would be stable for at least 1 week on dry ice. The current study was designed to test the K181 study material (S1 and S2) stability under additional and extended transportation conditions at ambient temperature incubation and of up to 14 days. Samples were incubated at 4 °C and 25 °C for 3, 7 and 14 days and compared to study material stored at -80 °C. Two vials were analyzed for each incubation temperature and time point. Measurements of ORF1ab, N and E coding regions were performed by RT-dPCR in duplicate.

Results of short-term stability studies

The RT-dPCR results, corrected for the suspected PCR efficiency bias, are shown in Table 2 and Figure 2. *T* test showed that S2 cannot be stable at 25ºC for 1 week or longer (p<0.0056).

Table 2: Results of STS study

Material	Coding region	Time (days)	Significant *		(*p*-value) *		Relative change in concentration (value/value reference temperature -80 °C)	
			4 °C	**25 °C**	**4 °C**	**25 °C**	**4 °C**	**25 °C**
S1	ORF 1ab	3	NO	NO	0.42	0.16	1.31%	-4.45%
		7	NO	NO	0.11	0.02	-3.34%	-8.46%
		14	NO	NO	0.03	0.97	-5.10%	-0.09%
	E	3	NO	NO	0.33	0.11	1.86%	-5.54%
		7	NO	NO	0.08	0.12	-3.72%	-4.87%
		14	NO	NO	0.10	0.16	-4.80%	4.20%
	N	3	NO	NO	0.69	0.24	0.59%	-1.85%
		7	NO	NO	0.66	0.31	-0.66%	-2.93%
		14	NO	YES	0.04	0.00	3.32%	10.77%
S2	ORF 1ab	3	NO	YES	0.06	0.00	6.67%	13.33%
		7	NO	NO	0.03	0.02	11.11%	-11.11%
		14	NO	YES	0.13	0.00	8.89%	-26.67%
	E	3	NO	NO	0.93	0.02	0.00%	14.29%
		7	NO	NO	0.26	0.13	4.08%	-12.24%
		14	NO	NO	0.88	0.21	0.00%	-10.20%
	N	3	NO	NO	0.76	0.66	1.92%	1.92%
		7	NO	YES	0.11	0.00	-7.69%	-19.23%
		14	NO	NO	0.11	0.01	-7.69%	-23.08%

*p-values are significant if $p < 0.0056$, corresponding to Type I error $\alpha = 0.05$, with Bonferroni's correction for multiple tests ($m = 9$) per material ($p = \alpha/m$).

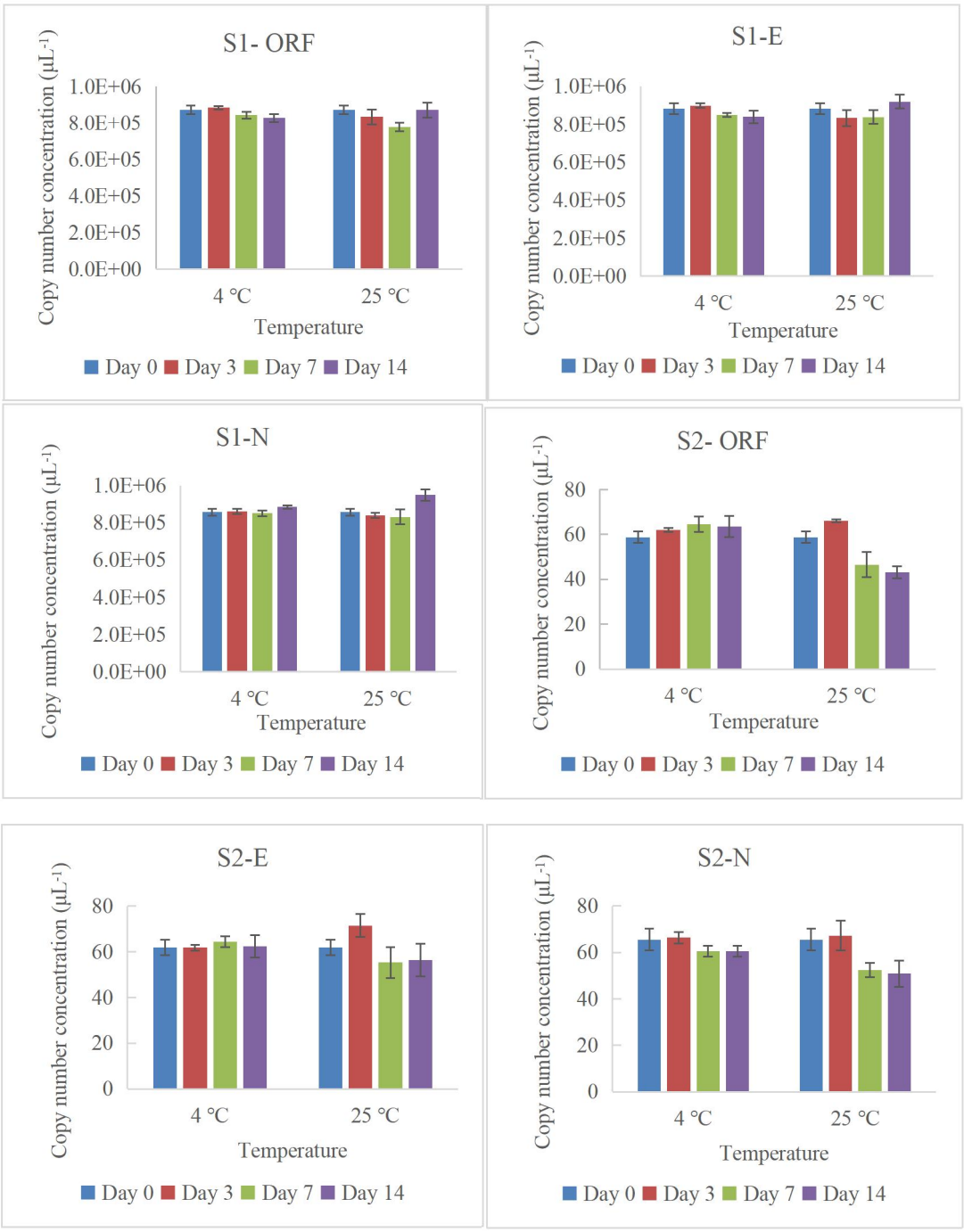


Figure 2. STS of the two unknown samples.

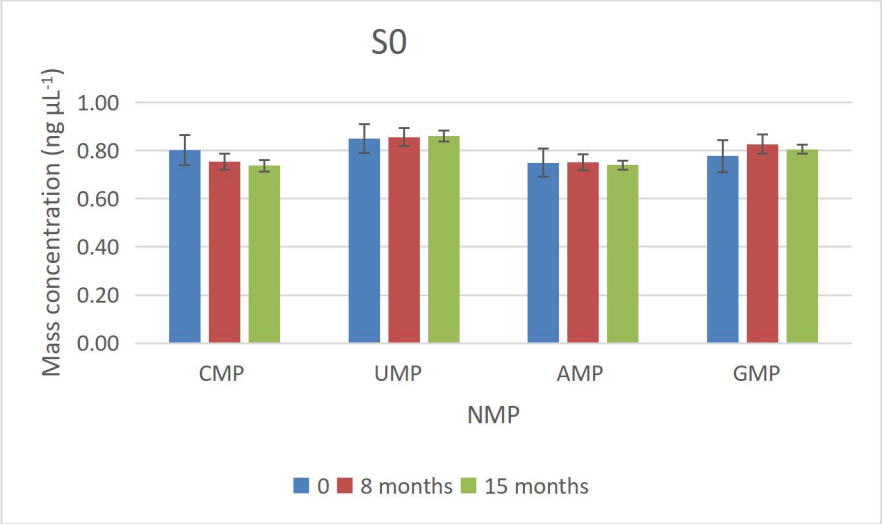
S1 and S2 were incubated at 4 °C and 25 °C for 3, 7 and 14 days, and RT-dPCR results of study materials stored at -80 °C were referred as day 0. Values of two vials with duplicates for each incubation temperature and time point were shown as mean ± SD (y-axis, copy number concentration).

Design of long-term stability studies

For long-term stability (LTS), S1 and S2 were evaluated by RT-dPCR at 0, 1, 2, 3 and 7 months post-production. Three units of S1 and two units of S2 were assessed. Triplicate measurements of each unit were performed with ORF 1ab, E and N RT-dPCR assays. S0 were evaluated by IDMS at 0, 8 and 15 months post-production. Two or three units were assessed. Each vial was tested twice by IDMS.

Results of long-term stability studies

The results of long-term stability studies are shown in Figure 3. LTS results were analyzed with linear regressions by plotting time points (x, months) and RNA concentration, which corresponding mass concentration for S0 (y, ng μL^{-1}) and corrected copy number concentration for S1 and S2 (y, μL^{-1}). The slope of the regression lines was tested for statistical significance (95% confidence level). No obvious trend was observed for any of the Study Materials.



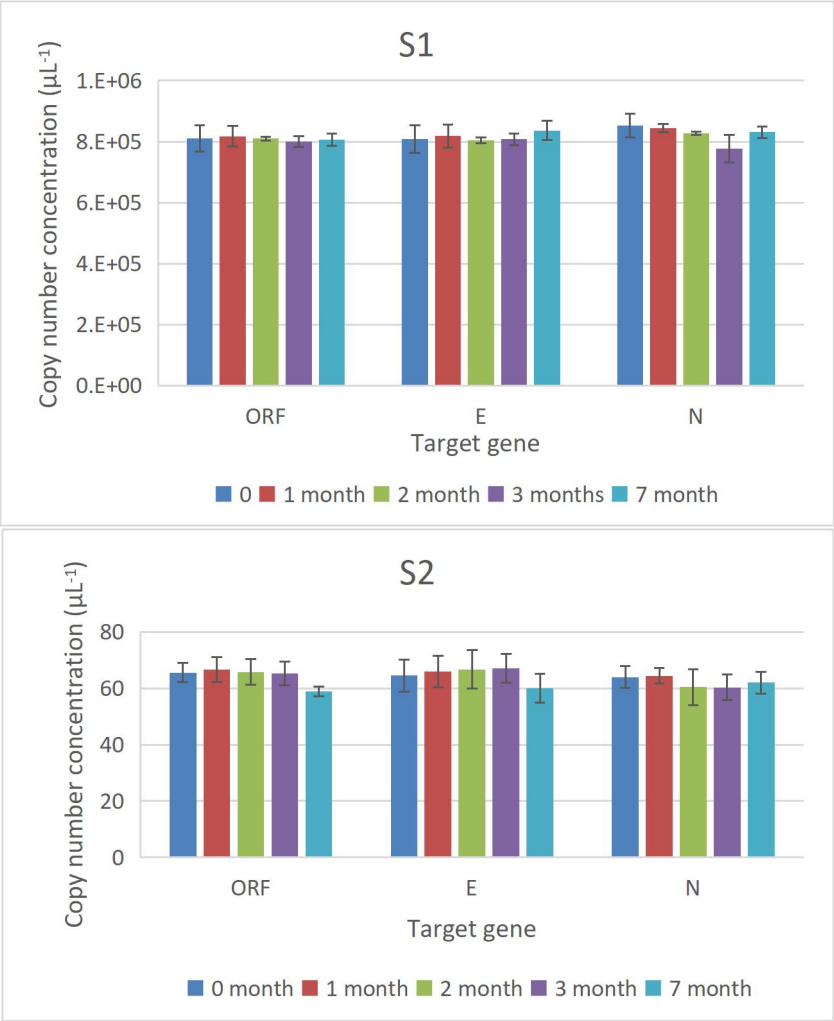


Figure 3. Results of the long-term stability assessment studies.
Concentration of S0 calculated from CMP, UMP, AMP and GMP and concentrations of S1 and S2 at different time points are shown as mean ± SD (*y*-axis, mass concentration for S0, corrected copy number concentrations for S1 and S2).

Coordinators' value assignment of Study Materials

Study Material S0

Values were assigned to **S0** based on IDMS analysis performed at NIM and NMIJ (see Results).

Study Materials S1 and S2

The coordinator's assigned values for S1, S2 are shown in Table 3. The concentration of S1 and S2 were determined by the three RT-dPCR assays of ORF 1ab, E and N coding regions. The RT-dPCR results on S1 and S2 were further corrected for each assay, according to the ratio of copy

number concentration of S0 converted from IDMS (C_{IDMS}) and copy number concentration of S0 measured by RT-dPCR ($C_{RT\text{-}dPCR}$). As example for the RT-dPCR results of S1 and S2 based on ORF 1ab, equation 1 was used:

$$RT - dPCR_{ORF1ab}\ corrected = RT - dPCR_{ORF1ab} / \frac{C_{RT-dPCR}}{C_{IDMS}} \quad (1)$$

The corrections were applied to compensate for a suspected not optimal efficiency of the reverse transcription step. The values of S1 and S2 were then calculated by the means of corrected results of three RT-dPCR assays. Contributions to the uncertainty in the assigned values are shown in Table 4.

Table 3: Coordinator's assigned values and uncertainties (S1 and S2)

	Reported value (S1)	Reported value (S2)
x /(μL^{-1})	8.1E+05	66
u /(μL^{-1})	3.1E+04	3.2
k	2	2
U /(μL^{-1})	6.3E+04	6.4
$U(x)/x$ (%)	8	10

Table 4: Uncertainty contributions to coordinator's assigned values (S1 and S2)

	S1			**S2**		
Factor	**ORF 1ab**	**E**	**N**	**ORF 1ab**	**E**	**N**
Method precision (%)	4.54	4.03	3.41	3.35	8.22	6.86
Dilution (%)	0.50	0.50	0.50	0.50	0.50	0.50
Partition volume* (%)	2.13	2.13	2.13	2.13	2.13	2.13
Correction for the RT-dPCR efficiency (%)	4.95	5.71	3.95	4.95	5.71	3.95
Relative standard uncertainty (%)	7.07	7.32	5.66	6.37	10.24	8.21
Standard uncertainty of each assay (u, μL^{-1})	5.55E+04	6.02E+04	4.62E+04	4.21	6.81	5.28
Standard uncertainty of S1 and S2** (u, μL^{-1})	3.13E+04			3.20		

*Partition volume was the average of the results of direct measurement by four laboratories in CCQM-P199b (0.76, 0.76, 0.782, and 0.793 nL), and the uncertainty of the partition volume was the RSD of the four results (CCQM-P199b study report).

**Standard uncertainty of S1 or S2 was calculated as: $u(\mathrm{S1/S2}) = \frac{1}{3}\sqrt{u^2{}_{ORF1ab} + u^2{}_E + u^2{}_N}$

PARTICIPANTS

The call for participation was distributed on 19 October 2022 with the intention to distribute samples in November and December 2022, receive results in April 2023, and discuss results at the CCQM NAWG April 2023 meeting. Appendix A reproduces the Call for Participation; Appendix C reproduces the Study Protocol.

Table 5: Institutions Registered for CCQM-K181.

Laboratory ID	NMI or DI	Code	Country	Main contact	Email	K181/P227
1	Physikalisch-Technische Bundesanstalt	PTB	Germany	Samreen Falak Esmeralda Valiente	samreen.falak@ptb.de esmeralda.valiente@ptb.de	K181
2	Inmetro – Instituto Nacional de Metrologia, Qualidade e Tecnologia (Brazil)	INMETRO	Brazil	Roberto B Flatschart	rbflatschart@inmetro.gov.br	K181
3	Korea Research Institute of Standards and Science	KRISS	Republic of Korea	Young Bae	ybae@kriss.re.kr	K181
4	National Institute of Metrology, China	NIM	P. R. China	Lianhua Dong	lianhuadong@126.com	K181
5	National Measurement Institute of Japan (NMIJ)/AIST	NMIJ	Japan	Sachie Shibayama	s-shibayama@aist.go.jp	K181
7	European Commission JRC GEEL	JRC-GEEL	EU	Philippe Corbisier	Philippe.corbisier@ec.europa.eu	K181
8	TUBITAK National Metrology Institute	TUBITAK UME	Turkiye	Sema AKYUREK	sema.akyurek@tubitak.gov.tr	K181
9	Instituto Nacional de Metrología de Colombia	INM-CO	Colombia	John Leguizamon Claudia Patricia Tere	jeleguizamon@inm.gov.co cptere@inm.gov.co	K181
10	Istituto Nazionale di Ricerca Metrologica	INRIM	Italy	Carla Divieto Jessica Petiti Mattia Pegoraro Laura Revel	c.divieto@inrim.it j.petiti@inrim.it m.pegoraro@inrim.it l.revel@inrim.it	K181
11	National Measurement Laboratory at LGC	NML_LGC	UK	Alison Devonshire	alison.devonshire@lgcgroup.com	K181
12	National Institute of Standards and Technology	NIST	USA	Megan Cleveland	megan.cleveland@nist.gov	K181
13	National Measurement Institute Australia	NMIA	Australia	Daniel Burke Leonardo Pinheiro	Daniel.Burke@measurement.gov.au Leo.Pinheiro@measurement.gov.au	K181
14	National Institute of Metrology Thailand	NIMT	Thailand	Phattarapornn Morris Sasithon Temisak	phattaraporn@nimt.or.th sasithont@nimt.or.th	K181
16	D.I. Mendelevev Institute for Metrology	VNIIM	Russia	Vonsky Maxim	m.s.vonsky@vniim.ru	K181

					a.l.runov@vniim.ru	
17	National Institute of Biology	NIB	Slovenia	Mojca Milavec	mojca.milavec@nib.si	K181
18*	Centro Nacional de Metrologia	CENAM	Mexico	Mercedes Herrera Norma González Melina Pérez-Urquiza	mherrera@cenam.mx ngonzale@cenam.mx meperez@cenam.mx	K181

*CENAM registered for participation and received Study Materials however did not submit study results due to sample arriving without dry ice and the expected results for a reference material sent along with the samples were not obtained

SAMPLE DISTRIBUTION

All Study Materials were shipped on dry ice. The majority of them were received with dry ice present and samples frozen, except for NIB and CENAM due to the unavailability of direct shipment from China. For NIB, result of S1 was nominated as it could be stable during the shipment based on the evidence of STS. CENAM did not submit result as they suspected a degradation occurred based on the CRM (GBW(E)091089) parallel shipped from NIM.

Table 6: Distribution of Study Materials for CCQM-K181.

ID	NMI/DI	Starting time	Arriving time	Duration (days)	Note
1	Physikalisch-Technische Bundesanstalt (PTB)	2022/11/16	2022/11/17	1	/
2	Inmetro – Instituto Nacional de Metrologia, Qualidade e Tecnologia (Brazil)	2022/11/15	2022/11/17	2	/
3	Korea Research Institute of Standards and Science	2022/11/16	2022/11/16	0	/
5	National Metrology Institute of Japan (NMIJ)/AIST	2022/11/15	2022/11/17	2	/
7	JRC (Joint Research Center) GEEL	2022/11/16	2022/11/17	1	/
8	TUBITAK UME	2022/12/9	2022/12/14	5	/
9	Instituto Nacional de Metrología	2022/12/5	2022/12/7	2	/
10	INRIM	2022/11/16	2022/11/21	5	/
11	National Measurement Laboratory (hosted at LGC)	2022/11/16	2022/11/17	1	/
12	NIST	2022/11/15	2022/11/17	2	/
13	National Measurement Institute, Australia	2023/2/20	2023/2/22	2	/
14	National Institute of Metrology (Thailand), NIMT	2022/11/15	2022/11/17	2	/
16	D.I. Mendeleyev Institute for Metrology (VNIIM)	2023/2/10	2023/2/17	7	/
17	National Institute of Biology (NIB)	2022/12/19	2022/12/27	8	In good condition on 2022/12/23, but no dry ice left when arrived on 2022/12/27, up to 4 days at between 4°C and 25°C
18	Centro Nacional de Metrología (CENAM)	2022/11/15	2022/11/25	10	No dry ice

TIMELINE

Table 7 shows the timeline for CCQM-K181.

Table 7: Timeline for CCQM-K181.

Date	Action
June 2022	Preparation of samples
August 2022	Homogeneity testing
Aug.-Oct. 2022	Stability testing
Oct. 2022	Invitation of participants
30 Oct. 2022	Deadline for registration
Nov. 2022	Dispatch of samples
10 March 2023	Deadline for submission of results
April 2023	Discussion of report at the CCQM NAWG
14 March 2024	Draft A Report circulated to Participants
24 September 2024	Draft B Report circulated to NAWG

RESULTS

Participants were requested to report an average value and expanded uncertainty for RNA copy number concentration result for each Materials.

In addition to the quantitative results (Form 3), participants were instructed to describe their experimental details, including analytical assay and dPCR platform used (Form 4).

CCQM-K181 results were received from 15 of the 16 institutions that received samples. CENAM did not submit results for any of the Study Materials received (no dry ice left when the samples arrived). One laboratory (NIB) received samples not frozen. According to the STS study, NIB's result for S1 was included but not the result for S2. All other 14 laboratories measured S1 and S2.Four laboratories from three institutions (NIM reported two results from two independent laboratories, NMIJ and NMIA) measured the additional Study Material S0 by IDMS and all reported copy number and mass concentration results of S0, except NMIA who reported only mass concentration result of S0. NMIA found a "impurity" on the bioanalyzer, so they did convert the mass concentration to copy number concentration as the molecule weight may not the same as assumed based on the intact molecule (Appendix A). Detailed information is provided in Appendix J.

Calibration Materials Used by Participants

For the analysis of S1 and S2, participants did not apply calibration materials as the analysis was performed by RT-dPCR that does not require a calibrator.

For the analysis of S0, laboratories performing IDMS applied calibration materials. Table 8 lists the CRMs that were used and how participants established traceability.

Table 8: Calibration Materials used for measurement of Study Material S0.

Laboratory ID	**CRM**	**Provider**	**Analyte**	**Certified value and uncertainty**[a]	**Method used to value assign CRM (basis for traceability)**
5	Calibration standards were prepared from analytical grade chemicals	NMIJ	Four nucleic bases	AMP: (1221 ± 16) nmol/g CMP: (1340 ± 26) nmol/g GMP: (1180 ± 29) nmol/g UMP: (1312 ± 15) nmol/g	^{1}H Quantitative NMR using potassium hydrogen phthalate (traceable to NMIJ CRM 3001-b as internal standard). Associated CCQM k55d
4	GBW(E) 100154 Purity of Adenosine-5′ monophosphoric acid GBW(E) 100067 Purity of Cytidine-5′monophosphoric acid GBW(E) 100068 Purity of Guanosine-5′ monophosphate disodium salt GBW(E) 100069 Purity of Uridine-5'monophosphate disodium salt	NIM	Four NMPs	AMP: 98.9%± 0.7% CMP: 99.3%± 0.6% GMP: 98.8%± 0.6% UMP: 99.4%± 0.4%	HPLC and HR-ICP-MS[HPC(B)-1,HPC(B)-2,HPC(B)-3,CCQM-P94;Refer CCQM-P20,K55b，K55d; CCQM-P20c,d]
13	Calibration standards were prepared from analytical grade chemicals	NMIA	Four NMPs	Nucleotide / nmol/g AMP 682.6 UMP 584.3 GMP 605.7 CMP 667.5	Quantitative NMR

[a] Stated as Value ± U_{95}(Value)

Methods Used by Participants

For the analysis of Study Materials 1 and 2, the majority of laboratories (14/15) used RT-droplet dPCR (Bio-Rad QX100/QX200 systems), with 14 of those laboratories using the One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad), one laboratory employing a 2-step RT-ddPCR approach (NMIJ) in their reported values.

For the analysis of Study Material S0, four laboratories reported results using IDMS. NMIJ measured mass fraction of S0 using an acidic hydrolysis followed by IDMS, in which mass fraction of four nucleic bases were measured. NIM and NMIA measured mass fraction of S0 after an enzymatic hydrolysis followed by IDMS, in which mass fraction of four NMPs (mononucleotide) were measured. Mass fraction of S0 were calculated from that of nucleic bases or NMPs. Copy number concentration of S0 were further calculated from its mass fraction by NMIJ and NMIA.

Further information on the analytical techniques and RT-dPCR methodological parameters are summarized in Appendix H. The participants' approaches to estimating uncertainty are provided in Appendix I.

Participant Results

Participant results for CCQM-K181 are detailed in Table 9 to Table 11 and presented graphically in Figures 4 to 5.

Table 9: CCQM-K181 participants' measurement results for S0.

Mass concentration					
Laboratory ID	***x*** **/(ng µL^{-1})**	***u*** **/(ng µL^{-1})**	***k***	***U*** **/(ng µL^{-1})**	***Rel U*** **(%)**
4(1)	0.81	0.03	2	0.06	7.4
4(2)	0.78	0.04	2	0.08	10.3
5	0.811	0.033	2	0.066	8.1
13	0.849	0.041	2.11	0.087	10
RNA copy number concentration					
Laboratory ID	***x*** **/(µL^{-1})**	***u*** **/(µL^{-1})**	***k***	***U*** **/(µL^{-1})**	***Rel U*** **(%)**
4(1)	3.86E+08	1.43E+07	2	2.86E+07	7.4
4(2)	3.75E+08	1.94E+07	2	3.88E+07	10.3
5	3.87E+08	0.15E+08	2	0.31E+08	8.10

Key: *x*, value; *u*, standard uncertainty; *k*, coverage factor; *U*, expanded uncertainty; *Rel U*, relative expanded uncertainty (%).

Table 10: CCQM-K181 participants' measurement results for S1.

