CCQM-K176

Breast cancer biomarker *HER2* copy number variation (CNV) measurement

Key Comparison

Final Report

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SUMMARY

Breast cancer is the most common malignant tumor in women all over the world. Breast cancers with amplification of the human epidermal growth factor receptor 2 (HER2) gene (referred to as HER2-positive breast cancer) accounts for about 20% ~ 30% of invasive breast cancer, and this type of tumor is characterized by high invasiveness, high risk of recurrence, rapid progression and poor prognosis. The determination of *HER2* gene expression or copy number variant (CNV) (*HER2* copy number normalized to cell number or a reference gene) in tumor cells of breast cancer patients is beneficial to the choice of treatment, through targeted therapies such as trastuzumab, and prognosis. Testing of the core competencies of laboratories to deliver services of CNV measurements for different genetic analytes has not been covered previously. Agreement was received in the October 2019 meeting of the NAWG in Torino to conduct the *HER2* CNV measurement as a key comparison. Evidence of successful participation in formal, relevant international comparisons is needed to support calibration and measurement capability claims (CMCs) made by national metrology institutes (NMIs) and designated institutes (DIs).

Ten National Metrology Institutions participated in the Track A Key Comparison: CCQM-K176 "Breast cancer biomarker *HER2* copy number variation (CNV) measurement". Participants were requested to evaluate the copy number concentration (expressed in [μL^{-1}]) and copy number ratio of defined genomic sequences in eukaryotic intact genomic DNA at an indicative concentration of 90~130 ng/μL. Participation in K176 required assay selection/design and quantitative detection of the analyte in the buffer. The objectives of the key comparison were to measure 1) *HER2* copy number concentration; 2) the copy number concentration of the single copy reference gene ribonuclease P RNA component H1 (*RPPH1*); 3) the *HER2*/ *RPPH1* copy number ratio, using two defined sequences representative of the two genes (*HER2* and *RPPH1*) in mixed genomic DNA samples, and 4) provide evidence for CMC claims by participating laboratories when measuring target sequence content in purified genomic DNA. All participants performed measurements by digital PCR (dPCR), with alternative assays and pre-treatment of samples (for example, restriction enzyme digestion). Interlaboratory reproducibility (%CV) was less than 13% and 5% for copy number concentration and *HER2*/*RPPH1* copy number ratio, respectively, demonstrating very high interlaboratory reproducibility in CNV measurements.

Successful participation in CCQM-K176 demonstrates the following measurement capabilities in determining copy number ratio (of a defined target sequence expressed relative to a reference gene sequence) in the range from 1.0 to 40.0 at a background of relatively high concentration of intact (non-fragmented) genomic DNA. The study will also support participants' claim for measurement of defined DNA sequence/gene copy number concentration in the range ~10^4-10^6 μL^{-1} (taking into account both reported reference gene and *HER2* copy number concentration values).

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ACRONYMS

CCQM	Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology
CMC	Calibration and Measurement Capability
CNV	copy number variant
CRM	certified reference material
CV	coefficient of variation, expressed in %: CV = 100·s/$\bar{x}$
DI	designated institute
DNA	deoxyribonucleic acid
DoE	degrees of equivalence
dPCR	digital PCR
DSL	DerSimonian Laird
FISH	fluorescence *in situ* hybridisation
gDNA	genomic DNA
HER2	human epidermal growth factor receptor 2 gene
IHC	immunohistochemistry
INDEL	insertion/deletion (genetic) variant
JCTLM	Joint Committee for Traceability in Laboratory Medicine
KC	Key Comparison
KCRV	Key Comparison Reference Value
MADe	median absolute deviation from the median (MAD)-based estimate of s: MADe = 1.4826·MAD, where MAD = median(|xi-median(xi)|)
MSD	Median scaled difference
NA	Nucleic Acid
NMI	national metrology institute
NAWG	Nucleic acid Analysis Working Group
PCR	polymerase chain reaction
qPCR	real-time quantitative PCR
REML	Restricted maximum likelihood
RMP	Reference Measurement Procedure
RNA	ribonucleic acid
RPPH1	human ribonuclease P RNA component H1 gene
SNV	single nucleotide variant
SV	structural (genetic) variant

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: $U(x) = k \cdot u(x)$

MSD median scaled difference: $\text{Med}\left(\left|x_i - x_j\right| \Big/ \sqrt{u_i^2 + u_j^2}\right)$

whereby value (x_i)/uncertainty(u_i) of laboratory (i) where j runs over all other reported values and u_j denotes the standard uncertainty associated with x_j.

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

$u(x_i)$' recalculated participant standard uncertainty for consistency in d_i (minimum value to be claimed in CMCs)

$\overline{u}(x)$ pooled uncertainty: $\overline{u}(x) = \sqrt{\sum_{i=1}^{n} u^2(x_i) / n}$

$U(x)$ expanded uncertainty

$U(x_i)$' recalculated participant expanded uncertainty for consistency in d_i (minimum value to be claimed in CMCs)

$U_{95}(x)$ 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

Breast cancer is the most common malignant tumor in women all over the world. Human epidermal growth factor receptor 2 (*HER2*)-positive breast cancer accounts for about 20% ~ 30% of invasive breast cancer, and this type of tumor is characterized by high invasiveness, high risk of recurrence, rapid progression and poor prognosis [1]. The *HER2* gene (NCBI Gene ID: 2064; official gene symbol *ERBB2*) is a proto-oncogene located on the long arm of human chromosome 17, coding a transmembrane glycoprotein with receptor tyrosine kinase activity. Over-expression caused by amplification of the *HER2* gene will lead to over-transmission of signals, which will stimulate the growth and metastasis of cancer cells [2].

The determination of *HER2* gene expression caused by increased gene copy number variant (CNV) in tumor cells of breast cancer patients is beneficial to the choice of treatment strategy through targeted therapies such as trastuzumab, and prognosis. *HER2* CNV is routinely measured by immunohistochemistry (IHC) or fluorescence in situ hybridization (FISH) in cancer tissue samples, however digital PCR (dPCR) has the benefit of providing quantitative results which can be informative in *HER2* equivocal tissue samples and liquid biopsy [3-6].