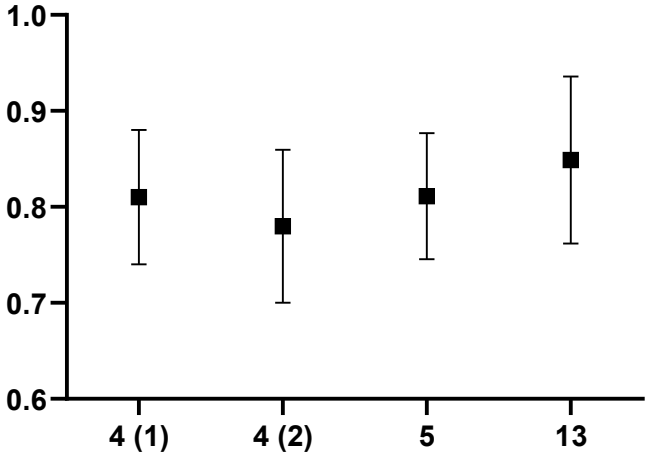
Lab ID	***x*** **/(μL^{-1})**	***u*** **/(μL^{-1})**	***k***	***U*** **/(μL^{-1})**	***Rel U*** **(%)**
1	570987.0	28149.4	2	57571.9	10.1
2	716E+03	40.2E+03	2.31	92.6E+03	12.9
3	6.4E+05	5.6E+04	2.11	1.2E+05	18.5
4	8.1E+05	3.1E+04	2	6.3E+04	8
5	8.5E+05	1.1E+05	2	2.2E+05	26.0
7	7.1E+05	0.42E+05	2	0.9E+05	12.0
8	694933	38136	2	76271	11
9	502558	45127	2	90255	18.0
10	806122	81299	2	162597	20.17
11	710000	46519	2.36	110000	15.5
12	624900	36900	2	73800	11.8
13	8.5E+05	7.6E+04	2.00	1.5E+05	18
14	5.60E+05	5.10E+04	2	1.00E+05	18.2
16	675395	48085	2	96171	14
17	553451	19573	2	39147	7.07

Key: *x*, value; *u*, standard uncertainty; *k*, coverage factor; *U*, expanded uncertainty; *Rel U*, relative expanded uncertainty (%).

Table 11: CCQM-K181 participants' measurement results for S2.

Lab ID	***x*** **/(µL^{-1})**	***u*** **/(µL^{-1})**	***k***	***U*** **/(µL^{-1})**	***Rel U*** **(%)**
1	46.9	4.3	2.2	9.4	20.0
2	53	7	1.96	13	24.3
3	51.5	4.2	2.12	9.0	17.5
4	66	3.2	2	6.4	10
5	75	24	2	47	63.1
7	50	1.9	2	4	7.6
8	56	4	2	7	13
9	46.87	7.54	2	15.28	32.6
10	53.3	3.65	2	7.3	13.71
11	50	3.38	2.36	8	16.0
12	53.82	4.02	2	8.04	14.9
13	66	7.5	1.99	15	23
14	40.3	4.5	2	9	22.4
16	44.1	3.26	2	6.5	15

Key: *x*, value; *u*, standard uncertainty; *k*, coverage factor; *U*, expanded uncertainty; *Rel U*, relative expanded uncertainty (%).


1.0
0.9
0.8
0.7
0.6
4 (1)
4 (2)
5
13

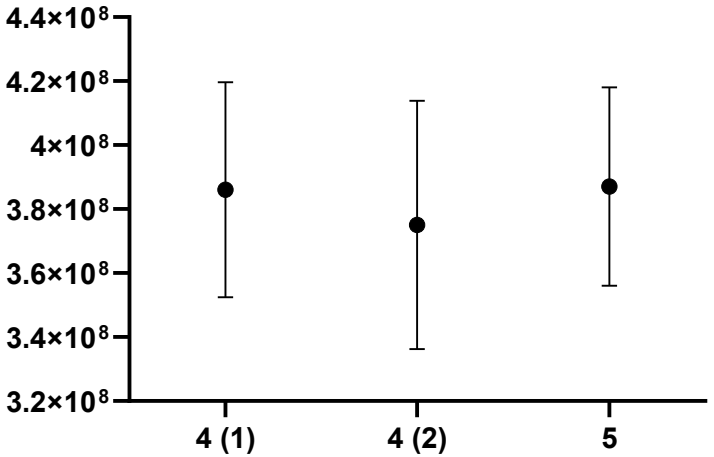
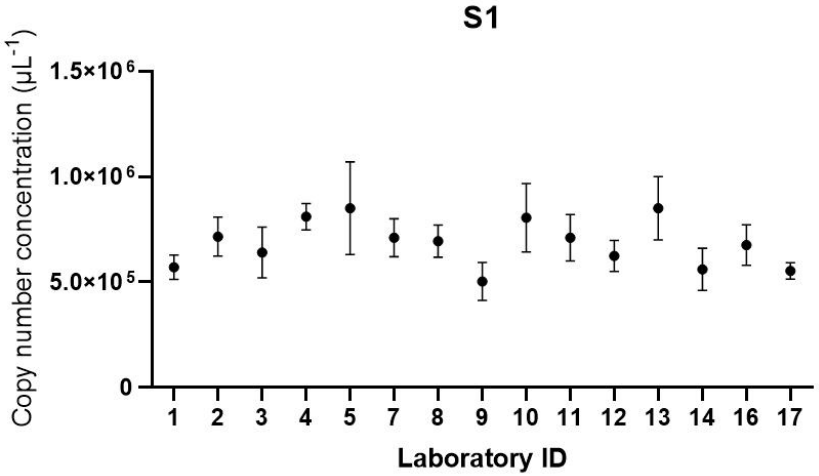


Figure 4. CCQM-K181 measurement results for S0.
Dots represent the reported values, x; bars their 95 % expanded uncertainties, $U(x)$.


S1
Copy number concentration (µL-1)
1.5×10^6
1.0×10^6
5.0×10^5
0
1 2 3 4 5 7 8 9 10 11 12 13 14 16 17
Laboratory ID

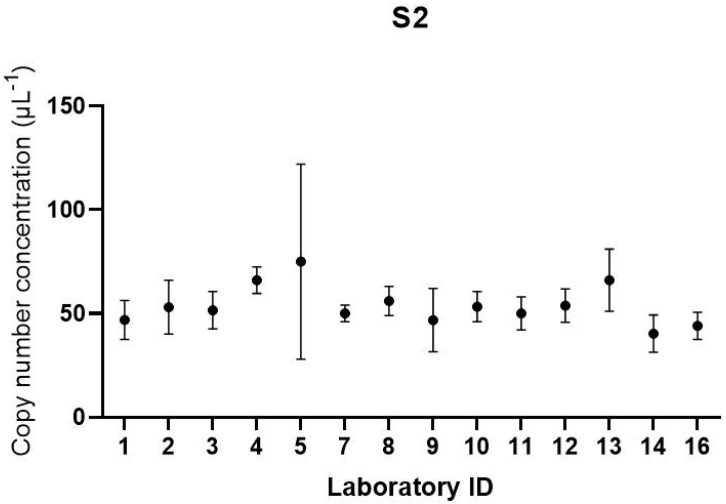

S2
Copy number concentration (µL-1)
150
100
50
0
1 2 3 4 5 7 8 9 10 11 12 13 14 16
Laboratory ID

Figure 5. CCQM-K181 participants' measurement results for S1 and S2.
Dots represent the reported values, x; bars their 95 % expanded uncertainties, $U(x)$.

Interlaboratory reproducibility and consistency

Table 122 provides a summary of the reproducibility of the study results according to Study Materials. For S0, three results of copy number concentration are included. For S1 and S2, all results of K181 are concluded. For S1 and S2, the reproducibility reflected by % CV were 16% and 18%, respectively. The reproducibility of IDMS results for the measurement of S0 was good, with an interlaboratory % CV of 1.7%.

Table 12: Summary of descriptive statistics for nominated results (K181).

Study Material	Median /(μL^{-1})	MADe/ (μL^{-1})	Relative MADe	Normal distrib. (Y/N) **	Mean /(μL^{-1})	SD/ (μL^{-1})	%CV
S0	*n* too small			N/A	3.83E+08	6.66E+06	1.7%
S1	6.95E+05	1.65E+05	24%	Y	6.85E+05	1.11E+05	16%
S2	52.3	6.8	13%	Y	53.8	9.4	18%

Median, MADe, Mean and SD describe RNA copy number concentration (μL^{-1}). MADe and SD are given to 3 s.f. for S0 and S1, with median or mean to the same order of magnitude. For S2, MADe and SD are given to 2 s.f. and median or mean to the same d.p. Relative variation (%MADe or %CV) are given to 2 s.f. for all Materials/Measurands. **Normality testing shows the result of Shapiro-Wilk test (alpha = 0.05). N/A: normality not assessed for subsets of Study Material S0 data as number of laboratories too small (n = 3).

KEY STUDY CONSENSUS REFERENCE VALUE (KCRV)

Purity evaluation and reference value calculation of Study Material S0

The consensus value for Study Material S0 was estimated following the CCQM guidance note CCQM13-22 and the weighted mean selected as the appropriate estimate (Table 13), due to the high level of consistency between IDMS results of Laboratories 4(1), 4(2) and 5 (Table 9; Figure 6). The uncertainty in the weighted mean was also more conservative (larger) than the standard error in the mean, if the latter were chosen as the consensus value (Table 13).

Table 13: Calculation of consensus value and uncertainty of S0.

Laboratory ID	***X*/ (µL^{-1})**	**w(i) * x(i)**	***u* / (µL^{-1})**	***1/u^2***	**weighting (w)**
4(1)	3.86E+08	1.57E+08	1.43E+07	4.89E-15	41%
4(2)	3.75E+08	8.31E+07	1.94E+07	2.66E-15	22%
5	3.87E+08	1.43E+08	1.50E+07	4.44E-15	37%
Weighted mean / (µL^{-1})	3.84E+08				

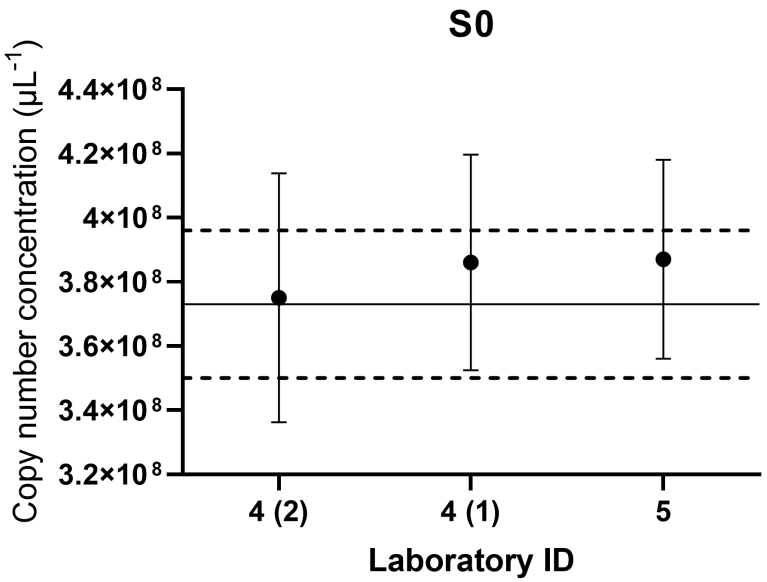


Figure 6. Study Material S0 consensus values, reported results and uncertainties.
Solid line shows the recommended consensus value and dotted line represents its expanded uncertainty (k=2).

The purity of S0 was checked by BioAnalyzer in triplicates and the size of the major peak was consistent with the theoretical size (Figure 7). There were two smaller peaks which could be considered as impurities with different lengths. The average percentage of the major peak was for 98.36% and their standard deviation was 0.31 % based on automatic analysis using the Agilent Bioanalyzer software. An in-house method was established to calculate the purity content as 97.14% (impurity 2.86%) (Appendix L), which was applied to calculate final concentration of S0.

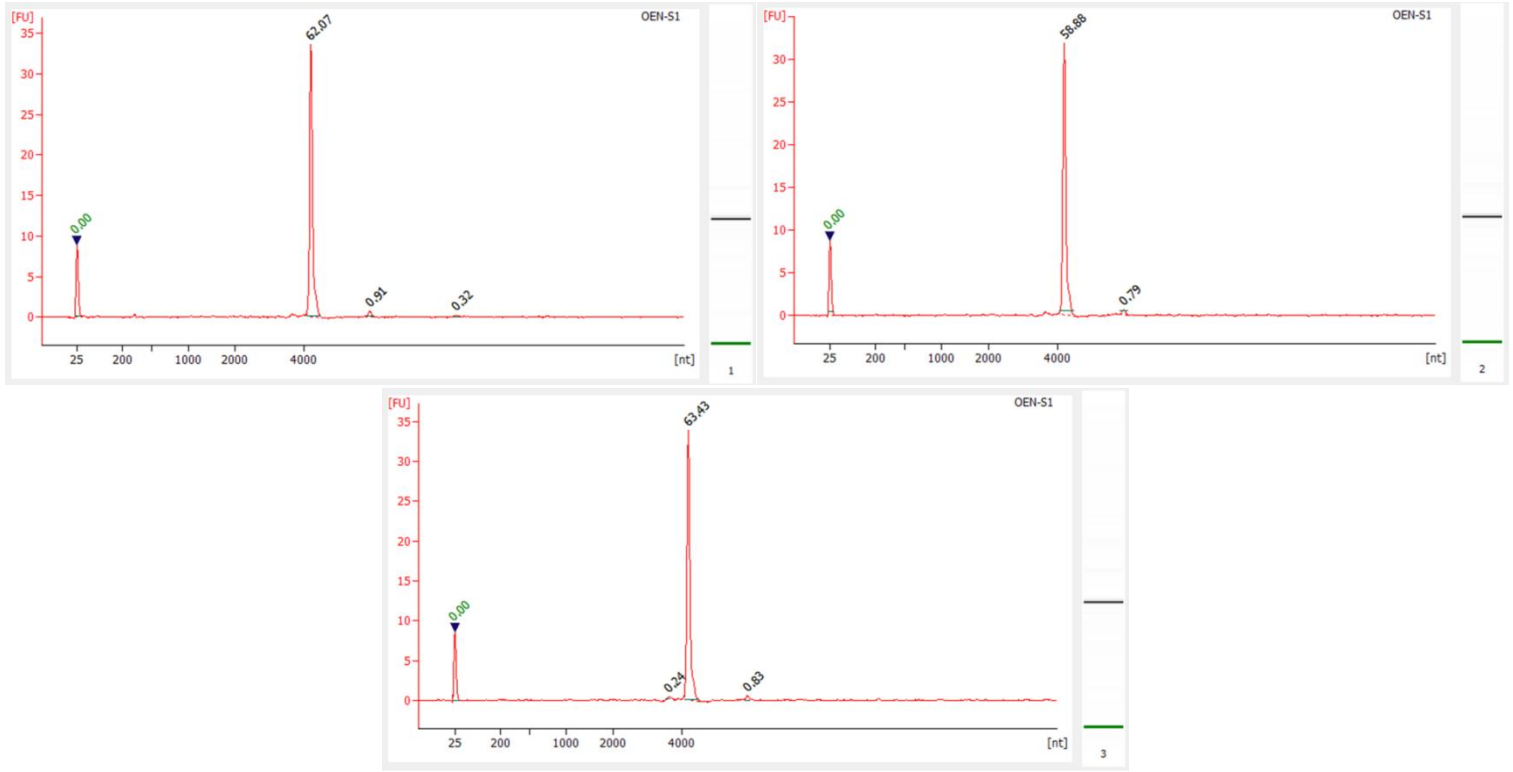


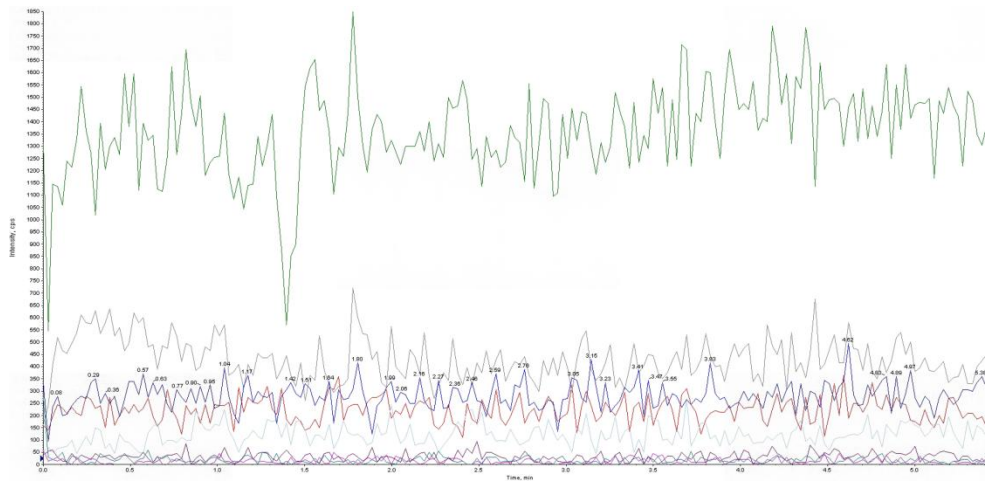
Figure 7. In vitro transcribed RNA assessed with the 2100 Bioanalyzer RNA 6000 Pico kit.

It confirmed the presence of ~4000 nt RNA fragment by three replicates.

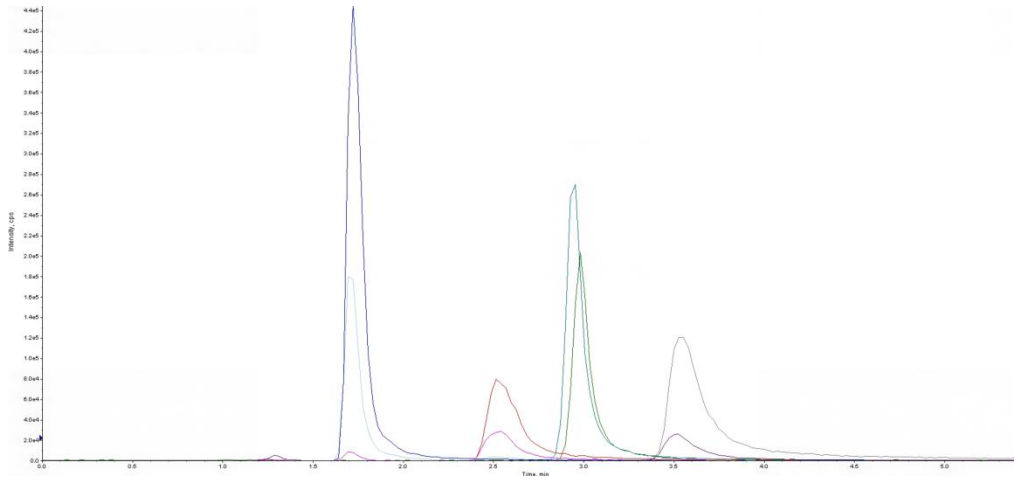
Free NMPs as a source of impurity in S0 was checked by mass spectrometry at NIM using undigested material. The result shows that no peak for NMPs was observed indicating no free NMPs in the S0 material (Figure 8). Additionally, the free NMPs as a source of impurity was also analyzed by size exclusion chromatography (SEC) at NMIJ. The chromatogram and UV spectrum of S0 measured by SEC are shown in Figure 9. In the S0, two peaks were detected, the main peak (RT=1.9 min) as target RNA and a small peak (RT=4.2 min) as an impurity. For the identification of impurity, the UV spectrums of the impurity and NMP monomer mix were

obtained and compared with each other. Regarding the analysis of impurity, there was one peak in the UV spectrum, and its maximum absorption wavelength was 214.6 nm. However, regarding the analysis of NMP monomer, there were two peaks in the UV spectrum, and their maximum absorption wavelength were 254.8 nm, respectively. Since the UV spectrums and the maximum absorption wavelength were inconsistent with each other, it was concluded that the impurity in the S0 was neither NMP monomers nor a similar structural compound related to nucleobases and therefore should not affect the IDMS result.

(a)



(b)



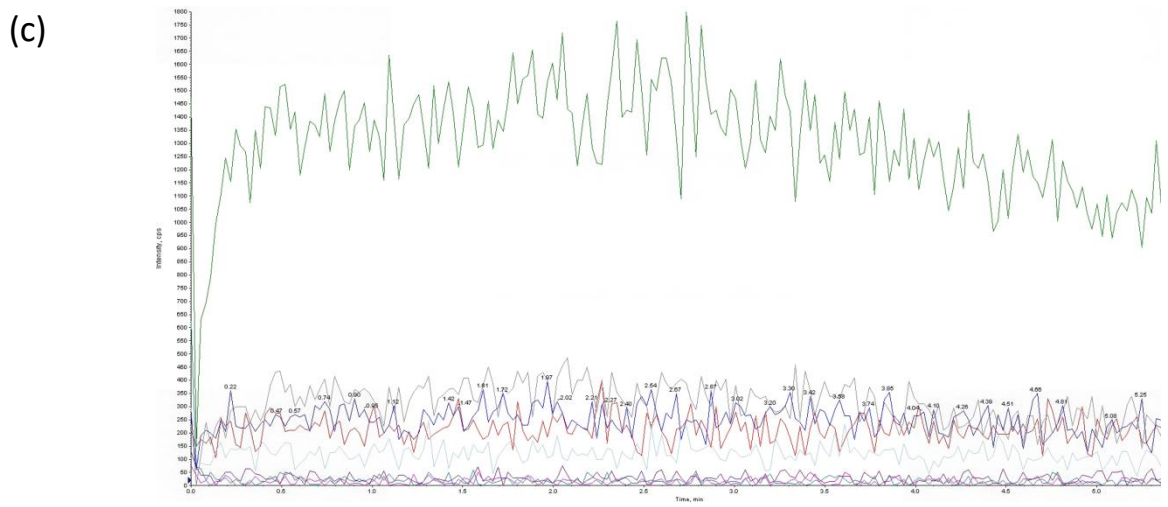
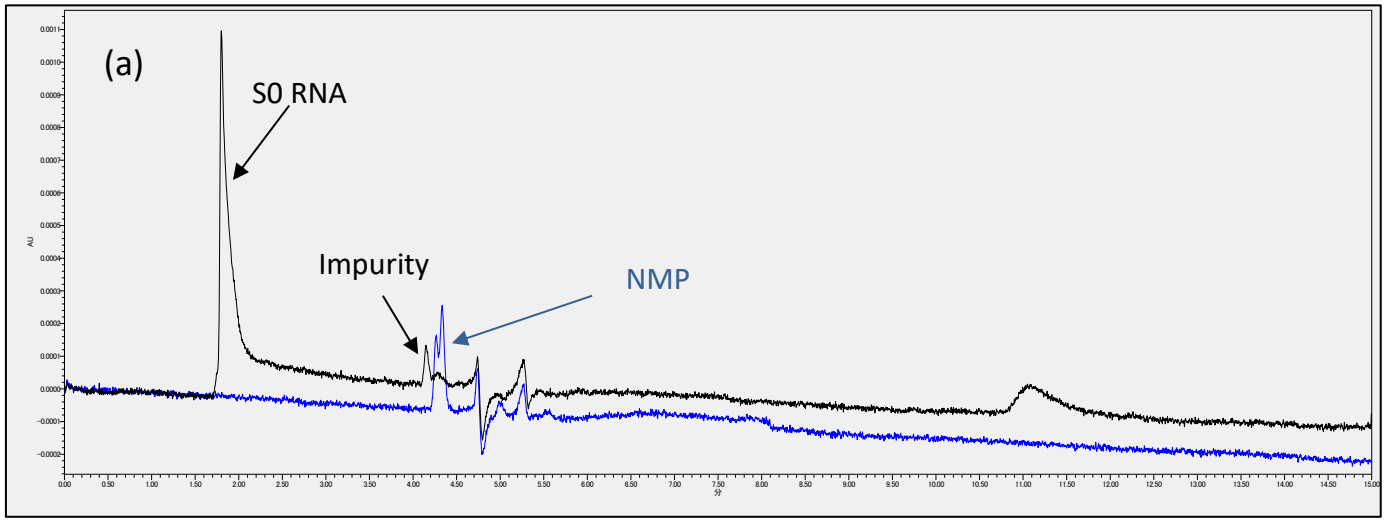


Figure 8. The total ion chromatogram (TIC) of no template control (a), NMP monomer mixture (b) and undigested S0 (c) measured by mass spectrometer (AB6500).