Testing of the core competencies of laboratories that deliver measurement services of CNVs and, in general, with **copy number ratio (of a defined target sequence relative to a reference sequence) with values** ≥1, has not been covered previously. The current study was proposed as a follow-up study to CCQM-P184 copy number concentration and fractional abundance of a mutation (single nucleotide variant (SNV) or insertion/deletion (INDEL))-containing template sequence mixed with wild-type DNA. This study aims to extend participants' capability from SNV and INDEL measurement to sequence structural variant (SV) measurement, focusing on gene copy number variation. SV were originally defined as insertions, deletions and inversions of sequences greater than 1 kb in size [7], but with the sequencing of human genomes now becoming routine, the operational spectrum of structural variants has widened to include events >50 bp in length [8]. CNVs are a type of SV where the number of repeats of a gene or genomic region varies compared to the reference genome and may result from deletion, insertion or duplication [9].

The current study also cements participating laboratories' capabilities to perform copy number concentration measurements, building on findings from CCQM-P154 that evaluated comparability of copy number concentration measurements of linearised plasmid DNA between dPCR and orthogonal techniques [10]. Evidence of successful participation in formal, relevant international comparisons is needed to support CMCs made by NMIs and DIs.

In October 2019, the Consultative Committee for Metrology in Chemistry and Biology (CCQM) approved the Key Comparison (KC) CCQM-K176 "Breast cancer biomarker *HER2* copy number variation (CNV) measurement". CCQM-K176 was designed to assess participants' capabilities

for copy number ratio and copy number concentration measurements of two target sequences in genomic DNA (gDNA).

The aim of the key comparison is to measure 1) *HER2* copy number concentration; 2) the copy number concentration of a single copy reference gene (ribonuclease P RNA component H1 (*RPPH1*); 3) the *HER2*/ *RPPH1* copy number ratio, using defined sequences representative of the two genes (*HER2* and *RPPH1*) in mixed gDNA samples, and 4) provide evidence for CMC claims by participating laboratories when measuring a target sequence in purified gDNA.

This study and K86 series studies on genetically modified organisms (GMOs) and food speciation support measurement of DNA copy number ratio of two defined target sequences. However, the difference is the range of copy number ratio is ≥1 in this study whereas it is normally between 0.1-100% (0.001-1) in GMO and food studies. However, both applications relate to the scenario whereby the reference gene (or endogenous gene in GMO studies) is expected to be stable (proportional to cell number) and forms the denominator in the copy number ratio.

The following sections of this report document the timeline of CCQM-K176, the measurands, study material, participants, results, and the measurement capability claims that participation in CCQM-K176 can support. The Appendices reproduce the official communication materials and summaries of information about the results provided by the participants.

MEASURANDS

The measurands of CCQM-K176 constitute the copy number concentrations and copy number ratio of *HER2* and *RPPH1* gene sequences in isolated human gDNA in buffered aqueous solution:

- Measurand 1 (M1): copy number concentration of *HER2* (μL^{-1})
- Measurand 2 (M2): copy number concentration of *RPPH1* (μL^{-1})
- Measurand 3 (M3): ratio of *HER2* copy number to *RPPH1* copy number (*HER2/RPPH1* copy number ratio, dimensionless quantity)

The *HER2* (*ERBB2*) gene spans a large region of gDNA (~40 kb) and contains 35 exons, therefore, selected exons (4, 16 and 24) were sequenced by the study coordinator and the sequences were provided to participants in the Study Protocol (Appendix C) to enable assay design. Likewise, the sequences of the reference gene *RPPH1* (340 bp region, single exon gene) were provided to participants.

STUDY MATERIALS

Background / Preparation of Study Material(s)

Two study materials (Sample 1 "S1" and Sample 2 "S2") with different *HER2* CNV levels prepared by mixing HER2 'normal' (2 *HER2* gene copies per diploid cell) and *HER2* 'positive' (*HER2*-amplified) gDNAs were provided by NIMC. The matrix is TE buffer. *HER2* 'positive' gDNA (*HER2*-amplified) was extracted from a HCC1954 cell line purchased from ATCC. *HER2* 'normal' gDNA was extracted from a cell line, PLCL7, originated from a healthy donor. The indicative range for the *HER2*/*RPPH1* of the analyte is 1.0~40, in a gDNA concentration of 90~130 ng/μL (based on Nanodrop 2000). The homogenized gDNA mixture was aliquoted into 200 bottles, with content of 100 μL each. Packing was in 1.5 mL microtube. Long term storage of the material at NIM is at -70 ºC.

For *RPPH1* sequence, two homozygous SNVs (g/a and c/t) occurred in the *HER2*-positive cell line, with the second SNV (c/t) also homozygous in *HER2*-normal cell line (see Appendix C).

Each participant received 2 samples and each sample with 3 vials each containing 100 μL. One vial is intended for method development and the other two are to be used for measurements. Samples should be stored at -70 ºC. A minimum sample intake of 5 μL is recommended.

Homogeneity Assessment of Study Material

From the batch of 200 vials of each test sample, 10 vials were randomly chosen for homogeneity testing by dPCR (QX200 system), three replicate measurements for each vial, Measurands 1-3 (*HER2*, *RPPH1*-related quantities) were measured to reflect homogeneity. The detailed method used by coordinator laboratory is described in Appendix B. The results of quantification are presented in **Figure 1** and **Table 1**. 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-vial differences in the measurands. The F-test values for *HER2* and *RPPH1* copy number concentration were bigger than the F critical at 0.05 confidence level, however the between-vial (homogeneity) uncertainty (u_{bb}) calculated based on the analysis, were smaller than 3% and deemed to be acceptable. The values of the relevant F-test for *HER2*/*RPPH1* copy number ratio was smaller than the F critical at 0.05 confidence level in both study materials, which indicated that the inhomogeneity of the ratio was insignificant.

Table 1: Results of the homogeneity assessment for Study Samples (Measurands 1-3).

Measurand (Number)	u_{bb}		F		$F_{0.05(10,22)}$	
	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
[*HER2*] (1)	2.7%	2.0%	8.99	6.74	2.39	2.39
[*RPPH1*] (2)	2.8%	1.2%	7.36	2.78	2.39	2.39
HER2/RPPH1 (3)	0.62%	1.2%	1.04	0.76	2.39	2.39

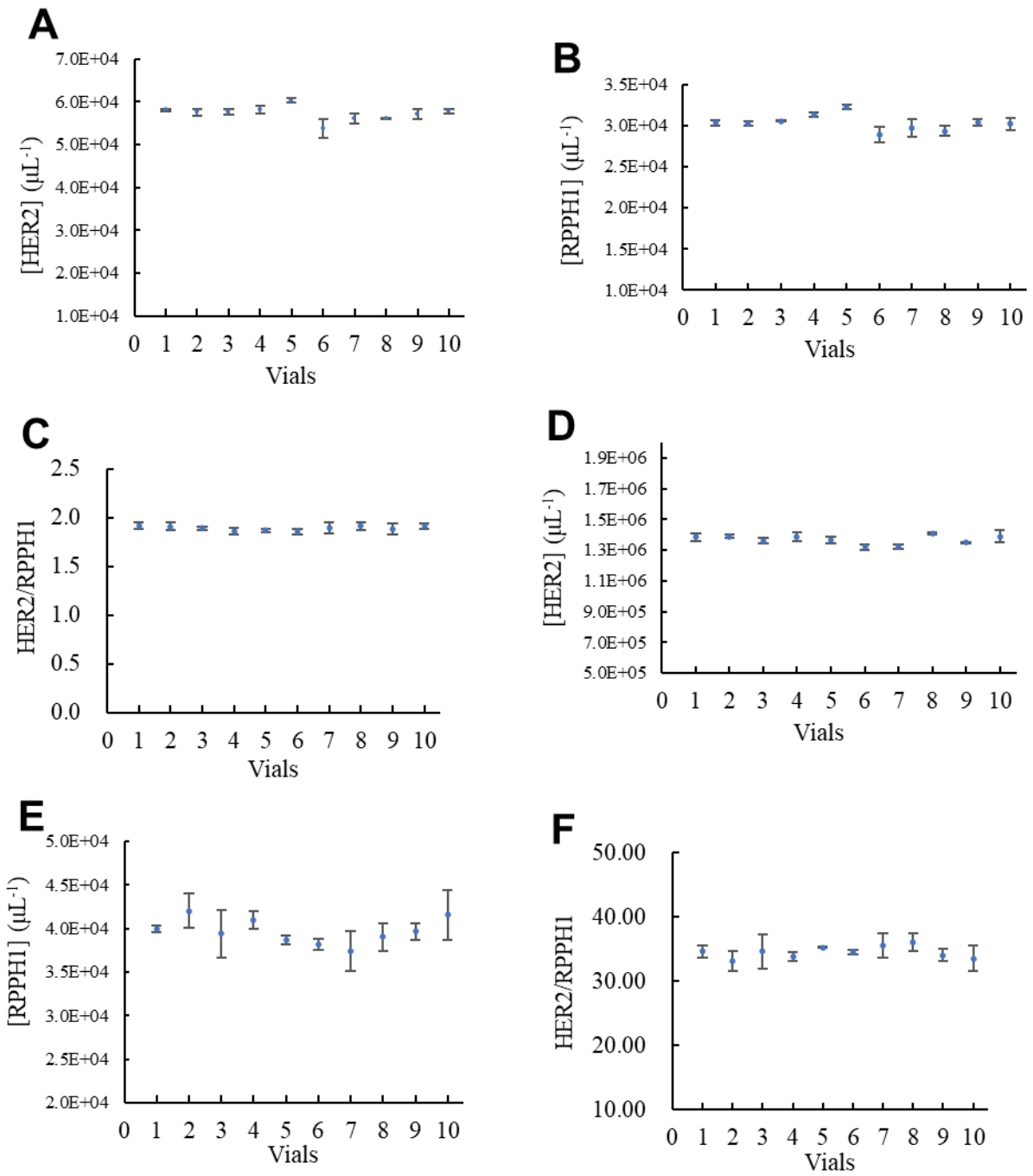

Figure 1: Results of the homogeneity assessment for Study Samples 1 and 2 (Measurands 1-3). (A) *HER2* copy number concentration in Sample 1; (B) *RPPH1* copy number concentration in Sample 1; (C) *HER2/RPPH1* copy number ratio in Sample 1; (D) *HER2* copy number concentration in Sample 2; (E) *RPPH1* copy number concentration in Sample 2; (F) *HER2/RPPH1* copy number ratio in Sample 2. Mean values for each unit ± SD are shown.

Stability Assessment of Study Material

Short-term stability was tested on the study material at 4 °C and 20 °C for 0, 4, 9 and 14 days. Three vials were analysed at each time point, and three replicate measurements for each vial. This study was designed to test the material stability under transportation conditions. The result is shown in **Figure 2**. The samples were demonstrated to be stable at 4 °C and 20 °C for 14 days.