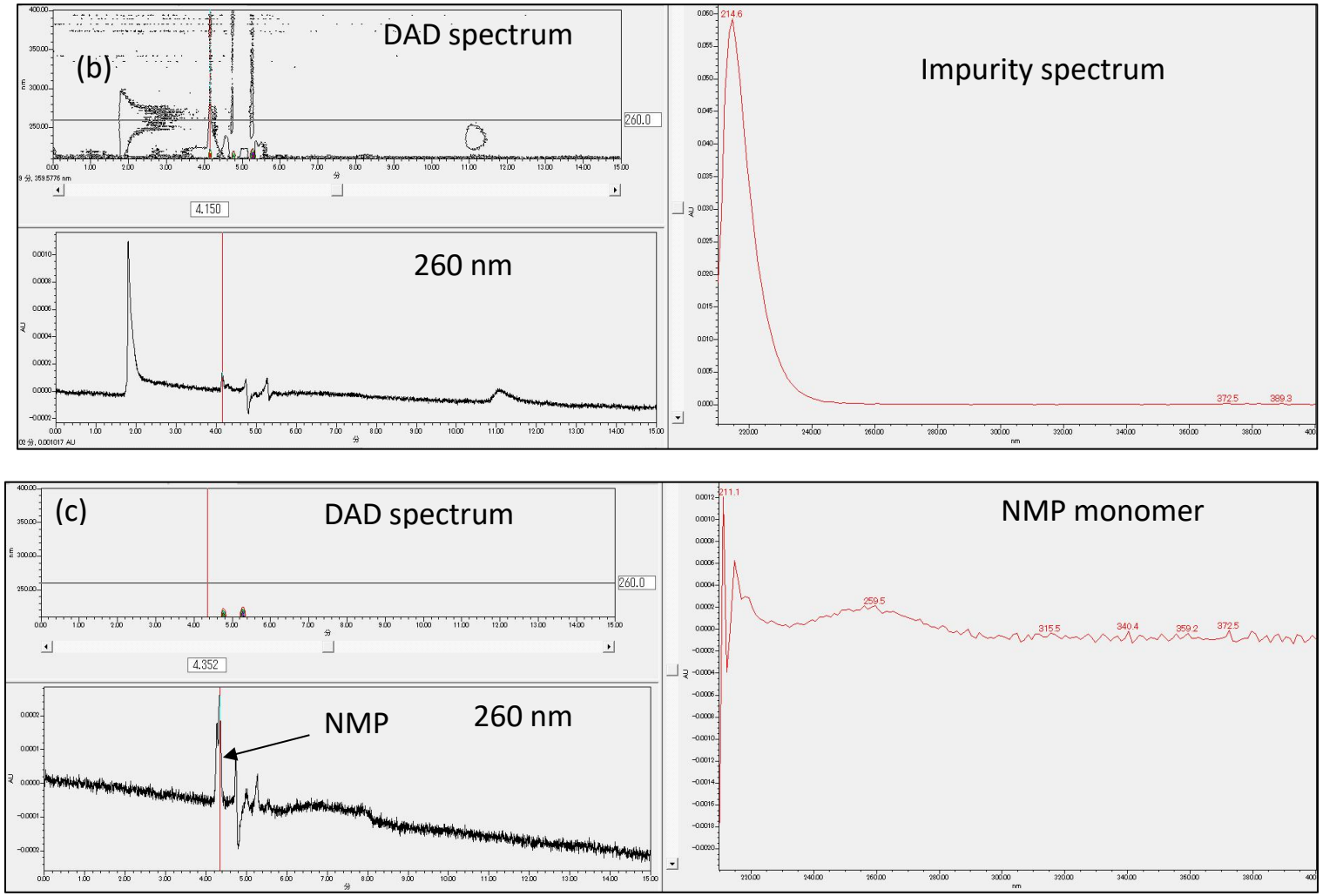


Figure 9. The chromatogram and UV spectrum of S0 and NMP monomer mix measured by SEC.

(a) The chromatogram of S0 and NMP monomer mix. The black line shows S0 analysis, and the blue line shows NMP monomer mix analysis. (b) The UV spectrum of S0 impurity. The maximum absorption wavelength was 214.6 nm. (c) The UV spectrum of NMP monomer mix.

The reference value for S0 was calculated by subtracting the maximum calculated value of the observed impurities (2.86%) from weighted mean. The reference value and uncertainty of S0 is shown in Table 14. Uncertainty in the S0 reference value was calculated by combining the uncertainty of weighted mean, the measurement of impurities and an allowance for the limit of blank (LOB) of the Bioanalyzer (possibility of multiple impurities below the limit of detection (LOD)).

Table 14: Reference value of Study Material S0

Study Material	**Estimator**	**Value/** (μL^{-1})	***u*** **/**(μL^{-1}) **(*rel. u*)**	**Degrees of freedom**	***k***	***U/*** (μL^{-1}) **(*rel. U*)**
S0	Weighted mean	3.73E+08	1.16E+07 (3.11%)	∞	2	2.32E+07 (6.22%)

Table 15: Uncertainty contributions to Study Material S0 reference value

Factor	**Uncertainty**	**Degrees of freedom**
Relative standard uncertainty of weighted mean $u_{\overline{x_u}}$(%)	2.45%	∞
Repeatability of measurement of observed impurity u_P(%)	0.92%	∞
LOB*	1.68%	∞
Relative standard uncertainty u_{S0}(%)	3.11%	∞
Standard uncertainty/ (μL^{-1})	1.16E+07	

* The specifications of the Agilent RNA Pico kit give a LOD of 50 pg/µL which is based on a "Signal-to-noise >3 (single peak)" [7] . Therefore the LOB is calculated to be 16.7 pg/mL. The possible scenario of the occurrence of 100 impurities across the measured size range equates to a concentration 1.67 ng μL^{-1}. The percentage contribution of the theoretical impurities compared to the measured concentration of the sample was calculated for the three replicate measurements (Figure 8) and an average of 1.68% calculated.

KCRV calculation of Study Materials S1 and S2

The KCRV of Study Material S1 was calculated by dividing reference value for Study Material S0 by the gravimetrical dilution factor applied to the preparation of Study Material S1. The Standard uncertainty of S1 was combined by the uncertainty of S0, stability of Study Material S1 (based on the STS study) and uncertainty from balance used in the preparation of the material. The KCRV of Study Material S2 was calculated by dividing KCRV of Study Material S1 by the gravimetrical dilution factor applied in the preparation of Study Material S2. The Standard uncertainty of S2 was based on the combining the uncertainty of Study Material S1, the stability of Study Material S2 (based on the STS study) and uncertainty derived from the balance used in the preparation of the material. Uncertainty contributions to the KCRV of Study Materials S1 and S2 are listed in Table 16 to Table 18.

Table 16 KCRV for Study Materials S1 and S2

Study Material	**Estimator**	**Value /(μL^{-1})**	***u* /(μL^{-1}) (*rel. u*)**	**Degrees of freedom**	***k***	***U*/ (μL^{-1}) (*rel. U*)**
S1	Calculated from Study Material S0	8.27E+05	0.29E+05 (3.51%)	∞	2	0.58E+05 (7.02%)
S2	Calculated from Study Material S1	64	3 (4.49%)	∞	2	6 (9.38%)

Table 17: Uncertainty contributions to KCRV of S1

Factor	**Uncertainty**	**Degrees of freedom**
Relative standard uncertainty of S0 u_{S0} (%)	3.11%	∞
Dilution factor u_D (%)*	0.71%	∞
STS (%)	1.32%	∞
Relative standard uncertainty u_{S1} (%)	3.45%	∞
Standard uncertainty/ (μL^{-1})	2.85E+04	

*Two weighing steps were performed for the dilution process, u_D (%) $=\sqrt{2 \times u^2_B}$, where uncertainty of balance u(B) = 0.50%

Table 18: Uncertainty contributions to KCRV of S2

Factor	**Uncertainty**	**Degrees of freedom**
Relative standard uncertainty of S1 u_{S1} (%)	3.45%	∞
Dilution factor u_D (%)*	1.50%	∞
STS (%)	2.38%	∞
Relative standard uncertainty u_{S2} (%)	4.46%	∞
Standard uncertainty /(μL^{-1})	2.83	

* As nine times of weighing were performed for the dilution process, u_D (%) $=\sqrt{9 \times u^2_B}$, where uncertainty of balance u(B) = 0.50%

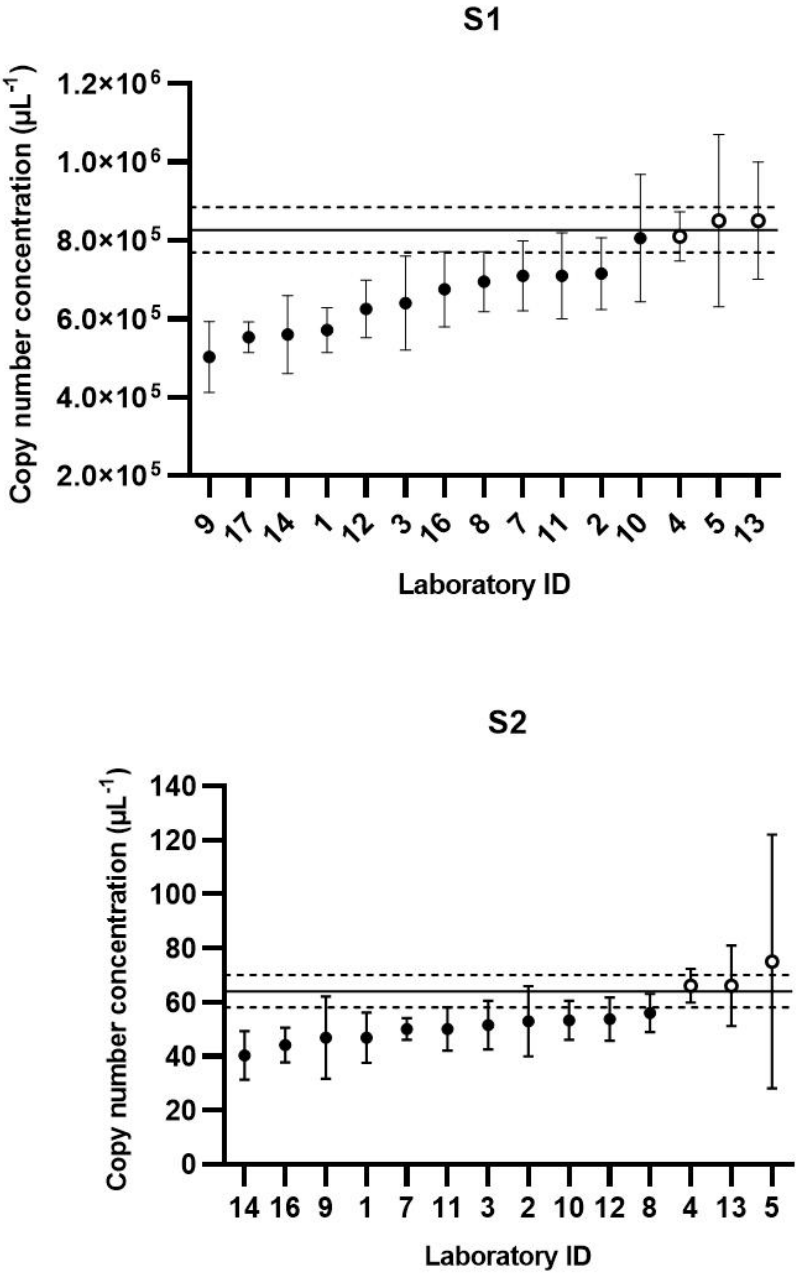


Figure 10.Study Material 1 and 2 consensus values, reported results and uncertainties. Solid line shows the recommended consensus value and dotted line represents its expanded uncertainty. Participants results are displayed as values (circles) and expanded uncertainty (k=2). Open circles represent RT-dPCR corrected for the suspected bias observed between RT-dPCR and IDMS results.

DEGREES OF EQUIVALENCE (DoE)

Degree of equivalence with respect to KCRV

The equivalence statements are calculated following the CCQM guidance note [8], which specifies that the degree of equivalence between an NMI result x_i and the x_{KCRV} is expressed as the difference D_i calculated using equation 2:

$$D_i = x_i - x_{KCRV} \tag{2}$$

The uncertainty associated with the difference was expressed in the form of an expanded uncertainty. The uncertainty of the degree of equivalence has been calculated using equation 3:

$$u^2(D_i) = u^2(x_{KCRV}) + u^2(x_i) \tag{3}$$

The degrees of equivalence calculated as above are shown in Table 19 and Table 20 and illustrated graphically in Figure 11 and Figure 12.

Table 19. Degrees of equivalence (DoE) for Study Material S1 with respect to KCRV

ID	Lab	Di / (µL^{-1})*	$u(Di)$ / (µL^{-1})	$U(Di)$ / (µL^{-1}), k=2
9	INM-CO	-3.24E+05	5.36E+04	1.07E+05
17	NIB	-2.74E+05	3.50E+04	7.00E+04
14	NIMT	-2.67E+05	5.87E+04	1.17E+05
1	PTB	-2.56E+05	4.04E+04	8.08E+04
12	NIST	-2.02E+05	4.69E+04	9.39E+04
3	KRISS	-1.87E+05	6.31E+04	1.26E+05
16	VNIIM	-1.52E+05	5.62E+04	1.12E+05
8	TUBITAK UME	-1.32E+05	4.79E+04	9.58E+04
7	JRC GEEL	-1.17E+05	5.10E+04	1.02E+05
11	NML_LGC	-1.17E+05	5.48E+04	1.10E+05
2	INMETRO	-1.11E+05	4.96E+04	9.91E+04
10	INRIM	-2.09E+04	8.63E+04	1.73E+05

4	NIM	-1.70E+04	4.24E+04	8.49E+04
5	NMIJ	2.30E+04	1.14E+05	2.28E+05
13	NMIA	2.30E+04	8.13E+04	1.63E+05

*Laboratories in red: $|Di| > U(Di)$

Table 20. Degrees of equivalence (DoE) for Study Material S2 with respect to KCRV

ID	Lab	Di / (μL^{-1})*	$u(Di)$ / (μL^{-1})	$U(Di)$ / (μL^{-1}), k=2
14	NIMT	-23.7	5.41	10.82
16	VNIIM	-19.9	4.43	8.86
9	INM-CO	-17.1	8.11	16.23
1	PTB	-17.1	5.24	10.49
7	JRC GEEL	-14.0	3.55	7.10
11	NML_LGC	-14.0	4.52	9.04
3	KRISS	-12.5	5.16	10.32
2	INMETRO	-11.0	7.62	15.23
10	INRIM	-10.7	4.72	9.45
12	NIST	-10.2	5.02	10.03
8	TUBITAK UME	-8.0	5.00	10.00
4	NIM	2.0	4.39	8.77
13	NMIA	2.0	8.08	16.16
5	NMIJ	11.0	24.19	48.37

*Laboratories in red: $|Di| > U(Di)$

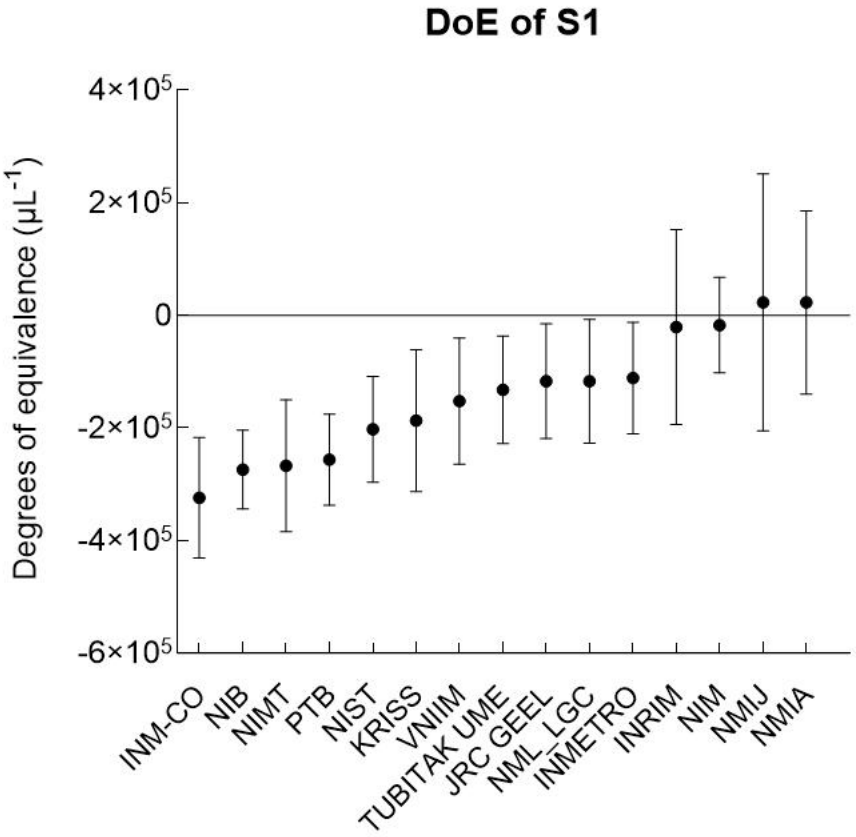


Figure 11. Degree of equivalence with respect to KCRV for Study Material S1 (k=2)

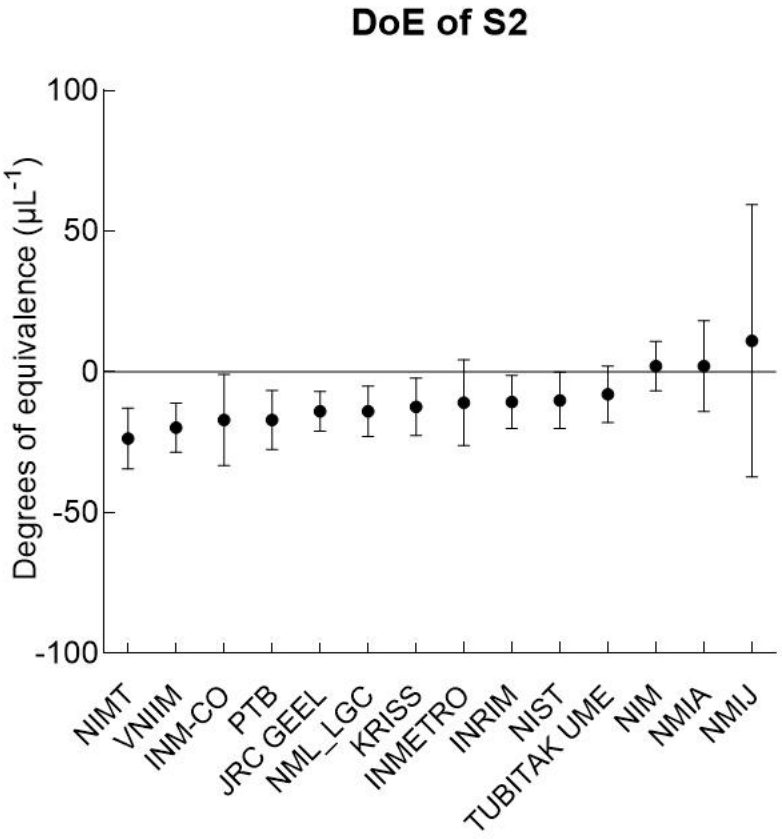


Figure 12. Degree of equivalence with respect to KCRV for Study Material S2 (k=2)

USE OF CCQM-K181 IN SUPPORT OF CALIBRATION AND MEASUREMENT CAPABILITY (CMC) CLAIMS

How Far the Light Shines

Based on degrees of equivalence, results of CCQM-K181 demonstrate a lack of comparability between the IDMS-based reference values and the majority of RT-dPCR results for Study Materials S1 and S2. These results indicate a potential negative bias associated with RT-dPCR, which may be due to RT efficiency (<100% conversion of RNA to cDNA) or other factors such as overestimation of dPCR partition volume or inefficiency affecting PCR amplification ("molecular dropout"). In contrast, RT-dPCR results using methods which had been optimised or corrected based on IDMS analysis showed agreement with the KCRVs.

Sources of molecular impurities which do not contain the target sequences measured by the RT-dPCR assay or are too short to be amplified would lead to a disrepancy between the RT-dPCR and IDMS values, therefore analysis of potential impurities (NMPs detectable by MS or SEC, or

larger RNA molecules detectable using capillary gel electrophoresis) was performed and included in the KCRV uncertainty, however in the future additional techniques such as sequencing may be applied to further characterise study material purity to a higher degree of certainty.

Due to the high reproducibility evidenced in this and previous (CCQM-P199b) studies, and SI-traceability to mass and purity of NMP standards, the IDMS-based reference values are considered to be reliable, therefore this KC study reflects the state-of-the-art in evaluation of trueness of RT-dPCR and provides a benchmark in assessing RT-dPCR performance and measurement uncertainty. The implication of these findings is that additional measurement uncertainties will be required by some laboratories claiming CMCs in the case that their reported result is not consistent with the KCRV. As assay efficiency is known to be sequence-specific, it is more conservative to restrict CMC claims for CCQM-K181 to the SARS-CoV-2 sequences measured in this study.

Therefore, participation in CCQM-K181 demonstrates calibration and measurement capability for determining RNA copy number concentration of SARS-CoV-2 sequence-containing molecules in the range from 10^1 μL^{-1} to 10^6 μL^{-1} in a non-target RNA matrix or as a single template in aqueous solution. CMC claims show evidence of measurement capabilities such as: (1) value assignment of primary reference material; (2) value assignment of calibration solutions; (3) measurement of RNA sequence copy number concentration using RT-dPCR.

Core Competency Statements and CMC support

Table 21 shows prototype CMCs which may be claimed by laboratories participating in CCQM-K181.

Table 21: CCQM-K181-specific CMC claims

Measurement service	Organic solutions
Measurement service sub-category	3.4
Matrix	Aqueous solution
Measurand	Analyte or Component: SARS-Co-V2 (NC_045512.2) specified RNA sequence Quantity: RNA copy number concentration Matrix: Aqueous solution
Dissemination range of measurement capability	From 10^1 – 10^6 Unit: μL^{-1}
Comments for publication	Labs should specify region/genomic coordinates measured

	e.g SARS-Co-V2 *N* gene (NC_045512.2: 28274-29533) SARS-Co-V2 *E* gene (NC_045512.2: 26245-26472) SARS-Co-V2 partial *Orf1ab* gene (NC_045512.2: 13201-15600)
Supporting Evidence	Successfully participated in CCQM-K181

Table 22 and Table 23 show the minimum expanded uncertainties ($U_{x(i)}$') which should be claimed by laboratories where the degree of equivalence with the KCRV was larger than the uncertainty in the degree of equivalence. This is calculated based on equation 4 and 5, which calculation of minimum uncertainty for laboratory results not in agreement with the KCRV

$$U_{x(i)}{}' = D_i \tag{4}$$

$$Relative\ U_{x(i)}{}' = \frac{U_{x(i)}{}'}{x_i} \tag{5}$$

Table 22: Minimum CMC uncertainties (Study Material S1)

ID	Lab	Expanded uncertainty / (μL^{-1})	Relative expanded uncertainty (%)
9	INM-CO	3.24E+05	65%
17	NIB	2.74E+05	49%
14	NIMT	2.67E+05	48%
1	PTB	2.56E+05	45%
12	NIST	2.02E+05	32%
3	KRISS	1.87E+05	29%
16	VNIIM	1.52E+05	22%
8	TUBITAK UME	1.32E+05	19%
7	JRC GEEL	1.17E+05	16%
11	NML_LGC	1.17E+05	16%
2	INMETRO	1.11E+05	16%
10	INRIM	As reported	As reported

4	NIM	As reported	As reported
5	NMIJ	As reported	As reported
13	NMIA	As reported	As reported

Table 23: Minimum CMC uncertainties (Study Material S2)

ID	Lab	Expanded uncertainty /(μL^{-1})	Relative expanded uncertainty (%)
14	NIMT	23.7	59%
16	VNIIM	19.9	45%
9	INM-CO	17.1	37%
1	PTB	17.1	36%
7	JRC GEEL	14.0	28%
11	NML_LGC	14.0	28%
3	KRISS	12.5	24%
2	INMETRO	As reported	As reported
10	INRIM	10.7	20%
12	NIST	10.2	19%
8	TUBITAK UME	As reported	As reported
4	NIM	As reported	As reported
13	NMIA	As reported	As reported
5	NMIJ	As reported	As reported

CONCLUSIONS

In CCQM-K181, IDMS of digested RNA was applied to provide a benchmark of RT-dPCR performance and, in keeping with its application in CCQM-P199b, showed a high degree of reproducibility and interlaboratory consistency. Similar to previous study CCQM-P199, assurance of the purity of *in vitro* transcribed RNA materials was highlighted as being critical to the comparison between techniques and use of IDMS value-assigned materials for RT-dPCR calibration.

CCQM-K181 RT-dPCR results ranged from 61% to 103% of the KCRV for Study Material S1 and from 63% to 117% of the KCRV for Study Material. This level of agreement (within 2-fold) shows unprecedented accuracy for "absolute quantification" measurements of RNA copy number concentration, including those of low concentration (<100 μL^{-1}). The majority of participants (73% Study Material 1 and 64% Study Material 2) showed a negative bias with respect to the KCRV which may be due to methodological factors such as reverse transcription and dPCR partition volume. Degrees of equivalence indicate that effective measurement uncertainties in the absence of calibration range from 16% to 65% (both materials) which is fit-for-purpose in performing value assignment of purified SARS-CoV-2 sequence-containing materials, in a diagnostic testing setting where performance is defined by order of magnitude-level quantities of viral RNA.

ACKNOWLEDGEMENTS

The study coordinators thank the participating laboratories for providing the requested information used in this study.

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7. Agilent Datasheet# 5991-7891EN Agilent RNA Kits for the Agilent 2100 Bioanalyz er System
8. CCQM Guidance note: Estimation of a consensus KCRV and associated Degree of

Equivalence.Version 10, Date: 2013-04-12.

APPENDIX A: Sequence information

Study Material 1

The sequence of the study material *in vitro* transcribed RNA construct contains three gene targets of SARS-CoV-2:

- NC_045512.2: 26245-26472 (*E*) corresponding to construct position 38-265
- NC_045512.2: 28274-29533 (*N*) corresponding to construct position 272-1531
- NC_045512.2: 13201-15600 (*ORF1ab*) corresponding to construct position 1538-3937

Underlined sequences correspond to the transcription initiation site of T7 RNA polymerase.

Italic sequences correspond to sequences of the vector.

Red sequences correspond to sites of restriction enzymes.