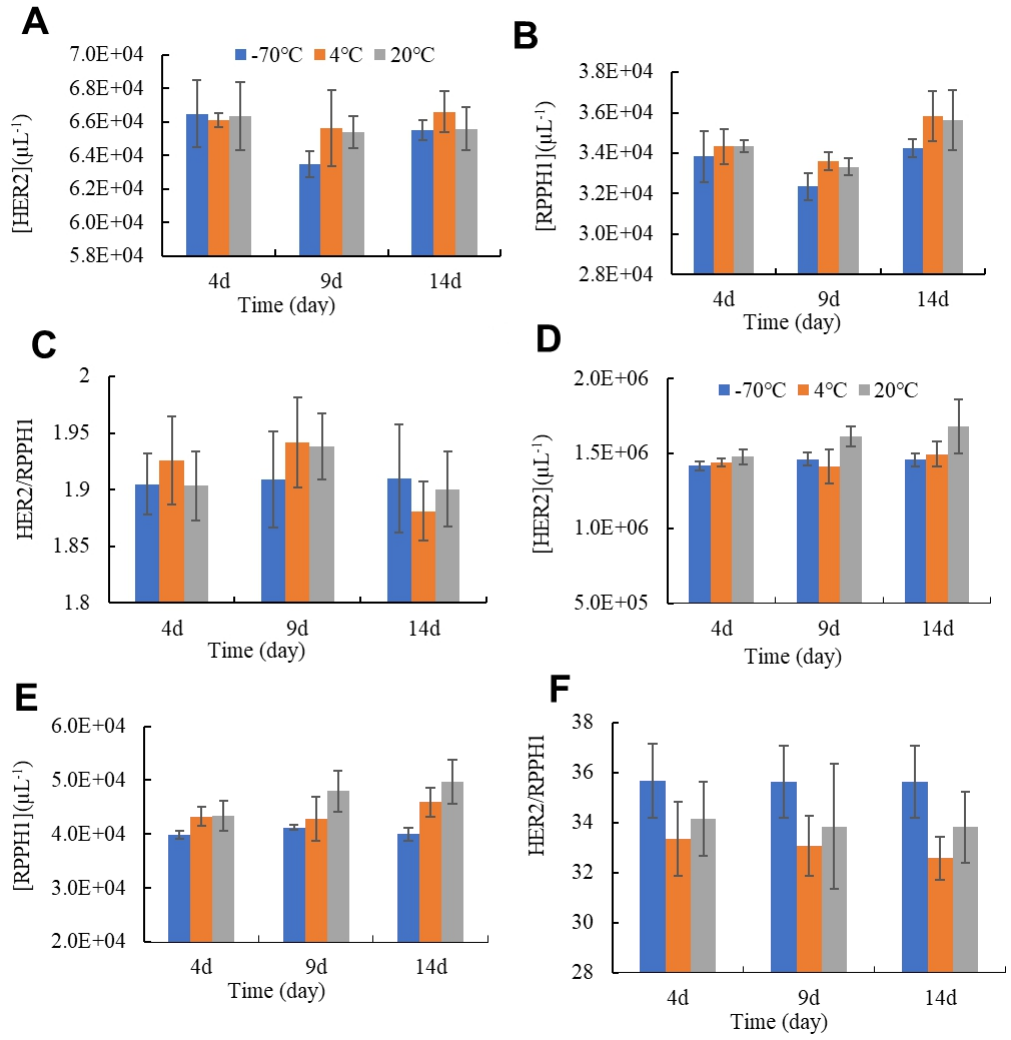

Figure 2: Results of the short-term stability assessment study for Study Samples 1 and 2 (Measurands 1-3). (A) *HER2* copy number concentration in Sample 1; (B) *RPPH1* copy number concentration in Sample 1; (C) *HER2/RPPH1* copy number ratio in Sample 1; (D) *HER2* copy number concentration in Sample 2; (E) *RPPH1* copy number concentration in Sample 2; (F) *HER2/RPPH1* copy number ratio in Sample 2. Results show mean ± SD, $n = 9$.

For evaluation of long-term stability, the study materials were tested at 0, 1, 2, 3, 6 and 12 months stored at -70 ºC. Three vials were taken at each time point and analysed using the same dPCR method as used in the short-term stability study. The results of long-term stability are shown in **Figure 3**. The trend-analysis was used to assess the stability according to ISO Guide 35. The statistical results indicated that no significant trend at 95% confidence level was detected. Hence, the instability of the material was insignificant at the study temperature over the study period.

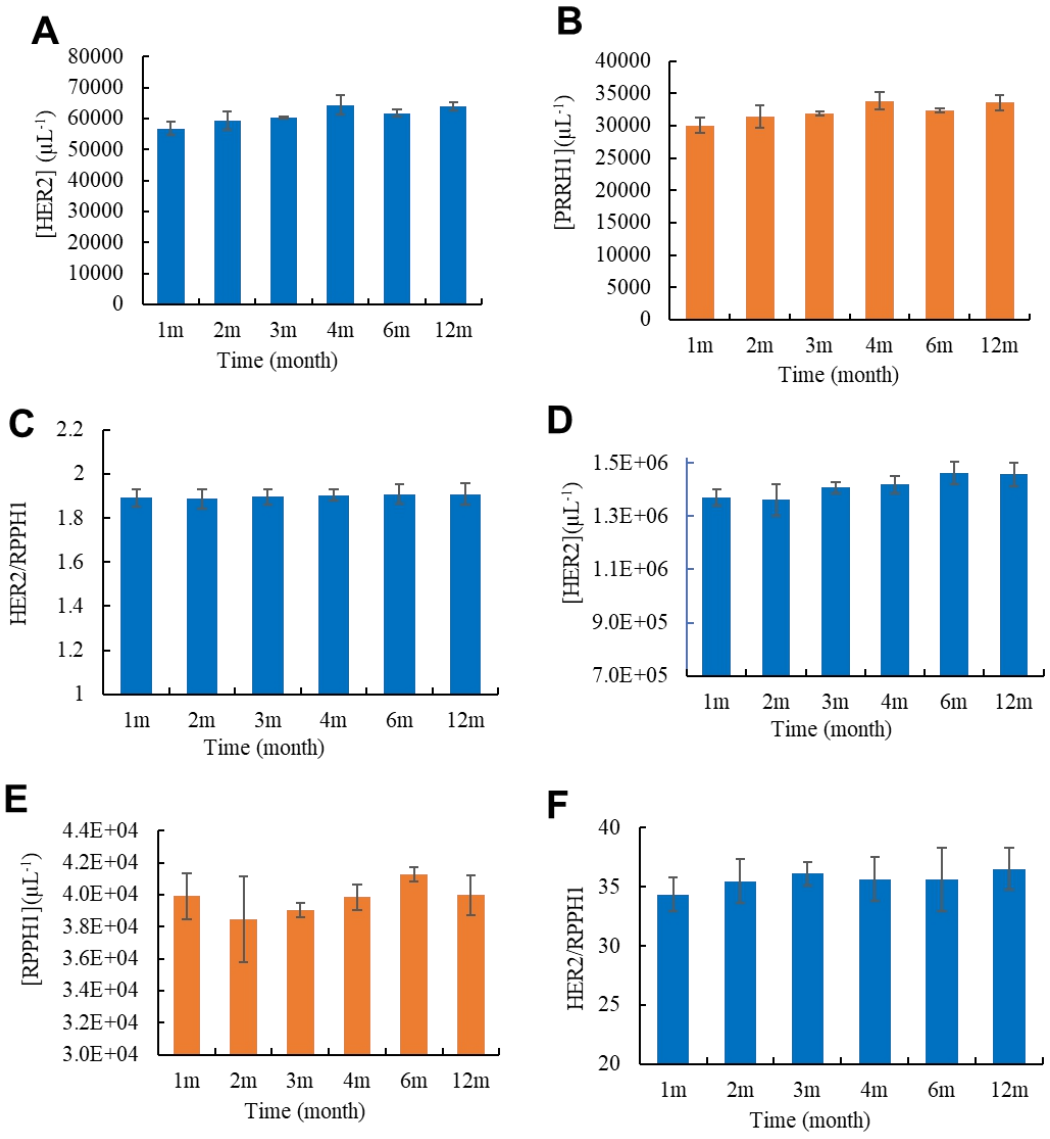

Figure 3: Results of the long-term stability assessment study for Study Samples 1 and 2 (Measurands 1-3). (A) *HER2* copy number concentration in Sample 1; (B) *RPPH1* copy number concentration in Sample 1; (C) *HER2*/*RPPH1* copy number ratio in Sample 1; (D) *HER2* copy number concentration in Sample 2; (E) *RPPH1* copy number concentration in Sample 2; (F) *HER2*/*RPPH1* copy number ratio in Sample 2. Results show mean ± SD, *n*=9.

PARTICIPANTS

The final Study Protocol (**Appendix C**) and call for participation was distributed in January 2021 with the intent to distribute samples in April-July 2021, receive results by 30 September 2021, and discuss results at the NAWG virtual meeting (Autumn 2021). Actual timelines are summarised in the Timelines section.

Ten institutes registered for CCQM-K176. **Table 2** lists the institutions that participated and submitted results for CCQM-K176.

Table 2: Participating laboratories in CCQM-K176

Laboratory ID	Institute / Organisation	Country	Contact
1	NIMT	Thailand	P. Morris
2	NML	United Kingdom	A. Devonshire
3	KRISS	South Korea	Y. Bae
4	VNIIM	Russia	M. Vonsky
5	NMIA	Australia	D. Burke
6	NIM	China	L. Dong
7	INMETRO	Brazil	R. Flatschart
8	INM	Colombia	J. Leguizamon
9	INRIM	Italy	C. Divieto
10	UME	Turkey	M. Akgöz

SAMPLE DISTRIBUTION

The coordinator delivered the samples to participants' door with 20 kg of dry ice. When all participants received the samples, there was still a lot of dry ice left as the coordinator asked the transportation agent to add dry ice every 3 days and kept the box in cold room to ensure the sample remaining frozen. Most participants received the sample within 8 days, excepting NMIA and INM. However the samples remained frozen when they received the samples.

Table 3: Distribution of Study Material Samples 1 and 2 for CCQM-K176

NMI or DI Code	**Country**	**Shipping date/time**	**Arrival time date/time**	**Days in transit**
NML	United Kingdom	2021.6.24	2021.6.29	5

NMIA	Australia	2021.6.24	2021.8.3	40
VNIIM	Russia	2021.7.20	2021.7.28	8
INMETRO	Brazil	2021.7.17	2021.7.22	5
NIMT	Thailand	2021.6.24	2021.6.28	4
KRISS	South Korea	2021.6.24	2021.6.25	1
INRIM	Italy	2021.7.17	2021.7.22	5
UME	Turkey	2021.8.11	2021.8.13	2
INM	Colombia	2021.7.21	2021.8.6	15

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TIMELINE

Table 4 lists the timeline for CCQM-K176.

Table 4: Timeline for CCQM-K176

Date	Action
October 2019	Agreement was received in the October 2019 meeting of the NAWG in Torino, Italy, to conduct the *HER2* CNV measurement as a key comparison.
May 2020	Draft protocol presented to NAWG as potential Track A Key Comparison
Nov 2020	NAWG authorized CCQM-K176 as a Track A Key Comparison; protocol approved
26 January 2021	Call for participation to NAWG members (Email from NAWG chair J. Huggett)
May-August 2021	Study samples shipped to participants. The range in shipping times reflects delays from shipping and customs.
30 November 2021	Final submission deadline for results to be sent coordinating laboratory
26 January 2022	Sharing of compiled study results with participating laboratories (Email from L. Dong)
March 2022	Study discussed at virtual CCQM-NAWG meeting
March 2023	Draft A report distributed to NAWG
October 2023	Draft B report distributed to NAWG
TBD	Final report approved by NAWG

RESULTS

Participants were requested to report the value (x), standard uncertainty (u), coverage factor (k), relative/expanded uncertainty (U) for each of the Measurands (Reply Form 3 in **Appendix F**).

In addition to the quantitative results, participants were instructed to describe their PCR assay and instrument (Reply Form 4 in Appendix G). At the virtual CCQM-NAWG meeting in March 2022, participants gave presentations on their analytical method and approach to uncertainty estimation.

CCQM-K176 results were received from 10 of the 11 institutions that received samples. NML (Laboratory 2) did not submit results for Measurands 1 and 2.

NIMT submitted two results within the deadline. The difference between the two submissions is that the first submission using *Hind*III **(Appendix J**) and the second result with a digesting of *Eco*RI. Since only one result can be submitted to the key study and they did not nominate which one should go to the key study, the study coordinator automatically treated the final data with *Eco*RI digestion as the nominated result. This has been discussed and agreed by NIMT.

Methods Used by Participants

Participants were permitted to use their own preferred methodology and assay for the quantification of the target sequences. The Study Protocol (**Appendix C**) provided information on restriction digestion using *Eco*RI which was recommended prior to analysis of the study materials.

All participants carried out their measurements by dPCR in CCQM-K176. **Appendix H** gives an overview of the sample processing (**Table H-1**), dPCR instrumentation, reagents (mastermix) and software (**Table H-2**) used by the participants. All participants used the same dPCR system (Bio-Rad Droplet Digital PCR System). Three participants used ddPCR Supermix for Probes *with* dUTP (Bio-Rad) and seven participants used ddPCR Supermix for Probes *without* dUTP (Bio-Rad). All participants designed their own assays. Primer and probe sequences and amplicon size information are listed in **Table H-3**, along with partition volume information (**Table H-4**) and PCR thermal cycling conditions (**Table H-5**).

The participants' approaches to estimating uncertainty are provided in **Appendix I**.

Participant Results for Measurand 1 to 3 of Sample 1 and 2

Table 5 gives an overview of the CCQM-K176 study results. Normality testing indicated that all datasets are normally distributed. For CCQM-K176, interlaboratory reproducibility (%CV) was between 9% - 13% for copy number concentration measurements and %CV values of 2.5% and 4.7% were observed for *HER2*/*RPPH1* copy number ratio values in the two study materials, demonstrating very high interlaboratory reproducibility in CNV measurements.

Tables 6-11 list individual participants' results for CCQM-K176 for each Measurand for Study Samples 1 and 2. Results are presented graphically in **Figures 4-5**.