RNA nucleotide bases (U) are shown as T for the purpose of primer design/alignment.

```
1       GGGCGAATTG GGTACCGGGC CCCCCCTCGA GGTCGACATG TACTCATTCG TTTCGGAAGA
61      GACAGGTACG TTAATAGTTA ATAGCGTACT TCTTTTTCTT GCTTTCGTGG TATTCTTGCT
121     AGTTACACTA GCCATCCTTA CTGCGCTTCG ATTGTGTGCG TACTGCTGCA ATATTGTTAA
181     CGTGAGTCTT GTAAAACCTT CTTTTTACGT TTACTCTCGT GTTAAAAATC TGAATTCTTC
241     TAGAGTTCCT GATCTTCTGG TCTAAATCGA TATGTCTGAT AATGGACCCC AAAATCAGCG
301     AAATGCACCC CGCATTACGT TTGGTGGACC CTCAGATTCA ACTGGCAGTA ACCAGAATGG
361     AGAACGCAGT GGGGCGCGAT CAAAACAACG TCGGCCCCAA GGTTTACCCA ATAATACTGC
421     GTCTTGGTTC ACCGCTCTCA CTCAACATGG CAAGGAAGAC CTTAAATTCC CTCGAGGACA
481     AGGCGTTCCA ATTAACACCA ATAGCAGTCC AGATGACCAA ATTGGCTACT ACCGAAGAGC
541     TACCAGACGA ATTCGTGGTG GTGACGGTAA AATGAAAGAT CTCAGTCCAA GATGGTATTT
601     CTACTACCTA GGAACTGGGC CAGAAGCTGG ACTTCCCTAT GGTGCTAACA AAGACGGCAT
661     CATATGGGTT GCAACTGAGG GAGCCTTGAA TACACCAAAA GATCACATTG GCACCCGCAA
721     TCCTGCTAAC AATGCTGCAA TCGTGCTACA ACTTCCTCAA GGAACAACAT TGCCAAAAGG
781     CTTCTACGCA GAAGGGAGCA GAGGCGGCAG TCAAGCCTCT TCTCGTTCCT CATCACGTAG
841     TCGCAACAGT TCAAGAAATT CAACTCCAGG CAGCAGTAGG GGAACTTCTC CTGCTAGAAT
901     GGCTGGCAAT GGCGGTGATG CTGCTCTTGC TTTGCTGCTG CTTGACAGAT TGAACCAGCT
961     TGAGAGCAAA ATGTCTGGTA AAGGCCAACA ACAACAAGGC CAAACTGTCA CTAAGAAATC
1021    TGCTGCTGAG GCTTCTAAGA AGCCTCGGCA AAAACGTACT GCCACTAAAG CATACAATGT
1081    AACACAAGCT TTCGGCAGAC GTGGTCCAGA ACAAACCCAA GGAAATTTTG GGGACCAGGA
1141    ACTAATCAGA CAAGGAACTG ATTACAAACA TTGGCCGCAA ATTGCACAAT TTGCCCCCAG
1201    CGCTTCAGCG TTCTTCGGAA TGTCGCGCAT TGGCATGGAA GTCACACCTT CGGGAACGTG
1261    GTTGACCTAC ACAGGTGCCA TCAAATTGGA TGACAAAGAT CCAAATTTCA AAGATCAAGT
1321    CATTTTGCTG AATAAGCATA TTGACGCATA CAAAACATTC CCACCAACAG AGCCTAAAAA
1381    GGACAAAAAG AAGAAGGCTG ATGAAACTCA AGCCTTACCG CAGAGACAGA AGAAACAGCA
1441    AACTGTGACT CTTCTTCCTG CTGCAGATTT GGATGATTTC TCCAAACAAT TGCAACAATC
1501    CATGAGCAGT GCTGACTCAA CTCAGGCCTA AGGATCCGGA AGCCAATATG GATCAAGAAT
1561    CCTTTGGTGG TGCATCGTGT TGTCTGTACT GCCGTTGCCA CATAGATCAT CCAAATCCTA
1621    AAGGATTTTG TGACTTAAAA GGTAAGTATG TACAAATACC TACAACTTGT GCTAATGACC
1681    CTGTGGGTTT TACACTTAAA AACACAGTCT GTACCGTCTG CGGTATGTGG AAAGGTTATG
1741    GCTGTAGTTG TGATCAACTC CGCGAACCCA TGCTTCAGTC AGCTGATGCA CAATCGTTTT
1801    TAAACGGGTT TGCGGTGTAA GTGCAGCCCG TCTTACACCG TGCGGCACAG GCACTAGTAC
1861    TGATGTCGTA TACAGGGCTT TTGACATCTA CAATGATAAA GTAGCTGGTT TTGCTAAATT
1921    CCTAAAAACT AATTGTTGTC GCTTCCAAGA AAAGGACGAA GATGACAATT TAATTGATTC
1981    TTACTTTGTA GTTAAGAGAC ACACTTTCTC TAACTACCAA CATGAAGAAA CAATTTATAA
2041    TTTACTTAAG GATTGTCCAG CTGTTGCTAA ACATGACTTC TTTAAGTTTA GAATAGACGG
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2101    TGACATGGTA CCACATATAT CACGTCAACG TCTTACTAAA TACACAATGG CAGACCTCGT
2161    CTATGCTTTA AGGCATTTTG ATGAAGGTAA TTGTGACACA TTAAAAGAAA TACTTGTCAC
2221    ATACAATTGT TGTGATGATG ATTATTTCAA TAAAAAGGAC TGGTATGATT TTGTAGAAAA
2281    CCCAGATATA TTACGCGTAT ACGCCAACTT AGGTGAACGT GTACGCCAAG CTTTGTTAAA
2341    AACAGTACAA TTCTGTGATG CCATGCGAAA TGCTGGTATT GTTGGTGTAC TGACATTAGA
2401    TAATCAAGAT CTCAATGGTA ACTGGTATGA TTTCGGTGAT TTCATACAAA CCACGCCAGG
2461    TAGTGGAGTT CCTGTTGTAG ATTCTTATTA TTCATTGTTA ATGCCTATAT TAACCTTGAC
2521    CAGGGCTTTA ACTGCAGAGT CACATGTTGA CACTGACTTA ACAAAGCCTT ACATTAAGTG
2581    GGATTTGTTA AAATATGACT TCACGGAAGA GAGGTTAAAA CTCTTTGACC GTTATTTTAA
2641    ATATTGGGAT CAGACATACC ACCCAAATTG TGTTAACTGT TTGGATGACA GATGCATTCT
2701    GCATTGTGCA AACTTTAATG TTTTATTCTC TACAGTGTTC CCACCTACAA GTTTTGGACC
2761    ACTAGTGAGA AAAATATTTG TTGATGGTGT TCCATTTGTA GTTTCAACTG GATACCACTT
2821    CAGAGAGCTA GGTGTTGTAC ATAATCAGGA TGTAAACTTA CATAGCTCTA GACTTAGTTT
2881    TAAGGAATTA CTTGTGTATG CTGCTGACCC TGCTATGCAC GCTGCTTCTG GTAATCTATT
2941    ACTAGATAAA CGCACTACGT GCTTTTCAGT AGCTGCACTT ACTAACAATG TTGCTTTTCA
3001    AACTGTCAAA CCCGGTAATT TTAACAAAGA CTTCTATGAC TTTGCTGTGT CTAAGGGTTT
3061    CTTTAAGGAA GGAAGTTCTG TTGAATTAAA ACACTTCTTC TTTGCTCAGG ATGGTAATGC
3121    TGCTATCAGC GATTATGACT ACTATCGTTA TAATCTACCA ACAATGTGTG ATATCAGACA
3181    ACTACTATTT GTAGTTGAAG TTGTTGATAA GTACTTTGAT TGTTACGATG GTGGCTGTAT
3241    TAATGCTAAC CAAGTCATCG TCAACAACCT AGACAAATCA GCTGGTTTTC CATTTAATAA
3301    ATGGGGTAAG GCTAGACTTT ATTATGATTC AATGAGTTAT GAGGATCAAG ATGCACTTTT
3361    CGCATATACA AAACGTAATG TCATCCCTAC TATAACTCAA ATGAATCTTA AGTATGCCAT
3421    TAGTGCAAAG AATAGAGCTC GCACCGTAGC TGGTGTCTCT ATCTGTAGTA CTATGACCAA
3481    TAGACAGTTT CATCAAAAAT TATTGAAATC AATAGCCGCC ACTAGAGGAG CTACTGTAGT
3541    AATTGGAACA AGCAAATTCT ATGGTGGTTG GCACAACATG TTAAAAACTG TTTATAGTGA
3601    TGTAGAAAAC CCTCACCTTA TGGGTTGGGA TTATCCTAAA TGTGATAGAG CCATGCCTAA
3661    CATGCTTAGA ATTATGGCCT CACTTGTTCT TGCTCGCAAA CATACAACGT GTTGTAGCTT
3721    GTCACACCGT TTCTATAGAT TAGCTAATGA GTGTGCTCAA GTATTGAGTG AAATGGTCAT
3781    GTGTGGCGGT TCACTATATG TTAAACCAGG TGGAACCTCA TCAGGAGATG CCACAACTGC
3841    TTATGCTAAT AGTGTTTTTA ACATTTGTCA AGCTGTCACG GCCAATGTTA ATGCACTTTT
3901   ATCTACTGAT GGTAACAAAA TTGCCGATAA GTATGTCGC
```

APPENDIX B: Coordinating laboratory methodology

Coordinating laboratory methodology NIM

Construct design

Sequences containing *E* gene (NC_045512.2:26245-26472) and *N* gene (NC_045512.2:28274-29533) of SARS-CoV-2 were synthesized by BGI (Beijing, China) to generate *in vitro* transcribed RNA molecules. These sequences was cloned into a pBluescript II SK(+) vector.

In vitro transcription of RNA

Four microgram of the SARS-CoV-2 ORF1ab, E and N gene plasmids were linearised with 15 U/µL *Bam*HI (1010S), 10X K buffer (1010S, both Takara) and nuclease-free water in a final reaction volume of 100 µL for 3 hour at 30 °C. The digest was seperated by gel electrophoresis and the corresponding bands were purified using the Universal DNA Purification Kit (DP214,TIANGEN BIOTECH (BEIJING) CO., LTD) with elution into 30 µL elution buffer. DNA concentration was estimated using Nanodrop.

To generate positive sense strand RNA *in vitro* transcription (IVT) was performed using the MEGAscript T7 kit (AM1334, Thermofisher). Two replicate reactions were included, each containing 7.5 mM of each of ATP, CTP, GTP and UTP, 1X Reaction Buffer, 2 µL T7 enzyme mix and 8 µL (approximately 0.2~1.1 µg) of plasmid. Incubation was performed at 37 °C for 4 hours followed by TURBO DNase treatment. The resulting RNA was purified using the MEGAclear™ Kit (AM 1908, ThermoFisher). RNA transcripts were eluted in 100 µL RNase-free water. An aliquot of RNA was diluted 10-fold in The RNA Storage Solution (Ambion) and the nucleic acid concentration estimated using Nanodrop. Successful *in vitro* transcription was confirmed by analysing the 1000-fold dilution with the 2100 Bioanalyzer RNA 6000 Pico kit (Agilent) (**Figure B-1**). Transcripts were expected to be 3939 nt in length. Total molecular weight (MW, g/mol) of the single stranded RNA transcript was estimated by multiplying the number of each nucleotide present (A, C, G, U) by the respective MW. Mass per RNA molecule (g) was calculated using the Avogadro number (6.022×10^{23} mol^{-1}). Copy number concentration in the stock RNA solution was calculated using Nanodrop results and the mass per RNA molecule in g. Diluted RNA solution were prepared at approximately 1E+10 μL^{-1} in RNA Storage Solution and stored at -80 °C along with the neat RNA stock.

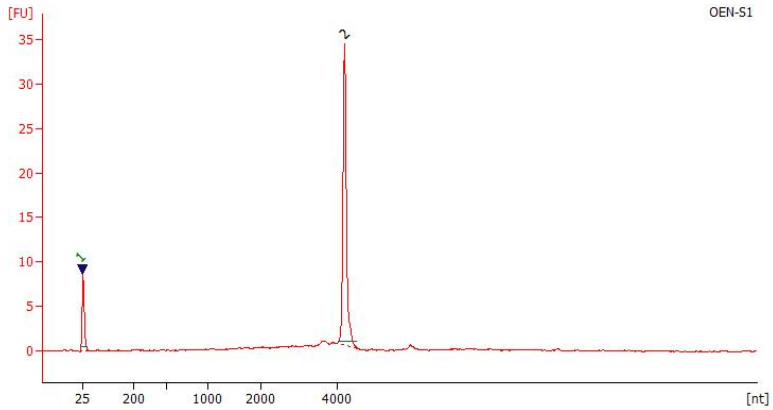


Figure B-1. In vitro transcribed RNA assessed with the 2100 Bioanalyzer RNA 6000 Pico kit (Agilent), confirming the presence of ~4000 nt RNA fragment

IDMS

S0 was digested to nucleotide mono-phosphates (NMPs) before IDMS analysis. The digestion mixture was gravimetrically prepared by adding 50 µL of RNA, 5 µL of LNMP mix solution and 1 µL of SVP (0.00023 U/µL). The mixture was incubated at 37 ℃ for 15 min and centrifuged at 13000 rpm for 2 min for subsequent IDMS analysis.

For the chromatographic separation of NMPs, high performance liquid chromatography (HPLC) system of Agilent 1200 series was used with an SB-AQ C_{18}, 3.5 µm particles, 2.1 mm × 100 mm analytical column (Agilent). The mobile phase was 0.1% formic acid (*v*/*v*) in a flow rate of 200 µL/min maintained at 30 ℃. The four NMPs were eluted after isocratic elution of 5.5 min. Sample aliquots of 3 µL were injected. Each sample was injected and analyzed for three replicates.

For quantification, signal detection was performed using SCIEX API 5500 QTrap MS/MS system in positive ion and multiple reaction monitoring (MRM) modes. The ionspray voltage was 5500 volts and source gas temperature was 600 ℃.

The final mass fraction of RNA concentration was calculated using Equation B1:

$$W_{\mathrm{RNA}} = \frac{W_{\mathrm{x}} \times M_{\mathrm{RNA}}}{M_{\mathrm{NMP}} \times N} \tag{B1}$$

Where W_{RNA} is the mass fraction of the RNA sample µg, W_{x} is the mass fraction of the selected NMP in the RNA sample, M_{RNA} is the molecular mass of the RNA molecule (1264317.80 was used), M_{NMP} is the molecular mass of the selected NMP and *N is* the number of the selected NMP in the RNA sample (812 for CMP, 1136 for UMP, 816 for GMP, 1175 for AMP).

The copy number of RNA (*n,* in copies/µL) was converted from mass fraction of RNA (W_{RNA}, in µg/g) using Equation B2:

$$n = \frac{W_{\mathrm{RNA}} \times D \times N_A}{M_{\mathrm{RNA}} \times 10^9} \tag{B2}$$

Where N_A is Avogadro's constant, and D is the density of the sample. A density of 1.00 g/mL was taken into account in the calculation.

RT-dPCR: Oligonucleotide sequences

Table B-1: Oligonucleotide sequences

Assay designation	**Genbank accession**	**Gene locus**	**Name**	**5′ to 3′**	Final (uM)
E	NC_045512.2	26269-26387	E-F1	ACAGGTACGTTAATAGTTAATAGCGT	0.6
			E-R2	ATATTGCAGCAGTACGCACACA	0.6
			E-P1	FAM-ACACTAGCCATCCTTACTGCGCTTCGBBQ	0.2
Orf1ab (duplex with E)	NC_045512.2	13342-13460	Orf-F1	CCCTGTGGGTTTTACACTTAA	0.6
			Orf-R2	ACGATTGTGCATCAGCTGA	0.6
			Orf-P1	5'-FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1-3'	0.05
US CDC N2	NC_045512.2	29164-29230	F	TTACAAACATTGGCCGCAAA	0.9
			R	GCGCGACATTCCGAAGAA	0.9
			P	5'-FAM-ACAATTTGCCCCCAGCGCTTCAGBHQ1-3'	0.25

RT-dPCR methodology

One-step RT-dPCR experiments were performed using the One-Step RT-ddPCR Advanced Kit for Probes (Cat no. 1864022, Bio-Rad). Reactions were prepared in a total volume of 22 µL containing 1X Supermix, 20 U/µL reverse transcriptase, 15 mM DTT, 4 µL of RNA template, and primers and probes at a concentration of 600 nM and 50 nM for E gene, 600 nM and 200nM for N gene, respectively. The probe was labelled with 5’ FAM and 3’ BHQ1. dPCR was performed using the QX200™ Droplet Digital™ PCR System (BioRad). 20 µL was pipetted into the sample well of a DG8 cartridge, and droplets generated as previously described. Thermocycling conditions were as follows: Reverse transcription at 45°C for 10 minutes, 5 minutes at 95 °C, 40 cycles of 95 °C for 15 s, and 58 °C for 30 s, followed by 98°C for 10 min and a 4 °C hold. The ramp rate for each step was 2°C/s. Droplets were read using the QX200 Droplet Reader, and the data were analyzed using QuantaSoft version 1.7.4.0917. No Template

Controls (NTCs) of nuclease-free water were employed as controls, and in all cases returned a negative result. A partition volume of 0.85 nL was used to calculate copy number concentration for preparation of the study materials. Data from dPCR experiments were subject to threshold and baseline setting in QuantaSoft software (Bio-Rad), and were exported as .csv files to be analysed in Microsoft Excel 2010.

S1 and S2 preparation

S1 was prepared by gravimetric dilution of S0 using a Mettler Toledo XP56 balance to 5 decimal places. It was diluted in a buffered solution (1 mM sodium citrate, pH 6.5 (RNA Storage Solution Thermo Fisher Scientific P/N AM7001) supplemented with ~5 ng/L yeast total RNA (purchased from Sigma). S2 was further prepared by gravimetric dilution of S1 using a Mettler Toledo XP56 balance to 5 decimal places. It was further diluted ~10^5 times in the same buffer (Table 2 and Table 3).

Table B-2: Preparation of S1

Sample	Mass of diluent /(mg)	Mass of sample /(mg)	Gravimetric DF
D1	29894.47	66.42	451.0824

Table B-3: Preparation of S2

Sample	Mass of tube /(mg)	Diluent vol. added/ (µL)	Mass of tube + diluent /(mg)	Mass of diluent /(mg)	Sample vol. added/ (µL)	Mass of tube + diluent + sample /(mg)	Mass of sample /(mg)	Volumetric DF	Gravimetric DF
D1	969.67	360	1328.73	359.06	20	1348.08	19.35	20	19.56
D2	965.47	360	1324.7	359.23	20	1344.05	19.35	20	19.56
D3	6664.31	9704.43	16359.42	9695.11	295.57	16652.99	293.57	33.83	34.02
total gravimetric DF			13018.31						

Homogeneity study

Homogeneity of Study Materials S1 and S2 was assessed by one-step RT-dPCR using the US CDC N2 assay (Table B-1) and a duplex assay to *Orf1ab*/*E*. Twelve units for S1 and eight units

for S2 were taken randomly, and analysis of triplicate sub-samples was carried out using RT-dPCR method.

The homogeneity of S0 was evaluated by IDMS.Ten units were taken randomly and measured at different days. Each unit was measured twice by IDMS and mass concentration of S0 was determined based on AMP, UMP, CMP an GMP.

Short-term stability study

Short-term stability (STS) of Study Materials S1 and S2 was assessed following incubation on dry ice, at 4°C and at 25°C for 3 ,7 and 14 days in comparison to a reference temperature of -80°C. Two units of each material were included per condition. Stability was assessed by one-step RT-dPCR using (n = 3) using a singleplex *N* assay and a duplex assay of *Orf1ab*/*E* (Table B-1).

Long-term stability study

For long term stability (LTS), S1 and S2 were evaluated by RT-dPCR at 0, 1, 2, 3 and 7 months post-production. Three units of S1 and two units of S2 were assessed. Triplicate RT-dPCR measurements of each unit were perfor med with ORF 1ab, E and N gene. S0 were evaluated by IDMS at 0, 8 and 15 months post-production. Two or three units were assessed. Each vial were tested twice by IDMS.

APPENDIX C: Protocol

CCQM-K181 / CCQM-P227

SARS-CoV-2 RNA copy number quantification

Coordinating Laboratories: NIM, NML, NIBSC, NIST

Study Protocol v1.0

16 August, 2022

Introduction

Quantitative viral genome copy measurements are important in a number of pathogens where viral load informs treatment, such has HIV or Hepatitis C. The diagnostic response to the COVID-19 pandemic also illustrated the importance of quantitative measurements in informing the performance of tests where the presence, and not quantity, of the pathogen is clinically required. This study will provide NMIs with a route to demonstrate the core competencies to deliver measurement services of RNA copy number concentration to respond to national needs in the global response to the SARS-CoV-2 pandemic. It is proposed to apply the aims and approach of the "CCQM-P154 Absolute Quantification of DNA" study to 'absolute' quantification of RNA and will follow on P199 and P199b studies.

The aim of CCQM-P154 was to assess the quantification of low-level amounts of DNA in an absolute manner without the aid of calibration using enumeration-based techniques (digital PCR and direct counting). The results reported for the low-level material by enumeration-based methods were consistent with values reported by laboratories using orthogonal methods (IDMS, UV-CE) for the approximately 100,000 times more concentrated high-level material from which the low-level material was prepared [1]. The close agreement between the mean results of the four alternative approaches tested (CV 1.8%) strongly supported the accuracy of more recently developed enumeration-based techniques.

Previous CCQM pilot studies have demonstrated NMI capabilities to perform accurate measurements of viral RNA copy number concentration (HIV-1 in P199 and SARS-CoV-2 in P199b). Reverse transcription-digital PCR (RT-dPCR) was used by most laboratories in these studies. Reported values for RNA copy number concentration were within ±40% (P199b). A good agreement between RT-dPCR and the orthogonal methods was observed in P199b. This provides evidence for the overall trueness in the RT-dPCR results however between-laboratory

variability (as reflected in reproducibility metrics of ~20% CV) suggests that sources of bias such as partition volume, RT efficiency or assay performance can add uncertainty to RT-dPCR-based measurement results. Therefore, an orthogonal method (IDMS) will be used to assign RNA copy number concentration values to highly concentrated *in vitro* transcribed RNA in this study, followed by gravimetric dilution of the highly concentrated RNA solution to prepare the study samples.

The aim of the key comparison is to demonstrate the ability to measure nucleocapsid (*N*), envelope (*E*) and open reading frame 1ab (*ORF1ab;* partial region) gene copy number concentration and provide evidence for CMC claims by participating laboratories when measuring purified RNA template in a mixed RNA matrix, containing non-target background (yeast total RNA).

Study Materials

There will be two study materials (Sample 1 "S1" and Sample 2 "S2") with different levels prepared by mixing target RNA with yeast total RNA. Target RNA is composed of a single *in vitro* transcribed SARS-CoV-2 RNA construct (**Appendix 1**) containing the complete *E* and *N* genes and a segment of *ORF1ab* at an approximate concentration of 10^0 ~10^5 /µL in a matrix of ~5 ng/µL yeast total RNA (purchased from Sigma) in buffered solution (1 mM sodium citrate, pH 6.5 (RNA Storage Solution Thermo Fisher Scientific P/N AM7001)). *In vitro* transcription was performed using MEGAscript™ T7 Transcription Kit (AM1334, Thermo Fisher Scientific, USA). RNA transcripts were purified with MEGAclear™ Kit (Thermo Fisher Scientific, USA). A total of 100 units of each study material, S1 and S2, were prepared, with each unit containing 50 µL.

Measurand

- Copy number concentration of *in vitro* transcribed RNA molecule containing SARS-CoV-2 *E, N* and partial *ORF1ab* genes *

**Genome accession and coordinates provided in Appendix 1*

Methods

The study will require assay design and/or selection, quantitative detection of the analyte in the buffer. Participants are anticipated to perform measurements by RT-dPCR (microfluidic dPCR or droplet dPCR).

Homogeneity

All samples were kept at the storage condition of -80 ºC by NIM. Twelve units for sample 1 (S1) and eight units for sample 2 (S2) were taken randomly, and analysis of triplicate sub-samples was carried out using RT-dPCR method [2]. Results are shown in Figure 1.

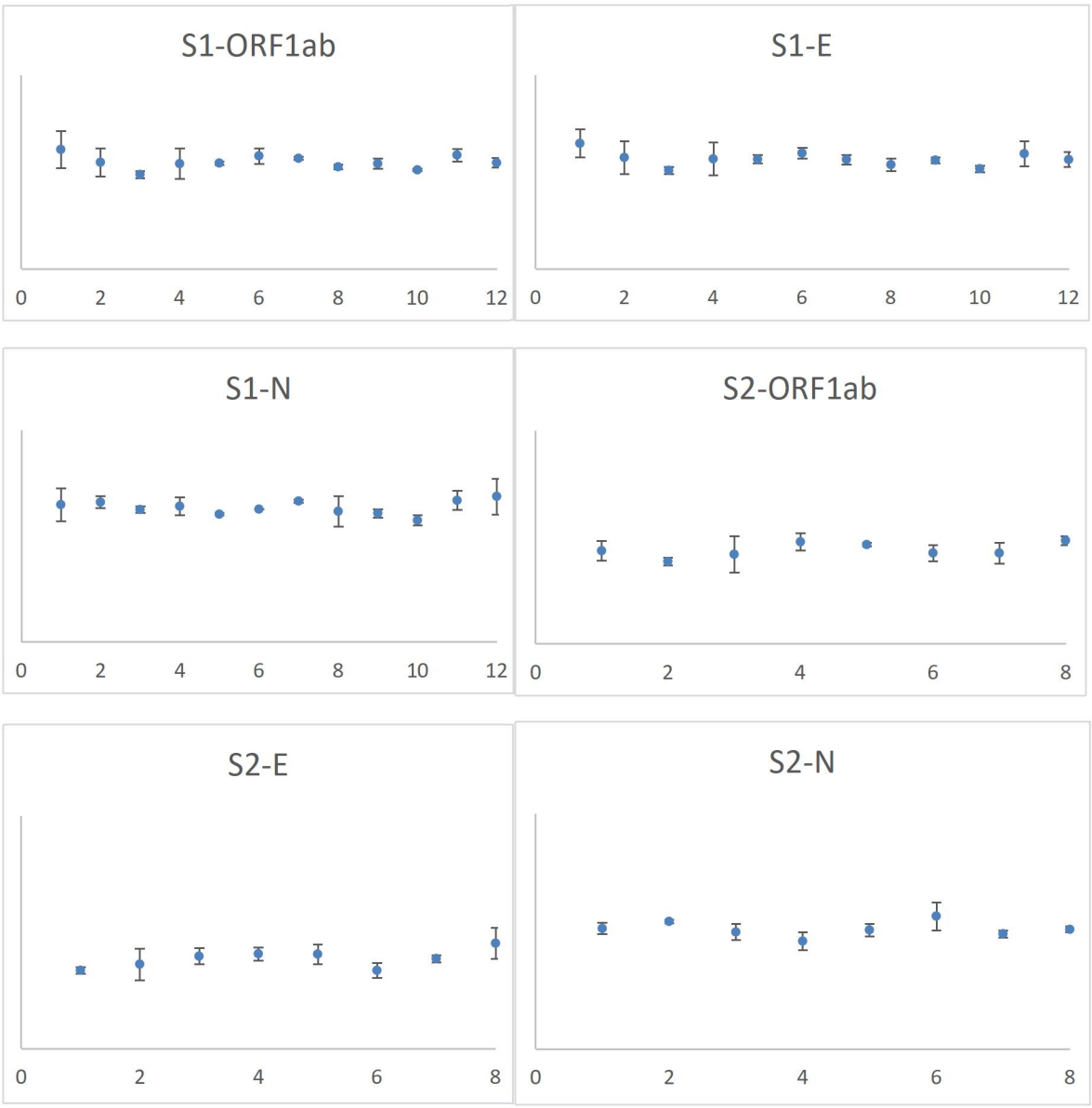


Figure 1 Homogeneity of the two unknown samples. Results are shown for 12 units of S1 and 8 units of S2 (x-axis) with values for each unit with its three replicates as mean±SD (y-axis, copy number concentration, scale blinded).