Table 5: Summary of CCQM-K176 participants' results

Measurand number	Number of values (*n*)	Shapiro-Wilk test *p*-value*	Median	Mean	SD	%CV
Study Sample 1						
1	9	0.8274	57600	58648	5716	9.8%
2	9	0.8525	30200	30499	2767	9.1%
3	10	0.2613	1.91	1.927	0.04785	2.5%
Study Sample 2						
1	9	0.6858	1300000	1351891	173474	12.8%
2	9	0.1669	37100	39054	3914	10.0%
3	10	0.0531	35.16	34.68	1.629	4.7%

*$p > 0.05$ indicates that the data are normally distributed

Table 6: CCQM-K176 participants' measurement results for Sample 1 Measurand 1

Laboratory ID	Reported results [*HER2*] (μL^{-1})	Standard uncertainty *u* (μL^{-1})	Coverage factor *k*	Expanded uncertainty *U* (μL^{-1})	Relative expanded uncertainty *U/x* (%)
1	4.8E+04	2.2E+03	2	4.4E+03	9
3	5.47E+04	4.0E+03	2	7.9E+03	14.5
4	56896	2591	2	5181	9.1
5	57500	1700	1.97	3300	5.8
6	57600	1700	2	3400	5.9
7	59264	2746	2.023	5555	9.373
8	61260	2319	2.2	5105	8.3
9	65205	3769	2	7538	11.6
10	67410	2976	2	5952	8.8

Table 7: CCQM-K176 participants' measurement results for Sample 1 Measurand 2

Laboratory ID	Reported results [*RPPH1*] (μL^{-1})	Standard uncertainty *u* (μL^{-1})	Coverage factor *k*	Expanded uncertainty *U* (μL^{-1})	Relative expanded uncertainty *U/x* (%)
1	2.6E+04	1.9E+03	2	3.8E+03	14
3	2.92E+04	2.3E+03	2	4.5E+03	15.6
4	28227	1248	2	2497	8.8
5	29400	910	1.98	1800	6.1
6	30200	900	2	1800	6.0
7	32016	1917	2.262	4336	13.54
8	30652	1135	2.2	2498	8.2
9	34143	1973	2	3947	11.6

Laboratory ID	Reported results [*RPPH1*] (μL^{-1})	Standard uncertainty *u* (μL^{-1})	Coverage factor *k*	Expanded uncertainty *U* (μL^{-1})	Relative expanded uncertainty *U/x* (%)
10	34654	1751	2	3502	10.1

Table 8: CCQM-K176 participants' measurement results for Sample 1 Measurand 3

Laboratory ID	Reported results *HER2*/*RPPH1*	Standard uncertainty *u*	Coverage factor *k*	Expanded uncertainty *U*	Relative expanded uncertainty *U/x* (%)
1	1.90	0.17	2	0.34	18
2	1.9	0.0314	3.18	0.1	5.26
3	1.90E+00	8.3E-02	2	1.7E-01	8.7
4	2.0	0.1	2	0.3	12.7
5	1.96	0.062	1.98	0.12	6.3
6	1.91	0.03	2	0.07	3.5
7	1.85	0.1196	1.985	0.2374	12.83
8	2.00	0.04	2.2	0.09	4.4
9	1.91	0.16	2	0.31	16.3
10	1.94	0.12	2	0.24	12.3

Table 9: CCQM-K176 participants' measurement results for Sample 2 Measurand 1

Laboratory ID	Reported results [*HER2*] (μL^{-1})	Standard uncertainty *u* (μL^{-1})	Coverage factor *k*	Expanded uncertainty *U* (μL^{-1})	Relative expanded uncertainty *U/x* (%)
1	1.2E+06	4.2E+04	2	8.4E+04	7
3	1.30E+06	1.1E+05	2	2.3E+05	17.3
4	1094440	47968	2	95937	8.8
5	1271000	41000	2.00	82000	6.5
6	1410000	39000	2	77000	5.5
7	1516291	100206	2.064	206815	13.64
8	1539486	68292	2.2	150309	9.8
9	1233721	103069	2	206138	16.7
10	1602085	164752	2	329502	20.6

Table 10: CCQM-K176 participants' measurement results for Sample 2 Measurand 2

Laboratory ID	Reported results [*RPPH1*] (μL^{-1})	Standard uncertainty *u* (μL^{-1})	Coverage factor *k*	Expanded uncertainty *U* (μL^{-1})	Relative expanded uncertainty *U/x* (%)
1	3.6E+04	1.4E+03	2	2.8E+03	7

Laboratory ID	Reported results [*RPPH1*] (μL^{-1})	Standard uncertainty *u* (μL^{-1})	Coverage factor *k*	Expanded uncertainty *U* (μL^{-1})	Relative expanded uncertainty *U/x* (%)
3	3.71E+04	3.4E+03	2	6.7E+03	18.1
4	35101	1557	2	3115	8.9
5	36400	1100	2.00	2200	6.0
6	**40100**	1300	2	2600	6.5
7	42747	3345	2.571	8600	20.12
8	41900	1990	2.2	4379	10.5
9	35790	2990	2	5980	16.7
10	46351	3433	2	6866	14.8

Table 11: CCQM-K176 participants' measurement results for Sample 2 Measurand 3

Laboratory ID	Reported results *HER2*/*RPPH1*	Standard uncertainty *u*	Coverage factor *k*	Expanded uncertainty *U*	Relative expanded uncertainty *U/x* (%)
1	32.5	1.7	2	3.4	10
2	35	0.628	3.18	2	5.71
3	35.7	1.8	2	3.7	10.3
4	31.2	1.9	2	3.9	12.5
5	34.9	1.2	2.01	2.4	6.8
6	35.4	1.4	2	2.8	7.9
7	35.47	3.633	2.020	7.337	20.69
8	36.75	1.35	2.2	2.98	8.1
9	35.31	4.07	2	8.15	23.1
10	34.6	2.8	2	5.6	16.0

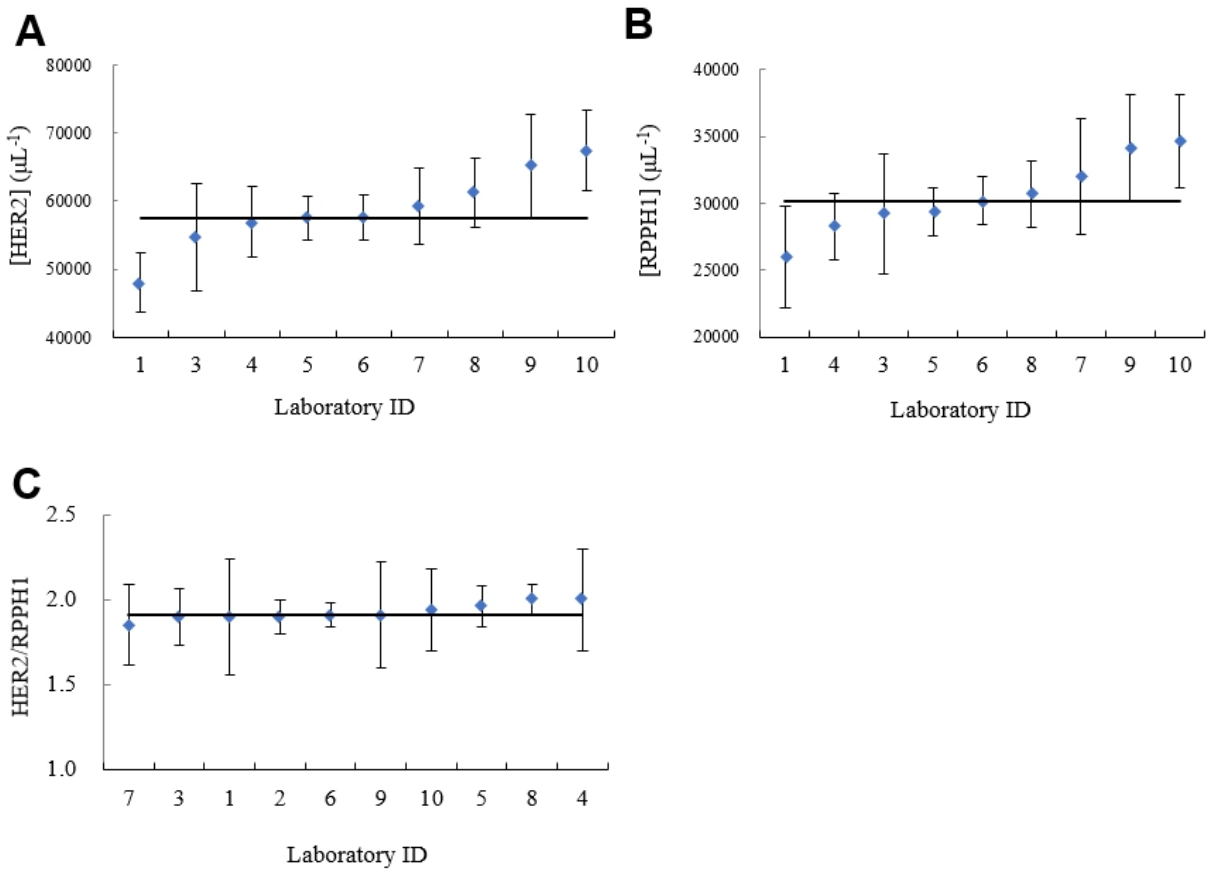

Figure 4: CCQM-K176 participants' measurement results for Sample 1. Panels A-C show results for Measurand 1-3 respectively. Results are shown in ascending order of reported value, with error bars showing expanded uncertainty. The solid horizontal line shows the median of all reported values.

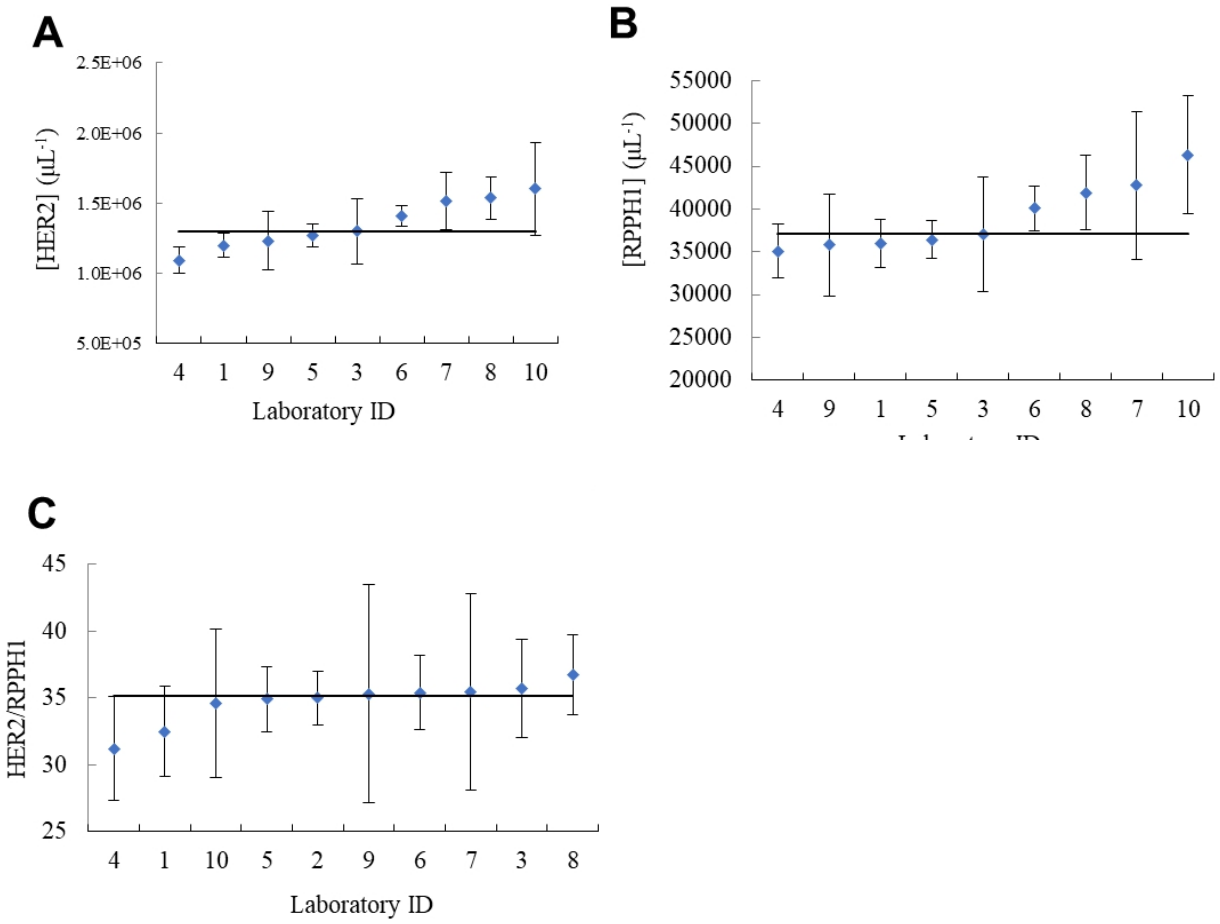

Figure 5: CCQM-K176 participants' measurement results for Sample 2. Panels A-C show results for Measurand 1-3 respectively. Results are shown in ascending order of reported value, with error bars showing expanded uncertainty. The solid horizontal line shows the median of all reported values.