One-way ANOVA with *F*-test in accordance with the requirements as stipulated in ISO Guide 35 was used to test whether there were significant between-unit differences in the copy number

concentration of the measurands (Table 1). The value of the relevant *F*-test is smaller than the *F* critical value at 0.05 confidence level, which indicates that the inhomogeneity of the study material was insignificant. Between-unit standard uncertainties (u_{bb}) are shown in Table 1.

Table 1 Summary of uncertainty evaluation for homogeneity

Study Material	Sample 1			Sample 2		
Target gene	***F*-value**	$F_{0.05(11,24)}$	u_{bb}	***F*-value**	$F_{0.05(7,16)}$	u_{bb}
N	1.60	2.25	1.1%	1.10	2.66	2.5%
E	1.70	2.25	1.2%	1.72	2.66	2.8%
ORF1ab	1.72	2.25	1.2%	1.55	2.66	2.3%

Stability

Short term stability (STS) was tested for similar RNA materials in P199b at 4 ºC and dry ice for 0, 3, and 7 days. This indicated that the materials would be stable for at least 1 week on dry ice. The current study was designed to test the K181 study material stability under additional and extended transportation conditions at ambient temperature incubation and of up to 14 days. Samples were incubated at 4°C and 25°C for 3, 7 and 14 days and compared to study material stored at -80°C (Two units). Two vials were analyzed for each incubation temperature and time point. The results are shown in Figure 2. Trend analysis showed that both S1 and S2 can be stable at 4°C and 25°C for 14 days. But *T* test showed that S2 can not be stable at 25ºC for 1 week (p<0.05).

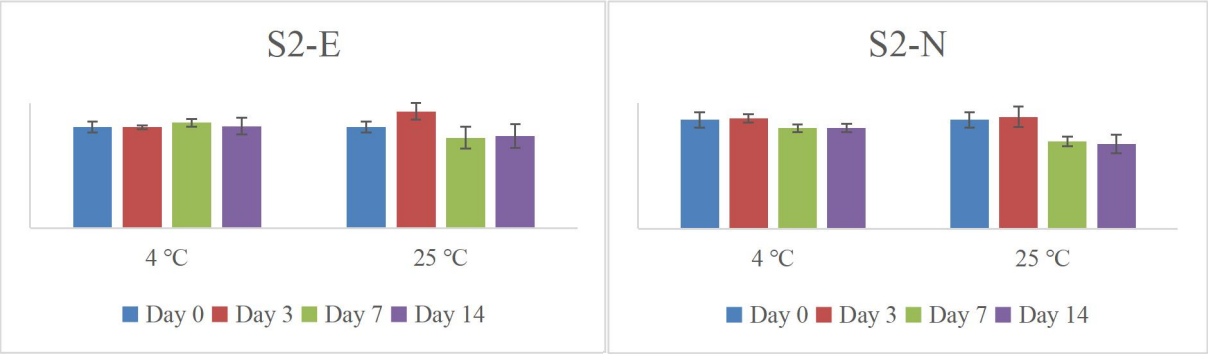


Figure 2 STS of the two unknown samples. S1 and S2 were incubated at 4°C and 25°C for 3, 7 and 14 days, and reluts of study materials stored at -80°C were referred as day 0. Values of two vials with duplicates for each incubation temperature and time point were shown as mean±SD (y-axis, copy number concentration, scale blinded).

For long term stability (LTS), similar RNA samples used in P199b were shown to be stable when stored at – 80 ºC and tested at 0, 4 and 9 months post-production. LTS of the K181 study materials will be measured at 2 months post-production.

Study Guidelines

Each participant will receive three vials (units) of study samples S1 and S2. Each vial contains 50 µL of RNA solution. One vial is intended for method development and the other two are to be used for determination of the reported results. Samples should be stored at -80ºC. A minimum sample intake of 5 µL is recommended. Participants may use their preferred laboratory procedures.

Reporting of Results

At the time of sample dispatch, a sample receipt form (Form 2 in Appendix 2) will be provided electronically to all participants and must be filled in and returned to the study coordinator on receipt of the shipments. The results reporting form (Form 3 in Appendix 2) will be provided to each participant and must be completed and returned to the study coordinator before the submission deadline.

The results should be reported in the unit of μL^{-1} and should include standard and expanded uncertainties (95 % level of confidence) for the mean of the replicate determinations. Information on the measurement procedure (assay design, primer and probe sequence, optimal concentration of primer and probe, RT approach (one or two step), dPCR mastermix, dPCR platform, quantification approach, partition volume) [3], any quality control materials, number of replicates, the calculation of the results and the estimation of measurement uncertainty should be included in Form 4 in Appendix 2.

Evaluation of Results

All the results of the pilot and key comparison will be evaluated against the key comparison reference value (KCRV). The KCRV will be determined from the results of the highly concentrated RNA sample characterized by NIM, NMIA and NMIJ using appropriately validated methods with demonstrated metrological traceability [4]. The Draft A report will provide candidate estimates of the KCRVs and their uncertainty for review and discussion by the NAWG.

How Far Does the Light Shine?

Successful participation in this key comparison CCQM-K181 "SARS-CoV-2 RNA copy number concentration measurement" will demonstrate participants' capabilities in determining RNA copy number concentration range from 10^0 μL^{-1} (with no upper limit) of defined target sequences (mRNA transcripts or gRNA regions <10 kb) in a non-target RNA matrix or as a single template in aqueous solution.

This may include measurement capabilities such as: (1) value assignment of primary reference standards; (2) value assignment of calibration solutions; (3) measurement of RNA sequence copy number concentration using RT-dPCR.

CMCs would not support measurement of whole viral RNA genome copy number concentration. CMCs may be extended to include measurement of RNA copy number concentration of target gene sequences in viral genomic RNA fragments in the context of biological materials through participation in analysis of relevant viral genomic materials in CCQM-P199 and/or CCQM-P199b. This would be judged on CMC-claimed measurement uncertainty being consistent with reported result compared to the consensus RV for the CCQM-P199/P199b material(s). However if uncertainties reported in CCQM-P199 or P199b were smaller than those which may be claimed on the basis of K181, the CMC for analysis of biological materials/whole virus extracts should claim a higher minimum uncertainty based on the performance in K181.

Study schedule

The time schedule for the various stages of the Key Comparison /Pilot Study is shown as follows:

Table 3 Study schedule for CCQM K181/P227

Event	Period
Preparation of sample	Jun 2022
Homogeneity testing	August2022
Stability testing	Aug-Oct 2022
Invitation of participants	Oct 2022
Deadline for registration	30 Oct 2022
Dispatch of samples	Nov 2022
Deadline for submission of results	10 March 2023
Discussion of report at the CCQM NAWG	April 2023

Contact information:

For enquiries, participants may wish to make contacts as follows:

Dr. Lianhua Dong, NIM, lianhuadong@126.com, donglh@nim.ac.cn

Dr. Chunyan Niu, NIM, niuchy@nim.ac.cn

References

[1] Yoo, H.-B. et al. International Comparison of Enumeration-Based Quantification of DNA Copy-Concentration Using Flow Cytometric Counting and Digital Polymerase Chain Reaction. Analytical Chemistry 88, 12169-12176 (2016).

[2] Dong L, Zhou J, Niu C, Wang Q, Pan Y, Wang X, *et al*. Highly accurate and sensitive diagnostic detection of SARS-CoV-2 by digital PCR. *medRxiv* 2020.https://doi.org/10.1101/2020.03.14.20036129.

[3] The dMIQE Group, Huggett J. The Digital MIQE Guidelines Update: Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020. Clin Chem. 2020;66(8):1012-29.

[4] Niu C, Wang X, et al, Accurate Quantification of SARS-CoV-2 RNA by Isotope Dilution Mass Spectrometry and Providing a Correction of Reverse Transcription Efficiency in Droplet Digital PCR, under review.

APPENDIX D: Registration Form

Form 1: Confirmation of Participation

Institute	
Contact person	
Email address	
Address for sample reception	
Phone number	
Any requirements on the airway bill and invoice	
Contact details (import broker or designated transportation person/company)	
K181 or P227 participation	☐ K181 ☐ P227

APPENDIX E: Study Material Receipt Form

Form 2: Receipt of Study Materials

Institute	
Contact person	
Email address	
Date and time of sample reception	
Dry ice present on receipt? (yes/no)	
Samples still frozen? (yes/no)	
Any sign of sample leakage (yes/no)	
Any mishaps during delivery? (yes/no)	If yes, please describe below:

APPENDIX F: Reporting Form

Form 3: Submission of Results

Institute	
Contact person	
Email address	

3.1 Result of Sample 1

	Reported value
x (μL^{-1})	
u (μL^{-1})	
k	
U (μL^{-1})	
$U(x)/x$ (%)	

3.2 Result of Sample 2

	Reported value
x (μL^{-1})	
u (μL^{-1})	
k	
U (μL^{-1})	
$U(x)/x$ (%)	

APPENDIX G: Experimental details form

Assays

Lab	Assay ID	Primer /probe	Oligonucleotide sequences (5′ → 3′)	Final (uM)	Amplicon size (bp)	Supplier & purification	PCR thermocycling condition	Technical replication *	Experiment replication#	Other information

*number of reaction replicates with a plate

#number of replicate plates

dPCR platform

Lab	dPCR platform	Mastermix	Thermal Cycler	Prepared reaction volume (µL)	Loaded reaction volume (µL)	Effective reaction volume (µL)	Mean observed accepted partition number (min-max)	Partition volume and uncertainty (nL)	Analysis Software

APPENDIX H: Summary of Participants' Analytical Information

The following Tables summarize the detailed information about the analytical procedures each participant provided in their "Experimental details" forms. The presentation of the information in many entries has been consolidated and standardized.

The participant's measurement uncertainty statements are provided verbatim in Appendix I.

Table H-1: PCR assay specifications CCQM-K181/P227

Lab	Assay ID	Primer/probe	Oligonucleotide sequences (5' → 3')	Final (uM)	Amplicon size (bp)	Supplier & purification	Technical replication *	Experiment replication #	Other information
PTB	Duplex assay (Sar E : ChinN)	Sarbeco E	Forward: ACAGGTACGTTAATAGTTAATAGCGT Reverse: ATATTGCAGCAGTACGCACACA Probe: HEX-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1	0,4 µM primers & 0,2 µM probe	113	Eurofins genomics & HPLC	3 to 6	1	Note: Sarbeco E and CDC N2 results are the main results. China N result is for supplementary information.
		China CDC N	"Forward: GGGGAACTTCTCCTGCTAGAAT Reverse: CAGACATTTTGCTCTCAAGCTG Probe: FAM-TTGCTGCTGCTTGACAGATT-BHQ1"	0,4 µM primers & 0,2 µM probe	99		3 to 6	1	
	Singleplex-N2	CDC N2	Forward: TTA CAA ACA TTG GCC GCA AA Reverse: GCG CGA CAT TCC GAA GAA	0,9 µM primers &	67		3 to 6	1	

			Probe: FAM-ACA ATT TGCCCC CAG CGC TTC AG-BHQ1	0,25µM probe					
INMETRO	E_Sarbeco	E_Sarbeco_Fw	ACAGGTACGTTAATAGTTAATAGCGT	1.125	113	Fiocruz (Brazil) HPLC	6 (SM1) 10 (SM2)	2 (SM1) 1 (SM2)	
		E_Sarbeco_Rv	ATATTGCAGCAGTACGCACACA	1.125					
		E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-NFQ	0.5626					
KRISS	N		forward 5'-CAGCAGTAGGGGAACTTCTC-3', reverse 5'-GCTGGTTCAATCTGTCAAGC-3', probe 5'- 6-FAM/TGATGCTGCTCTTGCTTTGCT/SFCQ1-3'	primer: 1uM each, probe 250 nM	88	SFC, HPLC	3	3	
	E		forward 5'-CGGAAGAGACAGGTACGTTAA-3', reverse 5'-GCAGTAAGGATGGCTAGTGT-3', probe 5'-6-FAM/TCTTGCTTTCGTGGTATTCTTGCT/SFCQ1-3'	primer: 1uM each, probe 250 nM	91	SFC, HPLC	3	3	
	E_s		forward 5'-ACAGGTACGTTAATAGTTAATAGCGT-3' reverse 5'- ATATTGCAGCAGTACGCACACA	primer: 1uM each, probe 250 nM	125	SFC, HPLC	3	3	

			probe 5'-6-FAM/ACACTAGCCATCCTTACTGCGCTT-SFCQ1-3'						
NIM	ORF	F	CCCTGTGGGTTTTACACTTAA	0.6	119	PAGE	3	1	
		R	ACGATTGTGCATCAGCTGA	0.6		PAGE			
		P	5'-FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1-3'	0.2		HPLC			
	E	F	ACAGGTACGTTAATAGTTAATAGCGT	0.6	113	PAGE	3	1	
		R	ATATTGCAGCAGTACGCACACA	0.6		PAGE			
		P	5'-VIC-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1-3'	0.05		HPLC			
	N2	F	TTACAAACATTGGCCGCAAA	0.9	67	PAGE	3	1	
		R	GCGCGACATTCCGAAGAA	0.9		PAGE			
		P	5'-FAM-ACAATTTGCCCCCAGCGCTTCAG BHQ1-3'	0.25		HPLC			
NMIJ	N1 NMIJ	F-primer	TGGCAGTAACCAGAATGGAGAAC	0.9	100	Thermo Fisher Scientific, HPLC	In each assay for S1, I prepared 2 RT-mixes, carried out 2 RT-reactions per 1 RT-	2 plates for quantification	Used for S1 and S2 quantification
		R-primer	AGTGAGAGCGGTGAACCAAGA	0.9					
		probe	VIC-CGCGATCAAAACAACGT-MGB	0.25					
	N2	F-primer	AAATTTTGGGGACCAGGAAC	0.9	158	Eurofines Genomics Primer: OPC			Used for only S1 quantificatio
		R-primer	TGGCACCTGTGTAGGTCAAC	0.9					

		probe	ATGTCGCGCATTGGCATGGA	0.25		Probe: HPLC	mix, and then 3 ddPCR measurements per 1 RT reaction were performed. So, totally 12 reaction replicates in a plate were performed for 1 assay.In each assay for S2, I prepared 2 RT-mixes, and carried out 1 RT reaction and 4 ddPCR measurement per 1 RT-mix. So, totally 8 reaction replicates in a plate were performed		n
	N2 CDC	F-primer	TTACAAACATTGGCCGCAAA	0.9	93	Eurofines Genomics Primer: OPC Probe: HPLC			Used for only S1 quantification
		R-primer	GCGCGACATTCCGAAGAA	0.9					
		probe	ACAATTTGCCCCCAGCGCTTCAG	0.25					
	ORF K181	F-primer	GTGARATGGTCATGTGTGGCGG	0.9	100	Eurofines Genomics Primer: OPC Probe: HPLC			Used for S1 and S2 quantification
		R-primer	CAAATGTTAAAAACACTATTAGCATA	0.9					
		probe	CAGGTGGAACCTCATCAGGAGATGC	0.25					
	ORF NMIJ	F-primer	GCCATGCGAAATGCTGGTA	0.9	100	Thermo Fisher Scientific, HPLC			Used for S1 and S2 quantification
		R-primer	CTGGCGTGGTTTGTATGAAATC	0.9					
		probe	TAGATAATCAAGATCTCAATGGT	0.25					
	E	F-primer	ACAGGTACGTTAATAGTTAATAGCGT	0.9	113	Eurofines Genomics Primer: OPC Probe: HPLC			Used for S1 and S2 quantification
		R-primer	ATATTGCAGCAGTACGCACACA	0.9					
		probe	ACACTAGCCATCCTTACTGCGCTTCG	0.25					

							for 1 assay.		
HSA	Duplex 1	China CDC ORF1ab forward primer	5' - CCC TGT GGG TTT TAC ACT TAA - 3'	0.9	119	HPLC purified primers were purchased fromGenScript Biotech (Singapore) PTE. LTD. HPLC purified double quencher probes were purchased from Integrated DNA Technologies PTE, Ltd.	4	2	The nominated results were the arithmetic mean of 3 targets (Charite E, US CDC N2, China CDC N) while the uncertainty from China CDC ORF was considered. The supplementary results were the arithmetic mean of all 4 targets after considering reverse transcription efficiency.
		China CDC ORF1ab reverse primer	5' - ACG ATT GTG CAT CAG CTG A - 3'	0.9					
		China CDC ORF1ab probe	5' FAM - ACA ATT TGC /ZEN/ CCC CAG CGC TTC AG - 3'IBkFQ	0.25					
		Charite E forward primer	5' - ACA GGT ACG TTA ATA GTT AAT AGC GT - 3'	0.9	113				
		Charite E reverse primer	5' - ATA TTG CAG CAG TAC GCA CAC A - 3'	0.9					
		Charite E probe	5' HEX - ACA CTA GCC /ZEN/ ATC CTT ACT GCG CTT CG - 3'IBkFQ	0.25					
	Duplex 2	US CDC N2 forward primer	5' - TTA CAA ACA TTG GCC GCA AA - 3'	0.9	67				
		US CDC N2 reverse primer	5' - GCG CGA CAT TCC GAA GAA - 3'	0.9					
		US CDC	5' FAM - ACA ATT	0.25					

		N2 probe	TGC /ZEN/ CCC CAG CGC TTC AG - 3'IBkFQ						
		China CDC N forward primer	5' - GGG GAA CTT CTC CTG CTA GAA T - 3'	0.9					
		China CDC N reverse primer	5' - CAG ACA TTT TGC TCT CAA GCT G - 3'	0.9					
		China CDC N probe	5' HEX - TTG CTG CTG /ZEN/ CTT GAC AGA TT - 3'IBkFQ	0.25	99				
JRC-GEEL	2019-nCoV_N2	F	5'-TTACAAACATTGGCCGCAAA-3'	2	67	Invitrogen/HPLC	Sample 1: 6 x 3 (vials) Sample 2: 8 x 3 (vials) Sample 1: 6 x 3 (vials) Sample 1: 6 x 3 (vials)	Sample 1: 2 Sample 2: 1 Sample 1: 2 Sample 1: 2	Sample1diluted 1:100 for measurement
		R	5'-GCGCGACATTCCGAAGAA-3'	2		Invitrogen/HPLC			Sample 2 measured undiluted
		P	5'-FAM-ACAATTTGCCCCCAGCGCTTCAG-QSY-3'	0.125		Applied Biosystems/HPLC			
	E gene / E_Sarbeco	F1	5'-ACAGGTACGTTAATAGTTAATAGCGT-3'	0.8	125	Invitrogen/HPLC			Sample 1 diluted 1:100 for measurement
		R2	5'-ATATTGCAGCAGTACGCACACA-3'	0.8		Invitrogen/HPLC			
		P1	5'-FAM-ACACTAGCCATCCTTACTGCGCTTCG-QSY-3'	0.2		Applied Biosystems/HPLC			
	China N	F	5'-GGGGAACTTCTCCTGCTAGAAT-3'	0.4		Invitrogen/HPLC			Sample 1 diluted 1:100 for meas
		R	5'-CAGACATTTTGCTCTCAAGCTG-3'	0.4		Invitrogen/HPLC			
		P	5'-FAM-TTGCTGCTGCTTGACAGATT-TAMRA-3'	0.2		Applied Biosystems/HPLC			

	RdRP gene	RdRP_SARSr-F2	5'-GTGARATGGTCATGTGTGGCGG-3'	0.6	100	Invitrogen/HPLC	Sample 1: 6 x 3 (vials)	Sample 1: 2	Sample 1 diluted 1:100 for meas
		RdRP_SARSr-R1	5'-CARATGTTAAASACACTATTAGCATA-3''	0.8		Invitrogen/HPLC			
		RdRP_SARSr-P2	5'-FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ-3'	0.2		Eurofins Genomic/HPLC			
	RdRp gene / nCoV_IP4	nCoV_IP4-14059Fw	5'-GGTAACTGGTATGATTTCG-3'	0.8	107	Invitrogen/HPLC			Sample 1 diluted 1:100 for meas
		nCoV_IP4-14146Rv	5'-CTGGTCAAGGTTAATATAGG-3'	0.8		Invitrogen/HPLC			
		nCoV_IP4-14084Probe (+)	5'-FAM-TCATACAAACCACGCCAGG-QSY-3'	0.2		Applied Biosystems/HPLC			
TUBITAK UME	N-Set 4 (in house assay)	F	CAACTGAGGGAGCCTTGAATAC	600	88	IDT/HPLC	Sample 1: 6 x 3 (vials)	Sample 1: 2	
		R	TGAGGAAGTTGTAGCACGATTG	600					
		P	FAM-CACCCGCAA/ZEN/TCCTGCTAACAATGC-IBFQ	50					
	China CDC ORF1ab	F	CCCTGTGGGTTTTACACTTAA	600	119	IDT/HPLC			
		R	ACGATTGTGCATCAGCTGA	600					
		P	FAM-CCGTCTGCG/ZEN/GTATGTGGAAAGGTTATGG-IBFQ	0					
INM-CO	E_INM	Forward	CTTGCTTTCGTGGTATTCTTG	0.8	86	Biosearch technologies Primers:RPC	RT: 50ºC/60 min	SM1:3 SM2:2	SM1:3 SM2:2
		Reverse	ACGTTAACAATATTGCAGCA	0.9					
		Probe	FAM-	0.3					

			CCTTACTGCGCTTCGATTGTGTGCGT - BHQ 1			Probe:Dual HPLC			
	N1 (China)	Forward	GGGGAACTTCTCCTGCTAGAAT	0.8	99			SM1:3 SM2:2	SM1:3 SM2:2
		Reverse	CAGACATTTTGCTCTCAAGCTG	0.9					
		Probe	FAM-TTGCTGCTGCTTGACAGATT-BHQ 1	0.3					
	RdRp (charite)	Forward	GTGAAATGGTCATGTGTGGCGG	0.8	100			SM1:3 SM2:2	SM1:3 SM2:2
		Reverse	CAAATGTTAAAAACACTATTAGCAT	0.9					
		Probe	HEX-CAGGTGGAACCTCATCAGGAGATGC-BHQ1	0.3					
INRIM	E3	Primer fw	GCTTTCGTGGTATTCTTGCTAGT	675	103	Metabion, desalted	3	8	Experiment replications were performed by 2 different operators (4 replications each)
	E3	Primer rv	AAGAAGGTTTTACAAGACTCACGTT	675		Metabion, desalted			
	E3	Probe FAM	AGCCATCCTTACTGCGCTTCGATTGTGT	187.5		Metabion, HPLC			
	RdRp3 (ORF1ab)	Primer fw	TGACCCTGTGGGTTTTACACTTAA	900	92	Eurofins genomics, HPSF	3	8	
	RdRp3 (ORF1ab)	Primer rv	GTTCGCGGAGTTGATCACAA	900		Eurofins genomics, HPSF			
	RdRp3(ORF1ab)	Probe HEX	CCGTCTGCGGTATGTGGAAAGGTTATGG	250		Eurofins genomics, HPLC			
	ITAN	Primer fw	CGATCAAAACAACGTCGGCC	1350	113	Eurofins genomics, HPSF	3	8	
	ITAN	Primer rv	GGAACGCCTTGTCCTCGA	1350		EurofinsgenomicsHPSF			
	ITAN	Probe FAM	CACCGCTCTCACTCAACATGGC	375		Eurofins genomics, HPLC			
NML_LGC	CDC N2	Forward	TTACAAACATTGGCCGCAAA	0.9	67	BioSearch & RPC	3 units x 3 replicates	3	

		Reverse	GCGCGACATTCCGAAGAA	0.9		BioSearch & RPC			
		Probe	FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQNova1	0.25		BioSearch & RP HPLC			
NIST	CDC N2	Probe	ACAATTTGCCCCCAGCGCTTCAG	.25	67	ThermoFisherTaqMan MGB, HPLC purified(catalog #4316034)	4	3	
		F	TTACAAACATTGGCCGCAAA	1					
		R	GCGCGACATTCCGAAGAA	1					
NMIA	Charite 2 E gene	E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT	900	113	Sigma_HPLC	24	1	
		E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA	900		Sigma_HPLC			
		E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1	250		Sigma_HPLC			
	USCDC2 N gene	2019-nCoV_N2F	TTACAAACATTGGCCGCAAA	900	67	Sigma_HPLC			
		2019-nCoV_N2R	GCGCGACATTCCGAAGAA	900		Sigma_HPLC			
		2019-nCoV_N2P	HEX-ACAATTTGCCCCCAGCGCTTCAG-BHQ1	250		Sigma_HPLC			
NIMT		nCoV_E-F	ACAGGTACGTTAATAGTTAATAGCGT	900	113	Macrogen/HPLC	4	4	
		nCoV_E-R	ATATTGCAGCAGTACGCACACA	900		Macrogen/HPLC			

	E gene	nCoV_E-P	Hex-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1	250		Macrogen/HPLC			
	CDC N1	nCoV-CDC_N1-F	GACCCCAAAATCAGCGAAAT	900	72	Macrogen/HPLC	4	4	
		nCoV-CDC_N1-R	TCTGGTTACTGCCAGTTGAATCTG	900		Macrogen/HPLC			
		nCoV-CDC_N1 P	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1	250		Macrogen/HPLC			
SNSU	ORF	F	CCCTGTGGGTTTTACACTTAA	900		Probe scale, HPLC Purified	3	1	
		R	ACGATTGTG CATCAGCTG A	900					
		P	5'-FAM-CCG-TCT GCG GTA TGT GGA AAG GTT ATG G-BHQ1-3'	100					
	N	F	GGGGAACTTCTCCTGCTAGAAT	900			3	1	Sampel 1 dilutions 750x
		R	CAGACATTTTGCTCTCAAGCTG	900					
		P	FAM-TTG CTG CTG CTT GAC AGA TT-BHQ1	250					
	E	F	ACAGGTACGTTAATAGTTAATAGCG T	500	113		3	1	Sampel 1 dilutions 750x
		R	ATATTGCAGCAGTACGCACACA	500					

		P	FAM-ACA CTA GCCATC CTT ACT GCG CTT CG-BHQ1-3’	200					
VNIIM	China E	E-F	ACAGGTACGTTAATAGTTAATAGCG T	1,2	113	Syntol Ltd., Moscow, Russia; HPLC	12-15 (S1) 4 -5 (S2)	2 out of 5 (S1) 3 (S2)	Results ofmeasurements for two tubes of S1 were rejected due to data inconsistency - potential RNA degradation during shipment
		E-R	ATATTGCAGCAGTACGCACACA	1,2					
		E-Probe	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1	0,2					
NIB	CDC, N2	2019-nCoV_N2 Forward primer	TTA CAA ACA TTG GCC GCA AA	0.5	67	IDT Probe – HPLC Primers – standard (STD)	6 (S1) / 3 (S2)	1	S1, 2 dilutions 100x and 1000x
		2019-nCoV_N2 Reverse primer	GCG CGA CAT TCC GAA GAA	0.5					
		2019-nCoV_N2 Probe	FAM-ACA ATT TGC /ZEN/ CCC CAG CGC TTC AG-BHQ1	0.125					
	Corman *et al.*, 2020; E_sarbeco	SARS-CoV-2 E1F	ACA GGT ACG TWA ATA GTT AAT AGC GT	0.4	125	IDT Probe – HPLC Primers – standard (STD)	6 (S1) / 3 (S2)	1	S1, 2 dilutions 100x and 1000x
		SARS-CoV-2 E1R	ATA TTG CAG CAS TAC GCA CAC A	0.4					
		E_sarbeco_P1 Probe	FAM- ACA CTA GCC ATC CTT ACT GCG CTT CG - ZEN/IowaBlack	0.2					

	ORF1ab Chinese Center for Disease Control and Prevention	nCoV-ORF1ab_F	CCC TGT GGG TTT TAC ACT TAA	0.25	Not stated	IDT Probe – HPLC Primers – standard (STD)	6 (S1) / 3 (S2)	1	S1, 2 dilutions 100x and 1000x
		nCoV-ORF1ab_R	ACG ATT GTG CAT CAG CTG A	0.25					
		nCoV-ORF1ab_P	FAM-CCG TCT GCG GTA TGT GGA AAG GTT ATG G- ZEN-3IABkFQ	0.1					
CENAM	China CDC N	Forward	GGGGAACTTCTCCTGCTAGAAT	0.9	99	T4Oligo, HPLC	4	1	The MRC from China, which arrived in the same condition as the samples, was measured to determine if there was any change due to the lack of dry ice.
		Reverse	CAGACATTTTGCTCTCAAGCTG	0.9					
		Probe	FAM-TTGCTGCTGCTTGACAGATT-BHQ1	0.25					
	Sarbeco E	Forward	ACAGGTACGTTAATAGTTAATAGCG T	0.9	113	T4Oligo, HPLC	4	1	
		Reverse	ATATTGCAGCAGTACGCACACA	0.9					
		Probe	FAM-ACACTAGCCATCCTTACTGCGCTTCG -BHQ1	0.25					

	Orf1ab	Forward	CCCTGTGGGTTTTACACTTAA	0.9	119	T4Oligo, HPLC	4	1	
		Reverse	ACGATTGTGCATCAGCTGA	0.9					
		Probe	FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1	0.25					

Table H-2：**dPCR platform**

Lab	dPCR platform	Mastermix	Thermal Cycler	Prepared reaction volume (µL)	Loaded reaction volume (µL)	Effective reaction volume (µL)	Mean observed accepted partition number (min-max)	Partition volume and uncertainty (nL)	Analysis Software
PTB	QX200	One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad) #1864022	C1000	20	20	20	15312 ± 1220	0.85 nL partition volume and (U - 0.06 nL)	Bio-Rad QuantaSoft version 1.0.596
INMETRO	QX-200 AutoDG BioRad	One-Step RT-ddPCR Advanced Kit for Probes #1864022 BioRad	C1000 Touch BioRad	23	20	20	15587 (13909-17416)	0.762 and 0.06	QuantaSoft Analysis Pro 1.0.596 BioRad

KRISS	QX200	Bio-rad one-step RT dPCR supermix Cat#1864022	ThermoFisher VeritiPro	20.1 ~ 20.2	20	20	18269 (15085-20639)	manufacturer's value std unc = 0.0442	Bio-Rad QuantaSoft 1.7.4.0917
NIM	QX200	Bio-rad one-step RT dPCR supermix Cat#1864021	Veritipro 96-well thermal Cycler	22	20	20	11061-16820	0.774, 0.0165	Bio-Rad QuantaSoft 1.7.4
NMIJ	QX200	ddPCR supermix for probes (no dUTP)	CT1000	80	20	20	S1:18544 (11726-21511) S2: 17746 (14268-19961)	0.786 ± 0.013	QX Manager 1.1
HSA	QX200	One-Step RT-ddPCR Advanced Kit for Probes	C1000 Touch	22	22	20	18563 (13808-20299)	0.731 ± 0.071	QX Manager Version 1.0.339.1125
JRC-GEEL	Bio-rad QX200	One-Step RT-ddPCR Advanced Kit for Probes	Bio-rad C1000 touch	22	20	20	12 500-18 500	0.797±0.010	Quantalife v. 1.7.4.0917
TUBITAK UME	QX200	One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad) #1864022	C1000	22	20	20	Min: 10582 Max: 18806 Mean:14860	0.776 – 0.040	Bio-Rad QuantaSoft Analysis Pro version 1.0.596

INM-CO	QX200 BioRad	One-StepRT ddPCR Adv kit forprobes 1864122 Lot 64460413	CFX96-BioRad	21 uL	21 uL	9.2 uL – 12.3 uL	12000 - 16000	x: 7.72 E-4 uL u: 2.3 E-5 uL	QuantaSoft V1.7.4
INRIM	QX200 Bio-Rad	One-Step RT-ddPCR Advanced kit for Probes Bio-Rad	T100 Bio-Rad	22	20	40	11724 (8253 - 15109)	Vol = 0.776* u (vol) = 0.035*	QuantaSoft™ Analysis Pro 1.0.596
NML_LGC	Bio-Rad QX200	One-Step RT-ddPCR Advanced kit for Probes (Bio-Rad Cat No: 186-4021, Lot No: 64512277)	Bio-Rad C1000	22	20	12.5	16065 (10873-19871)	0.776 ± 0.0403	QuantaSoft 1.7.4.0917
NIST	Bio-Rad QX200	One Step RT-ddPCR supermix	ProFlex	22	22	10.3	13803 (10989-16632)	.7472 +/- .013083	Quantasoft 1.7.4.0917 & Microsoft Excel
NMIA	BioRad QX200	One-Step RT-ddPCR Advanced Kit for Probes	BioRad C1000 Touch Thermocycler	600	22	20	Mean 17250 Min 13,40 Max 19,190	0.760 ± 0.015	BioRad QuantaSoft Version 1.7.4.0917
NIMT	BioRad QX200	One-Step RT-ddPCR Advanced Kit for Probes	BioRad C1000 Touch Thermocycler	25	20	20	18780 (16105-22326)	0.85 ± 0.021	Bio-Rad Quantasoft 1.7.4.0917
SNSU	Bio-Rad QX200	One Step RT-ddPCR Advanced Kit for Probes	CFX96-BioRad	22	20	20	15512-17963	0.85 nL partition volume	QuantaSoft Analysis Pro 1.0.596 BioRad

VNIIM	Bio-Rad QX200	Bio-rad, One-step RT-ddPCR advanced kit for probes, cat #1864021	Bio-Rad C1000 Touch	360 (S1) 100 (S2)	20	7	9054 – 13244 (S1) 9110 – 12915 (S2)	0,6820 0,0037	Bio-Rad Quantasoft 1.7.4.0917
NIB	QX200, BioRad	1-Step RT-ddPCR Advanced Kit for Probes, BioRad	T100, BioRad	22	20	13.39 (8.85 – 16.27)*	15757 (10406-19143)	0.85 nL ± 2.9% with 95 CI	QuantaSoft 1.7.4
CENAM	QX200, BioRad	One-step RT-ddPCR advanced kit for probes, BioRad	GENEAMP PCR 9700	21	20	20	Mean 14453 Max 16959 Min 10081	0.85 nL	Bio-Rad QuantaSoft version 1.7.4.0917

Table H-3：Reverse transcription and thermal cycling parameters for CCQM K181 RT-dPCR

Institute	RT temp (°C)	RT time (min)	PCR intial step temp	PCR intial step time (min)	PCR cycling temp 1 (°C)	PCR cycling time 1 (sec)	PCR cycling temp 2 (°C)	PCR cycling time 2 (sec)	Cycle number	PCR final incubation	Ramp rate (ddPCR only)
PTB	50	15	95	5	95	30	55	30	45	98 °C for 5 min; 4 °C end"	
	50	60	95	5	95	30	55	60	45	98 °C for 5 min; 4 °C end"	
INMETRO	49	60	95	10	95	30	58	60	40	98°C 10 min,16°C hold	
KRISS	46	60	95	10	95	30	59	60	50	98 °C 10 min	1.2°C/s
NIM	45	60	95	10	94	30	60	60	40	98 °C 10 min	2 °C/s
NMIJ			95	10	95	30	58	60	50	98 °C for 10 min and 4 °C.	
HSA	50	60	95	10	95	30	53	60	40	98 °C for 10 min; 4 °C hold.	2 °C/s

JRC-GEEL	50	60	95	10	95	30	60	60	45	98 ºC 10 min 4°C~	
TUBITAKUME	50	60	95	10	95	30	58	60	60	Enzy stab:98 ˚C-10 min 4 ˚C ~	
INM-CO	50	60	95	10	95	15	59	30	45	98 ºC 10 min	0.5 °C/s
INRIM	42	60	95	10	95	30	60	60	40	98 ºC 10 min 4°C~	2 °C/s
NML_LGC	47.5	60	95	10	95	30	55	60	40	10 min at 98 °C.	2 °C/s
NIST	50	60	95	10	95	30	55	60	60	10 min at 98 °C Stage 5, Hold 4 °C	
NMIA	45	60	95	10	95	30	57.5	30	40	98°C, 10 min	
NIMT	45	60	95	10	94	30	56	60	40	98 °C for 10 min; 4 °C Hold"	2 °C/s
SNSU	45	60	95	10	95	30	50	60	40	98ºC 10 min 4 °C hold,	2 °C/s
VNIIM	50	60	95	10	95	30	57	60	60	98 °C 10 min, 4 °C hold,	2 °C/s
NIB	lid temperature = 105 °C 50	60	95	10	95	30	55	60	45	10 min 98 °C ∞ 4 °C	2 °C/s
CENAM	50	For E gen 60	95	10	95	30	57	60	45	98°C/10 min	2 °C/s
		For N and Orf1ab genes 10	95	5	95	15	58	20	45	4°C hold	2 °C/s

APPENDIX I: Summary of Participants' Uncertainty Estimation Approaches

The following are text excerpts and/or pictures of the uncertainty-related information provided by the participants in the reporting form. Information is grouped by participant.

NIM

Submitted values of S1 and S2 ($C_{S1/S2}$) were calculated using equation below:

$$C_{S1/S2} = \frac{\frac{C_{RT-dPCR,ORF}}{RT_{ORF}} + \frac{C_{RT-dPCR,N}}{RT_N} + \frac{C_{RT-dPCR,E}}{RT_E}}{3}$$

Where $C_{RT\text{-}dPCR}$ is copy number concentration of *ORF 1ab*, *N* and *E* gene measured by RT-dPCR, and *RT* is reverse transcription efficiency calculated from S0 using equation below:

$$RT = \frac{C_{RT-dPCR}}{C_{IDMS}}$$

Where C_{IDMS} is copy number concentration of S0 converted from IDMS.

The uncertainty budget for S1 is:

Gene	ORF 1ab	E	N
x/ (μL^{-1})	7.85E+05	8.22E+05	8.16E+05
Repeatability $u_{A,rel}$ (%)	4.54	4.03	3.41
Dilution factor $u_{D,rel}$ (%)	0.50	0.50	0.50
Partition volume $u_{vp,rel}$ (%)	2.13	2.13	2.13
RT efficiency $u_{RT,rel}$ (%)	4.95	5.71	3.95
Relative Combined uncertainty $u_{c,rel}$ (%)	7.07	7.32	5.66
Combined uncertainty u_c / (μL^{-1})	5.55E+04	6.02E+04	4.62E+04
Combined uncertainty of S1 u / (μL^{-1})	3.13E+04		
k	2		

Expanded uncertainty U/ (μL^{-1})	6.26E+04
Relative expanded uncertainty U_{rel} (%)	7.76

$$u = \frac{1}{3}\sqrt{u^2_{c,ORF} + u^2_{c,E} + u^2_{c,N}}$$

The uncertainty budget for S2 is:

Gene	ORF 1ab	E	N
x/ (μL^{-1})	66.1	66.5	64.3
Repeatability $u_{A,rel}$ (%)	3.35	8.22	6.86
Dilution factor $u_{D,rel}$ (%)	0.50	0.50	0.50
Partition volume $u_{vp,rel}$ (%)	2.13	2.13	2.13
RT efficiency $u_{RT,rel}$ (%)	4.95	5.71	3.95
Relative Combined uncertainty $u_{c,rel}$ (%)	6.37	10.24	8.21
Combined uncertainty u_c/ (μL^{-1})	4.21	6.81	5.28
Combined uncertainty of S1 u/ (μL^{-1})	3.20		
k	2		
Expanded uncertainty U/ (μL^{-1})	6.39		
Relative expanded uncertainty U_{rel} (%)	9.74		

$$u = \frac{1}{3}\sqrt{u^2_{c,ORF} + u^2_{c,E} + u^2_{c,N}}$$

NML (LGC)

Measurement uncertainty for Study Materials 1 and 2 was calculated using the following equation:

$$\mu_c{}^2 = \mu_A{}^2 + \mu_{V_p}{}^2 + \mu_d{}^2 + \mu_{bb}{}^2$$

Type A uncertainty (u_A) was calculated based on triplicate analysis of each of three units of Study Materials 1 and 2 across three experiments (n = 9 measurements per unit). Partition volume uncertainty (u_{Vp}) was based on the standard deviation of four published results[1]. Pipetting uncertainty (u_d) included covariance associated with volumetric dilution steps (200-fold dilution Study Material 1; 2.2-fold Study Material 2). Homogeneity (u_{bb}) was based on the maximum reported values for each Study Material in the study protocol.

The table below summarizes the uncertainty budgets for Study Materials 1 and 2:

	Study Material 1	Study Material 2
x /(μL^{-1})	710000	50
Precision $u_{A,rel}$ (%)	1.25	3.02
Partition volume $u_{Vp,rel}$ (%))	5.19	5.19
Pipetting $u_{d,rel}$ (%	3.54	0.87
Homogeneity $u_{bb,rel}$ (%)	1.20	2.80
Relative Combined uncertainty $u_{c,rel}$ (%)	6.52	6.68
Combined uncertainty u_c / (μL^{-1})	46519	3.38
k	2.36	2.36
Expanded uncertainty U/ (μL^{-1})	110000	8
Relative expanded uncertainty U_{rel} (%)	15.5	16.0

PTB

The submitted values of S1 and S2 were calculated using equation below:

Eq.1 $$\lambda = -\ln(1 - P/N)$$

Eq.2 $$C = D \times \frac{\lambda}{V}$$

[1] Dagata *et al*., (2016) doi: 10.6028/NIST.SP. 260-184; Pinheiro *et al*., (2017) doi: 10.1021/acs.analchem.6b 05032; Kosir *et al*., (2017) doi: 10.1007/s00216-017-0625-y.

In Eq.1 the λ is the average number of targets per droplet, P is the number of positive and N is the total number of droplets. The final copy number concentration C was calculated using Eq.2 which includes λ , the dilution factor D and the droplet size V (taken to be 0.85 nL). The droplet size can be measured in principle, but the measurement of droplet size was not considered being part of this comparison.

For the measurement uncertainty (MU) calculations, factors affecting the measured concentration the final concentration (of the starting material) were included in the uncertainty budget. For each factor the relative uncertainty $u(r)$ and the expansion factor v were determined. The combined uncertainty and effective expansion factors were calculated using the Welch-Satterthwaite formula (GUM JCGM 1000 Appendix, G.4.1) with the following equations, where pipetting error corresponds to $u_{r,1}$, and the relative uncertainty $u_{r,2}$ results from standard deviation of replicate observations.

Eq.1 $$u_{r,c} = \sqrt{u_{r,1}^2 + u_{r,2}^2}$$

Eq.2 $$\nu_{\text{eff}} = \frac{u_{r,c}^4}{\frac{u_{r,1}^4}{\nu_1} + \frac{u_{r,2}^4}{\nu_2}}$$

Eq.3 $$k_{\text{eff}} = T_{\text{inv}}(0.05, \nu_{\text{eff}} - 1)$$

The expanded uncertainty (U) was calculated using the following equation:

Eq.4: $$U = k_{\text{eff}} \times u$$

The uncertainty budget for S1 is:

Gene	Sarbeco E	CDC N2	China N
C /(μL^{-1})	571000	590000	368000
Standard uncertainty u /(μL^{-1})	28000	23000	36000
Expanded uncertainty U/ (μL^{-1})	58000	48000	74000
k_{eff}	2.05	2.13	2.06
Relative expanded uncertainty U_{rel} (%)	10.1	8.1	20.2

The uncertainty budget for S2 is:

Gene	Sarbeco E	CDC N2	China N
C /(μL^{-1})	46.9	44.3	30.8
Standard uncertainty u /(μL^{-1})	4.3	4.1	5.1
Expanded uncertainty U/ (μL^{-1})	9.4	8.8	11.3
k_{eff}	2.20	2.37	2.20
Relative expanded uncertainty U_{rel} (%)	20.0	19.9	36.6

KRISS

The uncertainty budget for S1 and S2 is:

Material	S1	S2
x /(μL^{-1})	6.4.E+05	51.5
Method repeatability $u_{rep,rel}$ (%)	2.24	2.24
Intermediate precision $u_{pre,\ rel}$ (%)	4.32	3.04
Between bottle homogeneity $u_{bb,rel}$ (%)	1.17	2.53
Partition volume $u_{vp,rel}$ (%)	5.47	5.47
Between assays $u_{assay,\ rel}$ (%)	3.89	3.89
Manual thresholding $u_{thre,rel}$ (%)	0.83	0.83
Weighing for dilution $u_{weighing,rel}$ (%)	2.41	NA
Relative Combined uncertainty $u_{c,rel}$ (%)	8.75	8.23
Combined uncertainty u_c (μL^{-1})	5.6.E+04	4.2.E+00
k	2.11	2.12
Expanded uncertainty U/ (μL^{-1})	1.2.E+05	9.0.E+00

Relative expanded uncertainty U_{rel} (%)	18.5	17.5

Uncertainty factors for each material are averaged by measured relative uncertainty for multiple targets. Type A (method repeatability and intermediate precision) and Type B (all the rest) are each combined before calculating the relative combined uncertainty.

NMIJ

Submitted values of S1 and S2 ($C_{S1/S2}$) were calculated using equation 1:

$$C_{total} = \frac{1}{3}(C_N + C_E + C_{ORF}) \quad \text{-Eq. 1}$$

Where C_N, C_E and C_{ORF} are copy number concentrations of *N*, *E* and *ORF 1ab* gene measured by RT-dPCR.

The uncertainty of S1 and S2 were calculated using equation 2 and equation 3:

$$u_{gene} = \sqrt{\left(\frac{u_{prep_RT}}{C_x}\right)^2 + \left(\frac{u_{RT}}{C_x}\right)^2 + \left(\frac{u_{dPCR}}{C_x}\right)^2 + \left(\frac{u_{weigh_RT}}{\overline{w_{RT}}}\right)^2 + \left(\frac{u_{weigh_dPCR}}{\overline{w_{dPCR}}}\right)^2 + \left(\frac{u_V}{\overline{V}}\right)^2 + \left(\frac{u_{assay}}{C_x}\right)^2} \quad \text{-Eq. 2}$$

$$u_{total} = \sqrt{\left(\frac{u_N}{3}\right)^2 + \left(\frac{u_E}{3}\right)^2 + \left(\frac{u_{ORF}}{3}\right)^2 + {u_{dif}}^2} \quad \text{-Eq. 3}$$

The uncertainty budget for S1 is:

Uncertainty components	Relative uncertainty (%)		
	N	ORF1ab	E
Preparation of RT reaction mixture (u_{prep_RT})	2.247	6.710	5.61
RT reaction (u_{RT})	0.951	1.893	3.10
dPCR measurement (u_{dPCR})	0.625	0.899	1.12
Partition volume (u_V)	0.981	1.202	1.70
Weighing for RT mixture (u_{weigh_RT})	0.120	0.146	0.21
Weighing for dPCR mixture (u_{weigh_dPCR})	0.298	0.365	0.52
Dilution of Sample (u_{weigh_sample})	0.046	0.057	0.08
Difference between assay (u_{assay})	20.35	20.35	20.35
Combined standard uncertainty (u_{gene})	20.5	21.6	21.4

The uncertainty budget for S2 is:

Uncertainty components	Relative uncertainty (%)		
	N	ORF1ab	E
Preparation of RT reaction mixture (u_{prep_RT}) RT reaction (u_{RT})	6.125	1.090	0.000
dPCR measurement (u_{dPCR})	5.171	4.861	3.44
Partition volume (u_V)	1.700	1.202	1.70
Weighing for RT mixture (u_{weigh_RT})	0.207	0.146	0.21
Weighing for dPCR mixture (u_{weigh_dPCR})	0.516	0.365	0.52
Dilution of Sample (u_{weigh_sample})	-	-	-
Difference between assay (u_{assay})	27.56	27.56	27.56
Combined standard uncertainty (u_{gene})	28.8	28.1	27.8

The final result of S1 and S2 are:

	S1	S2
Value /(μL^{-1})	8.5×10^5	75
u_N (%)	20.5	28.8
u_E (%)	21.6	28.0
u_{ORF} (%)	21.5	27.8
u_{dif} (%)	4.4	31.6
Conbined uncertainty (u_{total}, %)	13.0	31.6
k	2	2
Expanded uncertainty U (%)	26.0	63.1
U /(μL^{-1})	2.2×10^5	47

JRC-Geel

$$c = \frac{d}{V * N} \frac{\ln\left(1 - \frac{p}{N}\right)}{\ln\left(1 - \frac{1}{N}\right)}$$

c copy number concentration in sample
d dilution factor
V volume of partition (V= 0.797 nL)
N total number of partitions
p positive number of partitions

$$u = \sqrt{u_{rep}^2 + u_{ip}^2}$$

u_r uncertainty from repeatability (experiment design)
u_{ip} uncertainty contribution from intermediate precision (experiment design)

UME

The copy number of both samples was determined using two different assays: one designed for the N gene and the other for the Orf1ab gene. The results from experiments conducted with both assays were averaged, and uncertainty calculations were performed based on these values. Only the dilution factors were used to calculate the copy number concentrations of the samples; the reverse transcription efficiency value was not taken into account in these calculations.

The uncertainty budget of the measurement results is composed of the parameters of repeatability, intermediate precision and partition volume uncertainty. Repeatability and intermediate precision were obtained through the analysis of the measurement results using the one-way ANOVA method. A coverage factor k=2 was used to expand combined uncertainty at 95% confidence interval. The following equations was employed in the calculations:

$$u_r = \frac{\sqrt{MS\ w}}{\sqrt{MS\ s(w)}} \qquad u_{ip} = \frac{\sqrt{MS\ w - MS\ b}}{\sqrt{MS\ s(b)}} \qquad u_c = \sqrt{(u_r)^2 + (u_{ip})^2}$$

Gene	ORF 1ab & N
x /(μL^{-1})	6.95E+05

Repeatability $u_{r,\,rel}$ (%)	0.97
Intermediate precision $u_{ip,\,rel}$ (%)	1.46
Partition volume $u_{vp,rel}$ (%)	5.2
Relative Combined uncertainty $u_{c,rel}$ (%)	5.49
Combined uncertainty u_c (μL^{-1})	3.81E+04
k	2
Expanded uncertainty U/ (μL^{-1})	7.63E+04
Relative expanded uncertainty U_{rel} (%)	11

where:
u_r: repeatability
u_{ip}: intermediate precision
u_{vp}: Partition volume
u_c: combined
s(w): number of within day replicate
s(b): number of between day replicates
U: Expanded uncertainty

INM-CO

Mathematical model for copy number concentration of study material 1 and 2 ($C_{S1/S2}$)

(Equation 1)

$$C_{S1/S2} = \frac{C_N + C_{ORF1ab}}{2} \qquad \text{Eq. 1}$$

Where:

$C_{N\,/\,ORF1ab}$: Copy number concentration of N or ORF1ab genes measured by RT-dPCR, calculated according to Equation 2

$$C_{N\ /\ ORF1ab} = \frac{\lambda}{v * D} * R \qquad \text{Eq. 2}$$

Where:

λ: Copy number per partition
v: Droplet volume
D: Gravimetric dilution
R: Precision

Uncertainty budget for S1 is

Gene	N	ORF1ab
x /(μL^{-1})	511675	493441
uλ (%)	1.50%	1.43%
u v (%)	2.94%	2.94%
u D (%)	0.08%	0.30%
u R (%)	8.08%	8.51%
Relative combined uncertainty (%)	8.73%	9.12%
Combined uncertainty	44657	44982
u bias N-ORF 1ab	5264	
Combined uncertainty for S1	45127	
k	2	
Expanded uncertainty	90255	
Relative expanded uncertainty	18%	

Uncertainty budget for S2 is

Gene	N	ORF1ab
x/ (μL^{-1})	49.028686	44.714261
uλ (%)	11.05%	13.49%
u v (%)	2.94%	2.94%
u D (%)	0.05%	0.06%
u R (%)	11.45%	8.10%
Relative combined uncertainty (%)	16.18%	16.01%
Combined uncertainty	7.93	7.16
u bias N-ORF 1ab	1.25	
Combined uncertainty for S1	7.54	
k	2.00	
Expanded uncertainty	15.09	

Relative expanded uncertainty	32%

INRIM

Submitted values of S1 and S2 ($C_{S1/S2}$) were calculated using equation below:

$$C_{S1/S2} = D_f \times -\frac{\ln\left(\frac{(N_{neg}/N)_N + (N_{neg}/N)_E + (N_{neg}/N)_{ORF}}{3}\right)}{V_d}$$

Where:
D_f = dilution factor;
V_d = droplet volume

The uncertainty budget for S1 is:

Component (y)	**value**	**Uncertainty u(y)**		**Sensibility coefficient** $c_y = \frac{\partial x}{\partial y}$	**Contribution to u(C_{HER2})** $c_y \times u(y)$
		source	Standard uncertainty		
D_f	4˙145	Pipette calibration	372.98	$-\frac{ln(\frac{N_{neg}}{N})}{V_d}$	72˙534
N_{neg} / N	0.860	Measurement reproducibility	0.047	$-\frac{D_f}{(\frac{N_{neg}}{N})\times V_d}$	-0.175
V_d	7.76x10^{-4}	Volume variability	3.54x10^{-5}	$\frac{D_f \times ln(\frac{N_{neg}}{N})}{V_d^2}$	-3.67x10^{4}
$u(C_{S1}) = \sqrt{(72\,534)^2 + (-0.175)^2 + (-3.67x10^4)^2}$					
		C_{s1} [µL^{-1}]	**u(C_{s1}) [µL^{-1}]**	**U(C_{s1}) [µL^{-1}], k=2**	**U(C_{s1}) [%], k=2**
		806˙122	**81˙299**	**162˙597**	**20.17**

The uncertainty budget for S2 is:

Component (y)	**value**	**Uncertainty u(y)**		**Sensibility coefficient** $c_y = \frac{\partial x}{\partial y}$	**Contribution to u(C_{HER2})** $c_y \times u(y)$
		source	Standard uncertainty		

D_f	20	Pipette calibration	1.04	$-\frac{ln(\frac{N_{neg}}{N})}{V_d}$	2.73
N_{neg} / N	0.9980	Measurement reproducibility	0.0008	$-\frac{D_f}{(\frac{N_{neg}}{N})\times V_d}$	-1.32 x10^{-5}
V_d	7.76x10^{-4}	Volume variability	3.45x10^{-5}	$\frac{D_f \times ln(\frac{N_{neg}}{N})}{V_d^2}$	-2.43
$u(C_{S2}) = \sqrt{(2.73)^2 + (-1.32x10^{-5})^2 + (-2.43)^2}$					
		C_{S2} [µL^{-1}]	**u(C_{S2}) [µL^{-1}]**	**U(C_{S2}) [µL^{-1}], k=2**	**U(C_{S2}) [%], k=2**
		53.31	**3.65**	**7.31**	**13.71**

NMIA

Submitted values of S1 and S2 ($C_{S1/S2}$) were calculated using equation below:

$$C_{S1/S2} = \frac{\frac{C_{RT-dPCR,E}}{E_{RT,E}} + \frac{C_{RT-dPCR,N}}{E_{RT,N}}}{2}$$

Where $C_{\text{RT-dPCR}}$ is copy number concentration of *E* and *N* gene measured by RT-dPCR, and *RT* is reverse transcription efficiency calculated from short synthetic RNA templates corresponding to each assay using the equation below:

$$RT = \frac{C_{\text{RT-dPCR}}}{C_{\text{IDMS}}}$$

Uncertainty budget for S1 individual assays:

Gene	E	N
x/ (µL^{-1})	8.97E+05	8.09E+05
Method precision *uprec,rel* (%)	3.69	3.34
Droplet volume (Type B) *uVd,rel* (%)	1.24	1.24
Subsample variance *uhom,rel* (%)	0.34	0.96

RT efficiency (%)	9.44	10.28
Gravimetric dilutions (%)	0.14	0.14
Assay bias (%)	3.04	3.04
RT efficiency commutability (%)	6.10	6.10
Combined relative standard uncertainty (%)	12.3	12.9
Standard uncertainty x /(μL^{-1})	1.10E+05	1.04E+05
Effective degrees of freedom	26.1	29.4

Uncertainty budget for S1 combined assays:

x /（μL^{-1}）	8.5E+05
Relative standard uncertainty u_{rel} (%)	8.9
Standard uncertainty u /(μL^{-1})	7.6E+04
Effective degrees of freedom	55.27
Coverage factor	2.00
Relative expanded uncertainty U_{rel} (%)	18
Expanded uncertainty U/（μL^{-1}）	1.5E+05

Uncertainty budget for S2 individual assays:

Gene	E	N
x /(μL^{-1})	69.30	61.61
Method precision *uprec,rel* (%)	10.60	9.96
Droplet volume (Type B) *uVd,rel* (%)	1.24	1.24

Subsample variance *uhom,rel* (%)	3.06	2.88
RT efficiency (%)	9.44	10.28
Gravimetric dilutions (%)	0.11	0.11
Assay bias (%)	3.04	3.04
RT efficiency commutability (%)	6.10	6.10
Combined relative standard uncertainty (%)	12.9	14.0
Standard uncertainty *x* /(μL^{-1})	8.95	8.62
Effective degrees of freedom	30.7	34.9

Uncertainty budget for S2 combined assays:

x / （μL^{-1}）	66
Relative standard uncertainty u_{rel} (%)	11.4%
Standard uncertainty *u*/ (μL^{-1})	7
Effective degrees of freedom	88.42
Coverage factor	1.99
Relative expanded uncertainty U_{rel} (%)	23%
Expanded uncertainty *U*/ （μL^{-1}）	15

VNIIM

Submitted values of C_S were calculated using equation:

$$C_s(D_F, N_T, N_P, V_{drop}) = D_F \times \left(\frac{1}{N_T \times V_{drop}}\right) \times \frac{\left(\log\left(1 - \frac{N_P}{N_T}\right)\right)}{\left(\log\left(1 - \frac{1}{N_T}\right)\right)}$$

The uncertainty were calculated using equation:

$$u = \sqrt[2]{u_{Df}^2 + u_{rep}^2 + u_{part}^2}$$

Uncertainty associated with RT was not taken into account.

Sourse of uncertainty (rel)	**S1**	**S2**
Repeatability, u_{rep} (%)	6,6	7,2
Dilution factor, u_{Df} (%)	2,5	1,4
Partition volume, u_{part} (%)	0,85	0,85
Relative Combined uncertainty (%)	7,1	7,38
Combined uncertainty, u /(µL^{-1})	48085	3,26
k		2
Expanded uncertainty, U/ (µL^{-1})	96171	6,52
Relative expanded uncertainty U_{rel} (%)	14	15

NIB

The uncertainty budget for S1 is:

Genes (ORF 1ab, E and N)	
x /(µL^{-1})	553451
Repeatability and intermediate precision uC_{prec} (%)	3.02
Partition volume	2.90

uD （%）	
Homogeneity *uH* （%）	1.2
Relative Combined uncertainty $u_{c,rel}$ （%）	3.54
Combined uncertainty u_c /（μL^{-1}）	19573
k	2
Expanded uncertainty *U*/（μL^{-1}）	39147
Relative expanded uncertainty U_{rel} %）	7.07

APPENDIX J: Additional results

Experimental details of NIM(1) for S0

(1) Experimental Design:

The IDMS method used by group 1 of NIM was as described in APPENDIX B: Coordinating laboratory methodology.

Five units of S0 were measured by IDMS at three separate days. Two units were measured on the first two days and one unit on the third day. Each unit was divided into two subsamples and enzymatically hydrolysed into NMP. The RNA concentration of each unit were calculated based on the concentration of each NMP.

(2) Measurement uncertainty.

The uncertainty of each NMP was evaluated, respectively. The uncertainty of reproducibility of the measurement was evaluated as type A uncertainty and calculated as the relative standard deviation of the three-day results. The uncertainty budget of each NMP quantification is shown Table J-1.

Table J-1: Uncertainty contributions to coordinator's assigned values (S0).

Uncertainty sources		CMP	UMP	AMP	GMP
Relative uncertainty	Reproducibility	0.029	0.045	0.036	0.053
	Weighing of samples	0.005	0.005	0.005	0.005
	Weighing of stock solutions of calibrator	0.004	0.004	0.004	0.004
	Weighing of high calibration blends	0.005	0.005	0.005	0.005
	Weighing of low calibration blends	0.005	0.005	0.005	0.005
	Purity of CRM	0.003	0.002	0.004	0.003
	Combined relative uncertainty	0.031	0.046	0.037	0.054
Combined uncertainty (u_{NMP}, ng μL^{-1})		0.025	0.040	0.029	0.043
$u_{(correlation)}$		0.0085			
Standard uncertainty of S0 (u, ng μL^{-1})*		0.029			

*Standard uncertainty of S0 was calculated by the following equation:

$$u(\mathrm{S0}) = \frac{1}{4}\sqrt{u^2{}_{\mathrm{CMP}} + u^2{}_{\mathrm{UMP}} + u^2{}_{\mathrm{AMP}} + u^2{}_{\mathrm{GMP}} + u_{correlation}}$$

$$u_{correlation} = 2u_{CMP}u_{UMP}r_{CMP,UMP} + 2u_{CMP}u_{AMP}r_{CMP,AMP} + 2u_{CMP}u_{GMP}r_{CMP,GMP} + 2u_{UMP}u_{AMP}r_{UMP,AMP} + 2u_{UMP}u_{GMP}r_{UMP,GMP} + 2u_{AMP}u_{GMP}r_{AMP,GMP}$$

Where *r* is the correlation coefficient.

Experimental details of NIM(2) for S0

(1) Experimental Design:

The IDMS method used by group 2 of NIM was as described in Analytical Information below. Two units of S0 were measured by IDMS. Each unit was divided into two subsamples and enzymatically hydrolysed into NMP. The RNA concentration of each unit were calculated based on the concentration of each NMP.

(2) Measurement uncertainty.

The uncertainty of each NMP was evaluated, respectively. The uncertainty of reproducibility of the measurement was evaluated as type A uncertainty and calculated as the relative standard deviation of seven independent results. The uncertainty budget of each NMP quantification is shown Table J-2.

Table J-2 The uncertainty budget of each NMP quantification.

Uncertainty component	Relative uncertainty(%)			
	Adenine (u_A)	Cytosine (u_C)	Guanine (u_G)	Uracil (u_U)
Method reproducibility	9.63	8.50	15.70	5.42
Weighing of enzyme	0.57	0.57	0.57	0.57
STD purity (u_{STD})	0.35	0.30	0.30	0.20
Weighing-STD preparition($u_{Std\text{-}prep}$)	0.11	0.11	0.11	0.11
Weighing-Sample preparition($u_{Sample\text{-}prep}$)	0.005	0.006	0.005	0.005
Combined relative uncertainty	9.70	8.50	15.70	5.50

$$\mathrm{u(S0)} = \frac{1}{4}\sqrt{u^2{}_{\mathrm{CMP}} + u^2{}_{\mathrm{UMP}} + u^2{}_{\mathrm{AMP}} + u^2{}_{\mathrm{GMP}}}$$

The optimized chromatograms are shown in Figure 1.

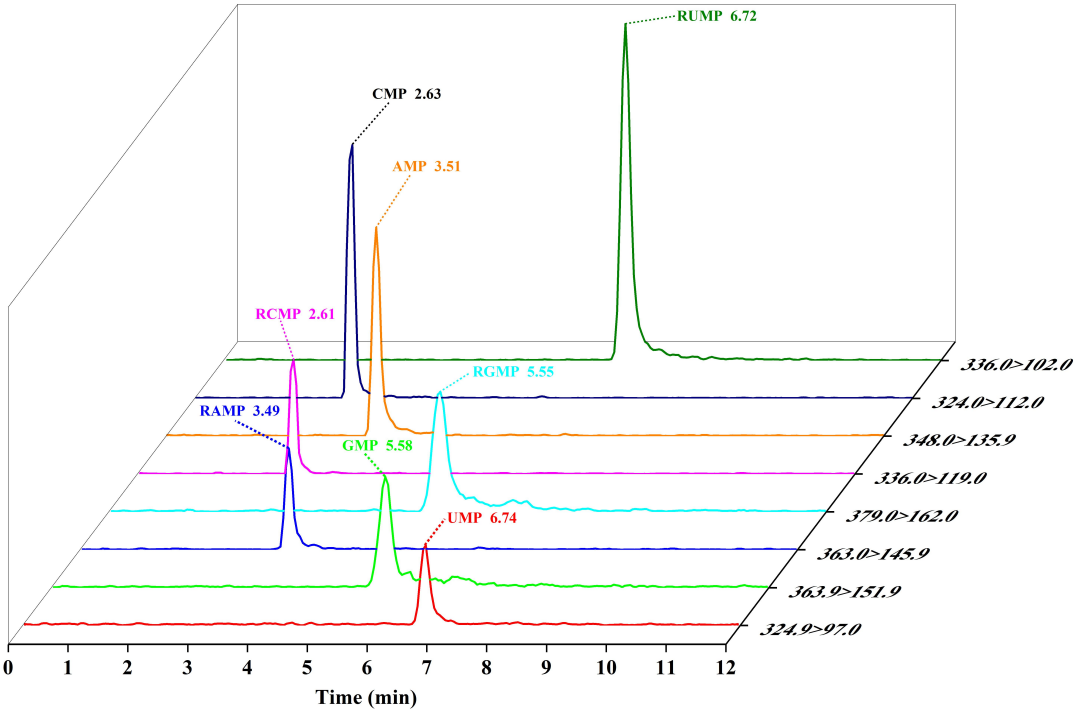


Figure J-1. LC-MS chromatograms of AMP, CMP, GMP and UMP

(3) Analytical Information

Sample preparation

Sample amount used for analysis (uL)		5
Method of enzymolysis	**Type** (e.g., Gas-phase, Liquid-phase, microwave-assisted, etc.)	Liquid-phase
	Conditions (e.g., temperature, time, acid, etc.)	In this experiment, 50 μL of samples, 5 μL of internal standard and 1 μL of enzyme were mixed evenly, then the temperature of PCR was set to 37℃ for

		15 min, and finally the temperature of PCR was set to 65℃ for 15 min to terminate the reaction.
Further preparation steps		The enzymolysis samples were centrifuged at high speed and then tested on the machine.

Analysis

Analytical instrumentation used (e.g., LC, GC, and more manufacturer, etc.)	The MS/MS acquisition was carried out using a home- made Q-LIT 6610 MS system (Mass Spectrometry Engineering Technology Research Center, Center for Advanced Measurement Science, National Institute of Metrology, Beijing, P.R. China). Chromatographic separation was performed on a HPLC system (Elite, Dalian, China).
Detection method used	Q-LIT 6610 mass spectrometer was operated in the positive ESI source using selected reaction monitoring (SRM) mode, which was used to determine the molar concentration of AMP, CMP, GMP and UMP.
Calibration method used (Quantification mode and calibration design)	Concentration determination by Internal standard method.
Chromatographic Column (i.e., specify type and manufacturer)	A Elite C_{18} column (150 mm×4.6 mm I.D., 5 μm particle size; Elite, China) was used for separation.

Chromatographic Conditions (e.g., gradient or temperature program)	Mobile phase A is water, containing 0.1% formic acid, and mobile phase B is methanol. Elution was performed isocratically at a flow rate of 600 µL/min, the injection volume was 5 µL, and the column oven temperature was maintained at 40 °C.
Calibration type/details	Internal calibration mode with bracket quantitative method
Calibration standards (e.g., source, purity, and traceability of standards)	CRMs of amino acid were produced by National Institute of Metrology, P.R. China. Each certified value was in the following: 5-AMP (GBW100154): 98.9% ± 0.7% (k = 2) 5-CMP (GBW100067): 99.3% ± 0.6% (k = 2) 5-GMPNa_2 (GBW100068): 98.8% ±0.6% (k = 2) 5-UMPNa_2 (GBW100069): 99.4% ± 0.4% (k = 2) Isotope labeled amino acid were purchased from Silantes Company, Germany. $^{13}C_9{}^{15}N_3$-CMP, $^{13}C_9{}^{15}N_2$-UMP, $^{13}C_{10}{}^{15}N_5$-AMP, $^{13}C_{10}{}^{15}N_5$-GMP were used as internal standard.
Indicate ion/MRM monitored in Mass Spec. (if applicable)	Precursor-to-fragment transitions of each analyte used were the following: 348.0 -> 135.9 (AMP), 363.0 -> 145.9 (rAMP); 323.9 -> 112.0 (CMP), 336.0 -> 119.0 (rCMP); 363.9 -> 151.9 (GMP), 379.0 -> 162.0 (rGMP);

	324.9 -> 97.0 (UMP) and 336.0 -> 102.0 (rUMP).
Additional Comments or Observations	The impurity had been checked .The related information was in the supporting information.

Experimental details of NIMA for S0

(1) Experimental information**:** Please complete CCQM-k181 supporting information.

(2) Experimental Design: Please describe or show in diagrammatic form the experimental design which was applied.

- One vial of S0 was analysed in triplicate by capillary electrophoresis (Bioanalyser) for purity evaluation. A typical electrophorogram is given below and shows that there were significant amounts of impurities; the average purity from triplicate analyses was 70.7% (time corrected area).
- For IDMS analysis, 1 subsample from 3 separate vials and 2 subsamples from another vial, each subsample was 50 μL, were enzymatically hydrolysed to nucleotide monophosphates with equivalent amounts of labelled analogues in a sample blend. Amounts of each nucleotide monophosphate were obtained by comparison to matching calibration blends prepared using nucleotide monophosphate solutions with concentration values measured by quantitative NMR.
- The procedure was essentially as given in Burke et al 2013.
- The amounts of each of the 4 nucleotide monophosphates (CMP, UMP, GMP, AMP) were measured in 5 independent subsamples and the total RNA concentration for each subsample was calculated as the sum of the 4 nucleotide monophosphate concentrations.

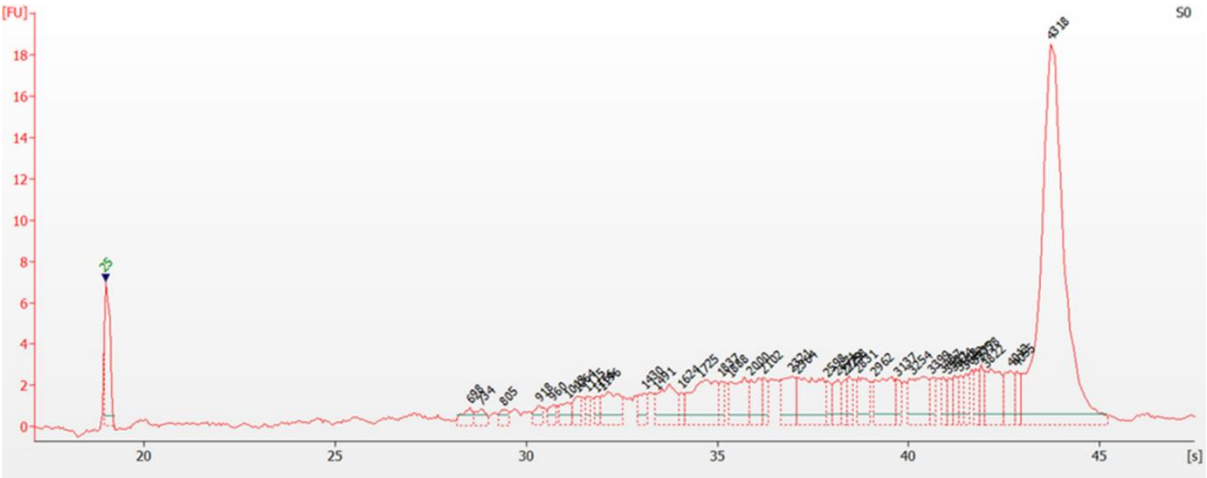


Figure J-2 Typical electrophorogram of S0 on Bioanalyser.

(3) Measurement uncertainty. Please summarise the calculation approach used and list the factors which were included

The major factors affecting uncertainty were between vial variance and within vials reproducibility of each NMP concentration and were calculates as the standard deviation of total RNA concentrations between vials and pooled standard deviation of concentrations of each NMP for each vial. The uncertainties of concentrations of calibration solutions were from the internal standard CRM uncertainty, precision of qNMR measurements and gravimetric preparation of the qNMR solutions.

The relative standard uncertainties for these factors were combined as the square root of the sum of the squares. Total effective degrees of freedom was calculated using the Welch-Satterthwaite equation and the coverage factor from the t-distribution.

Table J-1. Components for IDMS measurement uncertainty budget.

Factor	u/x	Proportion of combined uncertainty
Between vials	3.17%	42.6%
Within NMPs	3.67%	56.9%
QNMR GMP	0.22%	0.2%
QNMR CMP	0.16%	0.1%
QNMR AMP	0.17%	0.1%
QNMR UMP	0.16%	0.1%

(4) Analytical Information

The experimental conditions were essentially as given in Burke *et al* 2013 with the following differences.

1. 0.05 g sample was digested instead of 0.1 g as published
2. Nucleotide monophosphate solutions were calibrated by quantitative NMR

Burke, D. G., *et al*. (2013). "Digital Polymerase Chain Reaction Measured pUC19 Marker as Calibrant for HPLC Measurement of DNA Quantity." Analytical Chemistry **85**(3): 1657-1664.

Table J-2 Analysis infromation

<table>
<tr><td>Calibration standards

(e.g., source, purity, and traceability of standards)</td><td>Calibration standards were prepared from analytical grade chemicals and concentrations were measured using quantitative NMR with dimethylsulphone CRM as internal standard

<table><tr><th>Nucleotide</th><th>nmol/g</th></tr><tr><td>AMP</td><td>682.6</td></tr><tr><td>UMP</td><td>584.3</td></tr><tr><td>GMP</td><td>605.7</td></tr><tr><td>CMP</td><td>667.5</td></tr></table></td></tr>
</table>

Experimental details of NMIJ for S0

(1) Experimental information**:** Please complete CCQM-k181 supporting information. Described below.

(2) Experimental Design: Please describe or show in diagrammatic form the experimental design which was applied.

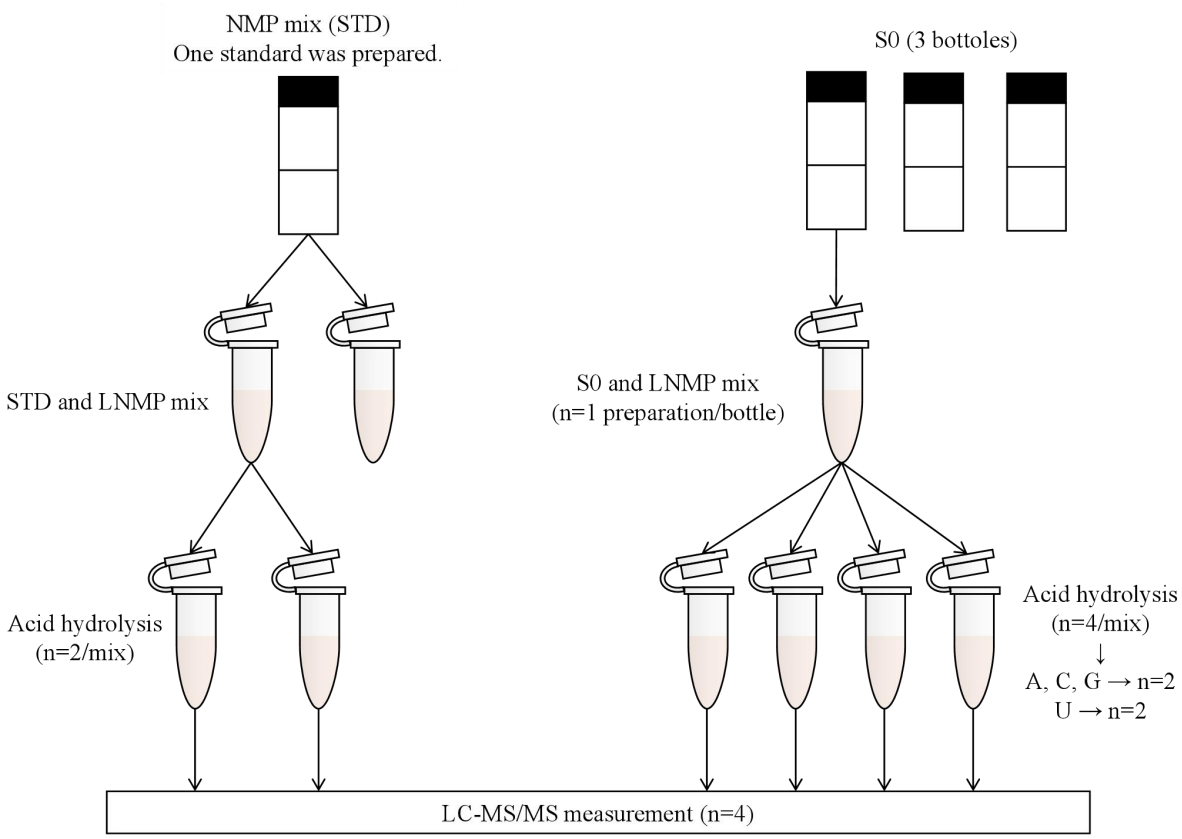


Figure J-2 Experimental design of S0 quantification by acid hydrolysis-LC-IDMS. One NMP standard mixture was prepared, divided into two bottles and mixed with LNMP mixture. Then, each standard mixture was subjected to two independent acid hydrolysis. Three bottles of S0 were mixed with LNMP mixture and divided into four bottles. One bottle of STD/LNMP mix and two of S0/LNMP mix were subjected to acid hydrolysis, 150 oC for 12 H to quantify Adenine, Cytosine and Guanine, and the others were applied to acid hydrolysis, 150 oC for 48 H to quantify Uracil. After hydrolysis, all STDs and samples were measured four times each by LC-MS/MS. The nucleobases were target measurand of acid hydrolysis-LC-IDMS.

(3) Measurement uncertainty.

To evaluate the measurement uncertainty of acid hydrolysis-LC-IDMS, the uncertainty of each nucleobase quantification (u_{base}) was evaluated, respectively. The uncertainty of the between vials (u_{vial}), between acid hydrolysis ($u_{ohydrolysis}$) and LC-MS/MS measurement ($u_{measurement}$) was evaluated by two-way ANOVA. Then, the uncertainty of NMP STD concentration (u_{STD}), weighing for STD preparation (u_{STD_prep}), and weighing for sample preparation (u_{Sample_prep}) were combined. The uncertainty budget of each nucleobase quantification is shown Table J-1.

Table J-3 The uncertainty budget of each nucleobase quantification.

Uncertainty component	Relative uncertainty (%)			
	Adenine (u_A)	Cytosine (u_C)	Guanine (u_G)	Uracil (u_U)
Vial (u_{vaial})	5.21	5.66	5.23	-
Acid hydrolysis ($u_{hydrolysis}$)	0.91	0.00	0.39	1.24
Measurement ($u_{measurement}$)	0.35	0.78	0.33	1.49
STD conc. (u_{STD})	0.64	0.97	1.21	0.58
Weighing_STD preparation (u_{STD_prep})	0.15	0.21	0.18	0.15
Weighing_Sample preparation (u_{Sample_prep})	0.05	0.05	0.05	0.05
Combined standard uncertainty	5.34	5.80	5.40	2.03

To estimate total S0 uncertainty, the equation 1 was used for calculation.

$$u_{S0} = \sqrt{\left({}^{u_A}/_4\right)^2 + \left({}^{u_c}/_4\right)^2 + \left({}^{u_G}/_4\right)^2 + \left({}^{u_U}/_4\right)^2 + u_{between_base}{}^2} \qquad \text{-Eq. J-1}$$

Here, $u_{between_base}$ was calculated from SD of the quantified value of each nucleobase, 3.22 %.

(4) Analytical Information

Table J-4 Sample preparation

Sample amount used for analysis (μL)		25 mL of sample was used for one acid hydrolysis
Method of hydrolysis	Type (e.g., Gas-phase, Liquid-phase, microwave-assisted, etc.)	Liquid-phase acid hydrolysis
	Conditions (e.g., temperature, time, acid, etc.)	Adenine, Cytosine, Guanine: 150 °C for 12 h by 88 % of formic acid Uracil: 150 °C for 48 h by 88 % of formic acid
Further preparation steps		

Table J-5 Analysis information

Analytical instrumentation used (e.g., LC, GC, and more manufacturer, etc.)	LC30 series and LCMS-8040 (Shimadzu) were used for quantification.
Detection method used	MRM mode
Calibration method used (Quantification mode and calibration design)	Internal standard method (isotope labelled-nucleobases were used as internal standards)
Chromatographic Column (i.e., specify type and manufacturer)	Kinetex XB-C18 (4.6 mm x 250 mm, Phenomenex)
Chromatographic Conditions (e.g., gradient or temperature program)	Isocratic elution (A) 0.1 % CH3COOH/(B) MeOH = 92/8 Flowrate: 0.5 mL/min Column temperature: 37 oC
Calibration type/details	Single-point calibration
Calibration standards (e.g., source, purity, and traceability of standards)	In-house NMP standard of which purity were determined by qNMR (Value ± U) nmol/g AMP: (1221 ± 16) nmol/g CMP: (1340 ± 26) nmol/g GMP: (1180 ± 29) nmol/g UMP: (1312 ± 15) nmol/g
Indicate ion/MRM monitored in Mass Spec. (if applicable)	Adenine: 136.10 → 119.00

	LAdenine: 146.10 → 128.00 Cytosine: 112.10 → 94.95 LCytosine: 119.10 → 101.00 Guanine: 152.10 → 135.00 LGuanine: 162.10 → 143.95 Uracil: 113.10 → 70.15 LUracil: 119.10 → 74.00
Additional Comments or Observations	

APPENDIX K: In-house integration method for impurity content measured by bioanalysis

1. Brief introduction

In order to accurately calculate the detection response signal of the Agilent 2100 bioanalyzer, we first exported the original CSV file of the data generated by the instrument software, which includes the detection time (time) and signal response value (value). Subsequently, the original spectrum was reconstructed and displayed using Python software, as shown in Figures 1-3 (in blue). There was no difference in the instrument response signals, but the instrument software generated the [nt] abscissa based on the relationship between the reference material and time; Due to the unclear relationship between the internal [nt] of the instrument and time, we directly display it using time as the x-axis, preserving the most primitive characteristics of the instrument's detection signal. From Figures 1-3, it can be seen that there is no significant difference in the image structure and area characteristics between our image reconstruction results and the original parsing software.

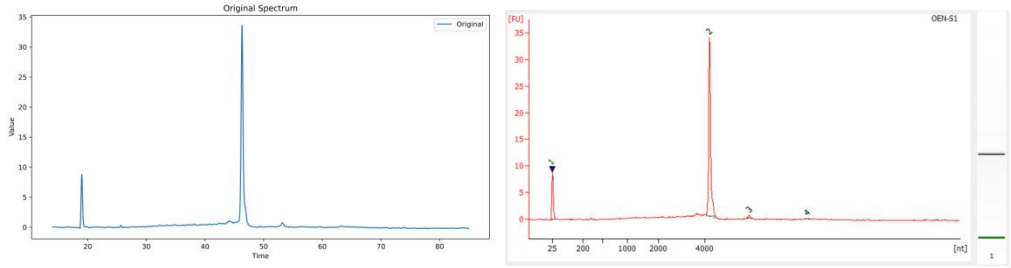


Figure 1.Rep-1 the relationship between time and signal response value(blue) and between size (nt) and signal response value(red)

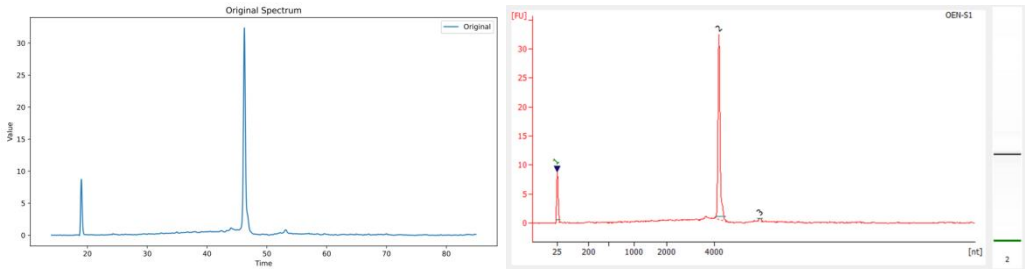


Figure 2. Rep-2 the relationship between time and signal response value(blue) and between size (nt) and signal response value(red)

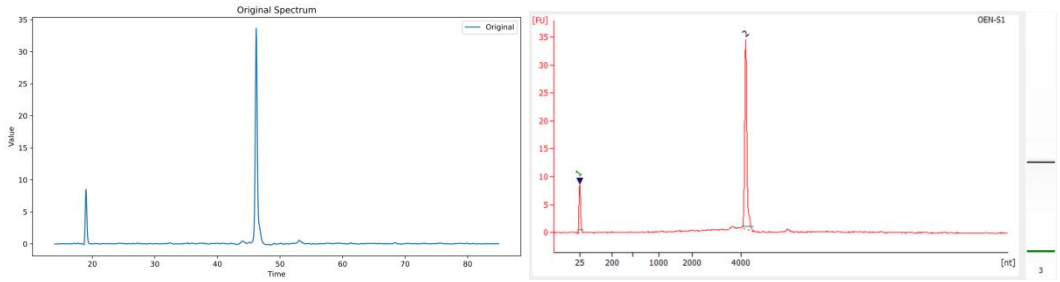


Figure 3. Rep-3 the relationship between time and signal response value(blue) and between size (nt) and signal response value(red)

Next, we will use Rep-3 as a case study to provide a detailed introduction to our data processing and final area extraction process.

2. Detail process of the integration

Step 1: Complete baseline calibration

The significance of baseline calibration is to remove baseline offset or drift caused by instruments, sample containers, or other factors, to ensure that the instrument detection results reflect the characteristics of the sample itself.

The main process of this study is to complete the baseline deduction method on the original data. The baseline is achieved by selecting data points from non peak regions and using polynomial equations. The fitted curve is shown in Figure 4. Meanwhile, based on the results of the linear equation, we corrected the baseline and plotted a reference standard line for the instrument's detection signal value response to 0, as shown in Figure 5. The response signal with material content should appear above 0 to be reasonable, which also provides an important basis for us to choose the integration region.

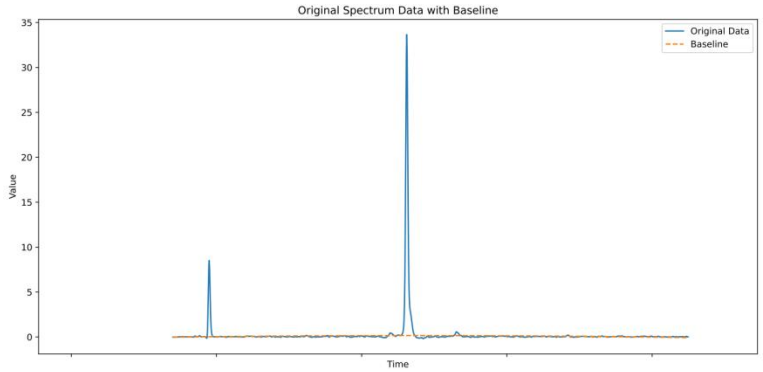


Figure 4. Baseline calibration. The yellow dashed line represents the linear equation result of polynomial fitting, and based on this linear model, the baseline is calibrated.

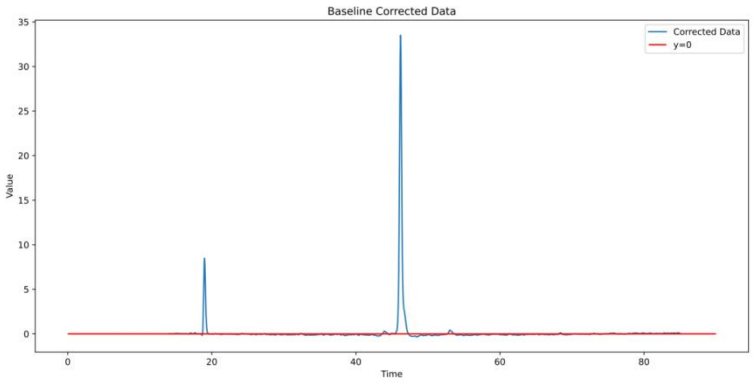
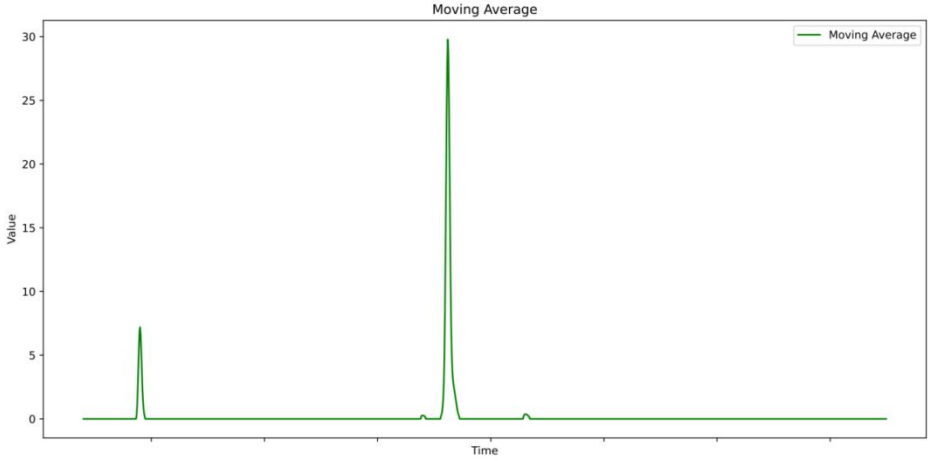


Figure 5. The corrected signal response result. The red line represents the zero value point of the signal response.

Step 2: Signal denoising and smoothing

The noise in the baseline corrected data is still quite noticeable, so we have performed noise reduction and smoothing on the data. Mainly using the Moving Average model, MA and Wavelet Denoising are two methods for denoising.

A. Moving Average model (MA), MA is a commonly used smoothing technique in signal processing and time series analysis. The moving average model smooths time series by averaging a certain number of data points (known as window size). Each average is calculated as the window slides from one end of the dataset to the other. By smoothing data, moving average can reduce random fluctuations, making trends and cyclical components more pronounced, thus helping to identify and locate peaks. It can effectively reduce random fluctuations in data and help reveal underlying trends. Disadvantage: Excessive smoothing may lead to the loss of useful signal details, especially at important features of the signal (such as abrupt changes or sharp peaks), which is not conducive to quantification. The results of the moving average model are shown in Figure 6. In addition to the standard reference material points, three spectral peak signals were identified, which has certain guiding significance in the selection of peaks.



Figue 6. Moving average model noise reduction results

B. Wavelet transform denoising: it is an efficient signal processing technique that utilizes the multi-scale decomposition ability of wavelet transform to separate and reduce noise components in data, while retaining important signal features, suitable for calculating spectral peak area. The results after noise reduction are shown in Figure 7. In Figure 8, we standardized the baseline value of 0. Under normal circumstances, the spectral peak response signal should not be negative, so the area in the negative part of the spectral peak should not be included in the integrated area. This result provides basic information reference for subsequent integration evaluation.

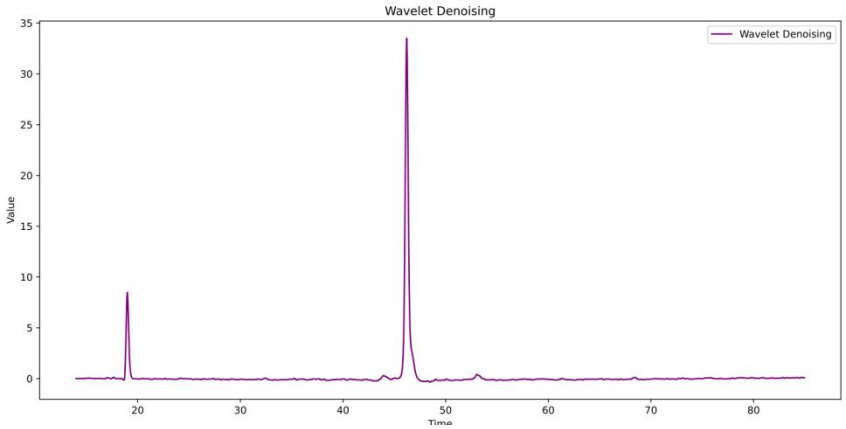


Figure 7. Wavelet transform denoising results.

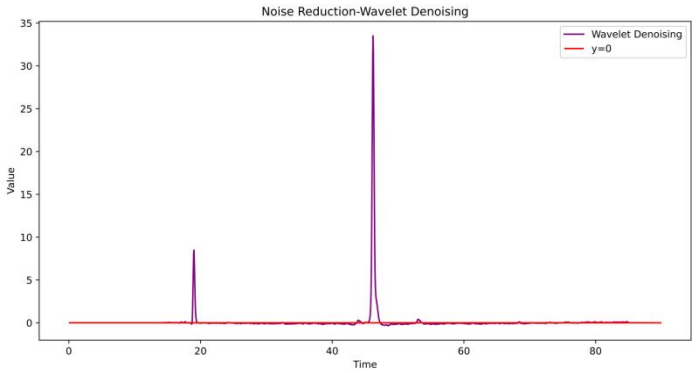


Figure 8. The result of wavelet transform denoising with a baseline value of 0.

Step 3: Determination of peaks

Find local maximum value：

This step involves searching for all data points that are higher than their adjacent points. These points are considered as candidate points for local maximum values.

Filter significance：

Use the "prominence" parameter as the filtering criterion. Significance is defined as the vertical distance between a peak and its nearest lower valley. Only retain local maximum values with significance higher than the set threshold. For example, the significance threshold can be set to a peak value that is at least 20% of its average relative to the local minimum value.

Filter height：

Use the height parameter to further filter for peaks. Height refers to the absolute height of a peak relative to its nearest lower trough. Only retain the peaks that reach at least the set height parameter value.

The Rep-3 spectral peak information is shown in Figure 9, and a total of four spectral peak signal points have been identified.

Integration:

In this study, we used the trapezoidal method (integration) to integrate the peak area. The integration result is shown in Figure 9.

Purity is: 13.42÷（13.42+0.12+0.14）=98.1%。

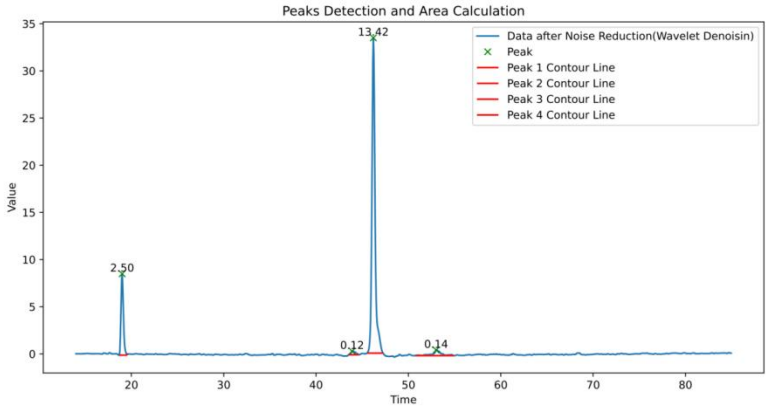


Figure 9. Detected peak and integration

3. Summary

Based on the above processing process, integrate the three plots (figure 10) to obtain the proportion of the main peak area as shown in the table below. This is not significantly different from the result base on the integration and calculated by using the original data exported by Agilent 2100 shown in table 2 and figure 11.

Table 1. Integration by using the above process

Detected peak	Peak area		
	Rep 1	Rep 2	Rep 3
1	0.05	0.35	0.12
2	0.4	13.17	13.42
3	13.96	0.06	0.14
4	0.08		
Total area	14.49	13.58	13.68
Proportion of the major peak area	0.96342305	0.969808542	0.980994152
Average Proportion	0.9714		
SD	0.0089		
RSD	0.0092		

Table 2.Integration by using the automatic exported data by Agilent 2100

Detected peak	Peak area		
	rep1	rep2	rep3
1	62.07	58.88	0.24
2	0.91	0.79	63.43
3	0.32		0.83
4	/	/	/

Total area	63.3	59.67	64.5
Proportion of the major peak area	0.98056872	0.986760516	0.983410853
Average Proportion	0.9836		
SD	0.0031		
RSD	0.0031		

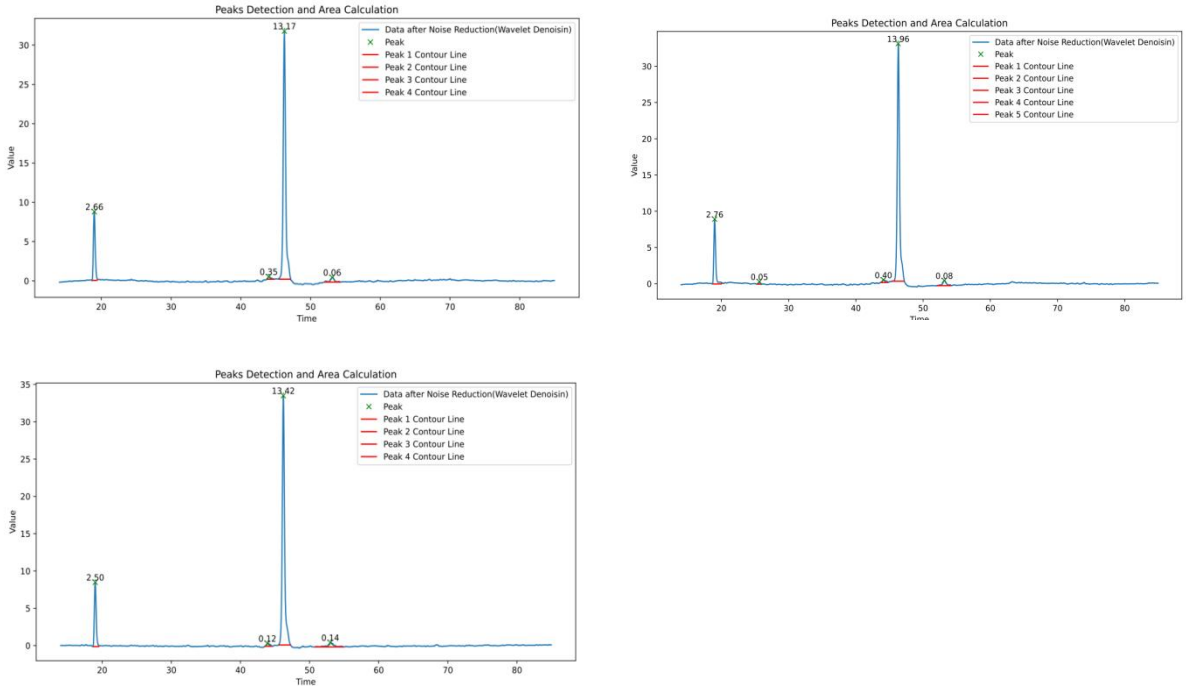


Figure 10. Graph and integration base on the above process

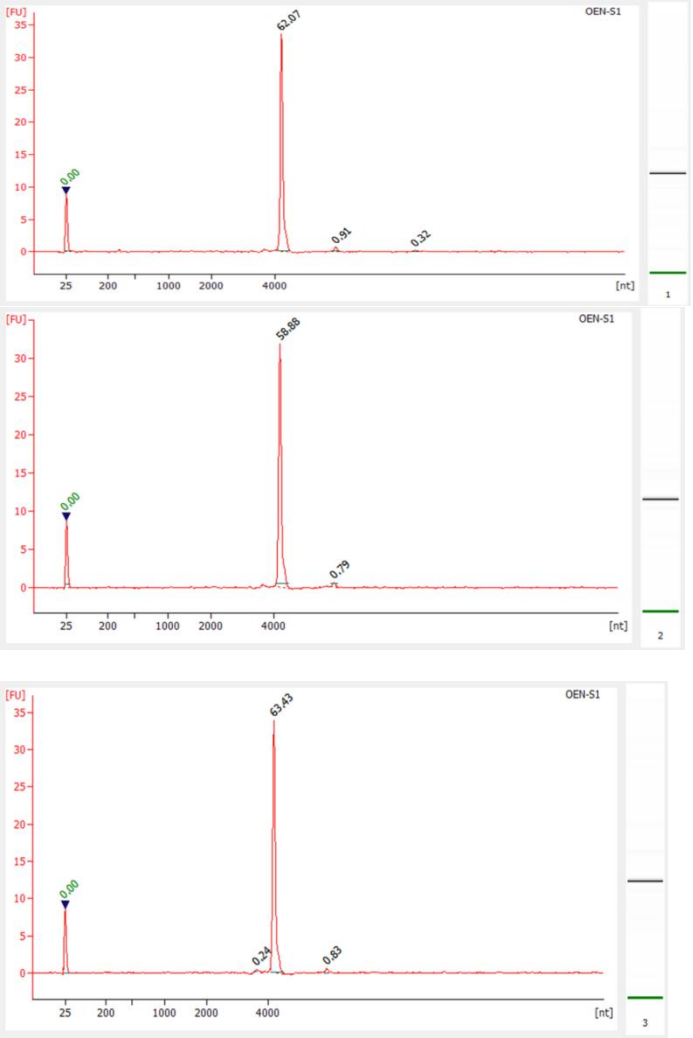


Figure 11. Graph and integration base on the automatic exported data by Agilent 2100