Within- and Between-Measurand Comparisons

For Measurand 1 and 2 in Sample 1, results from Laboratories 1 (NIMT), 3 (KRISS), 4 (VNIIM) were lower than the median and results from Lab 9 (INRIM) and 10 (TUBITAK UME) were higher than the median. A similar trend for Measurand 1 and 2 in Sample 2 was observed. Results from Laboratories 1 and 4 were lower than the median, and results from Laboratories 7 (INMETRO), 8 (INM) and 10 (TUBITAK) were higher than the median. This demonstrates a possible systematic negative bias in Laboratories 1 and 4, and a possible positive bias in Laboratory 10 when measuring absolute copy number of *HER2* or *RPPH1*. Laboratory 4 also reported the lowest HER2/RPPH1 ratio for Study Material 2, whilst no trends were observed in Laboratories 1 and 10's measurements of copy number ratio of *HER2*/*RPPH1*, suggesting that the potential biases in *HER2* and *RPPH1* were of similar magnitude and cancelled out in these two cases.

Discussion of Results

All ten participants submitted the results for CCQM-K176, with the exception of Laboratory 2 (NML) not submitting Measurand 1 and 2 for CCQM-K176, as high between-experiment measurement variability was observed during study participation for absolute copy number concentration data. NML further compared different sample preparation workflows and confirmed that pre-treatment could cause the variability. For additional information please refer **Appendix J**.

This study focuses on both absolute copy number concentration and copy number ratio measurements of target sequences in gDNA. The overall consistency in copy number concentration greatly improved when the study was compared with that in the previous study (CCQM-P154) for plasmid DNA copy number concentration. The copy number ratio measurement also significantly improved when it was compared with those in the previous studies (CCQM K86 series), which may be due to variability due to pre-analytical processing (e.g. DNA extraction) being absent in this study.

KEY COMPARISON REFERENCE VALUE (KCRV)

CCQM-K176 results were evaluated according to CCQM/13-22 [11]. As no technical anomalies were reported, all submitted results were analysed and included in KCRV, with results for CCQM-K176 (Measurands 1-3) shown in the following. Each dataset was analysed for overdispersion relative to reported uncertainties by plotting as median-scaled difference (MSD) [12] (**Figure 6**). Consistency tests for differences between pairs of laboratories [13] are shown in **Figure 7**. Broadly, copy number concentrations (Measurands 1 and 2 4) tend to show appreciable overdispersion, while ratio measurements (Measurands 3) agree well within reported uncertainties. There are, however, no outlying values and the distributions show little or no evidence of asymmetry (**Table 5**).

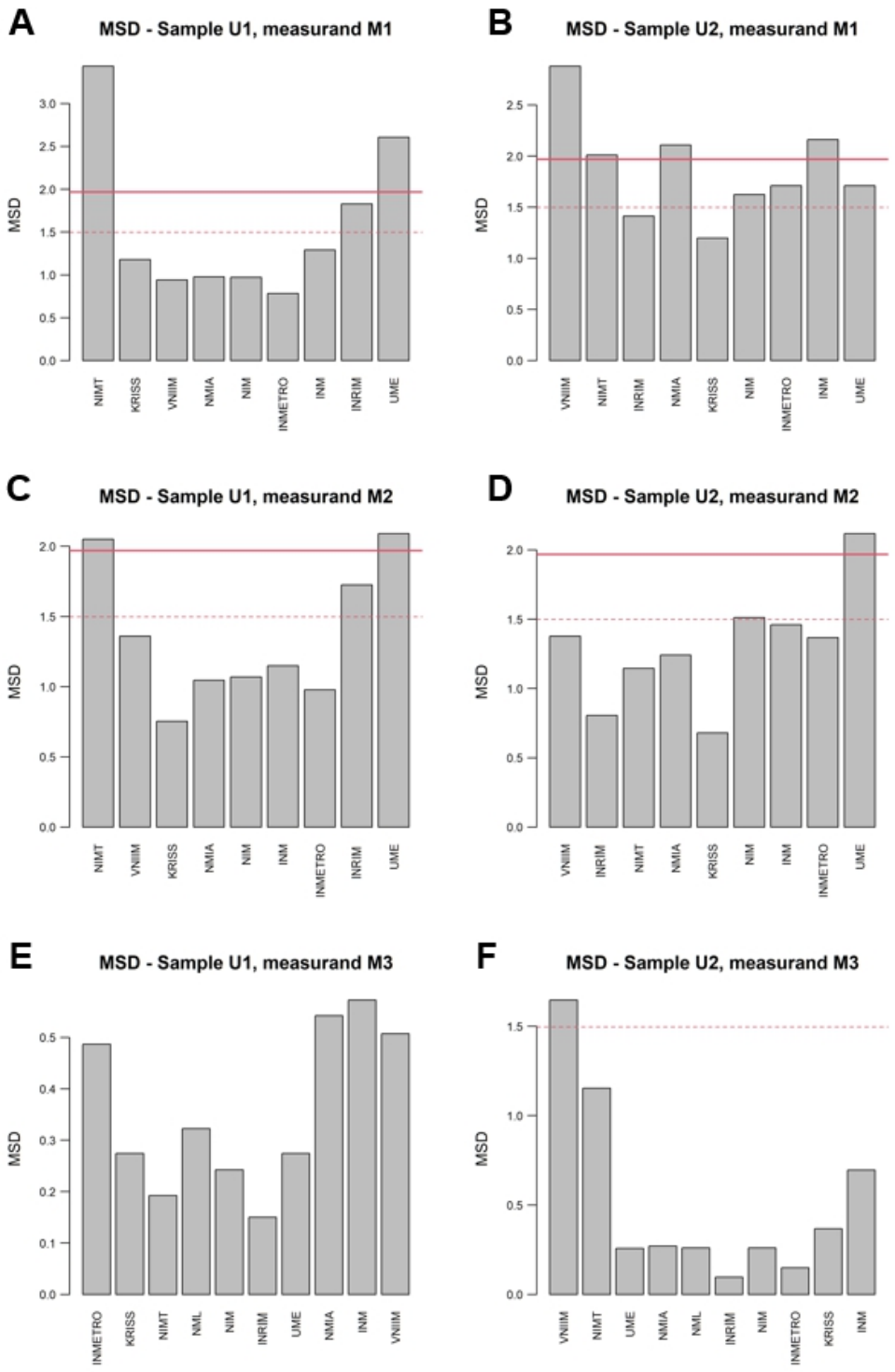

Figure 6: Median-scaled difference of participants' results for CCQM-K176. MSD values for each laboratory in ascending order of reported value (x) are shown for Measurands 1-3 for Sample 1 (U1) (left) and Sample 2 (U2) (right). The horizontal red lines are at approximate 95% (dashed) and 99% (solid) upper limits, corrected for multiple comparisons using a Holm correction and indicate results which differ significantly to other laboratories relative to their uncertainties.

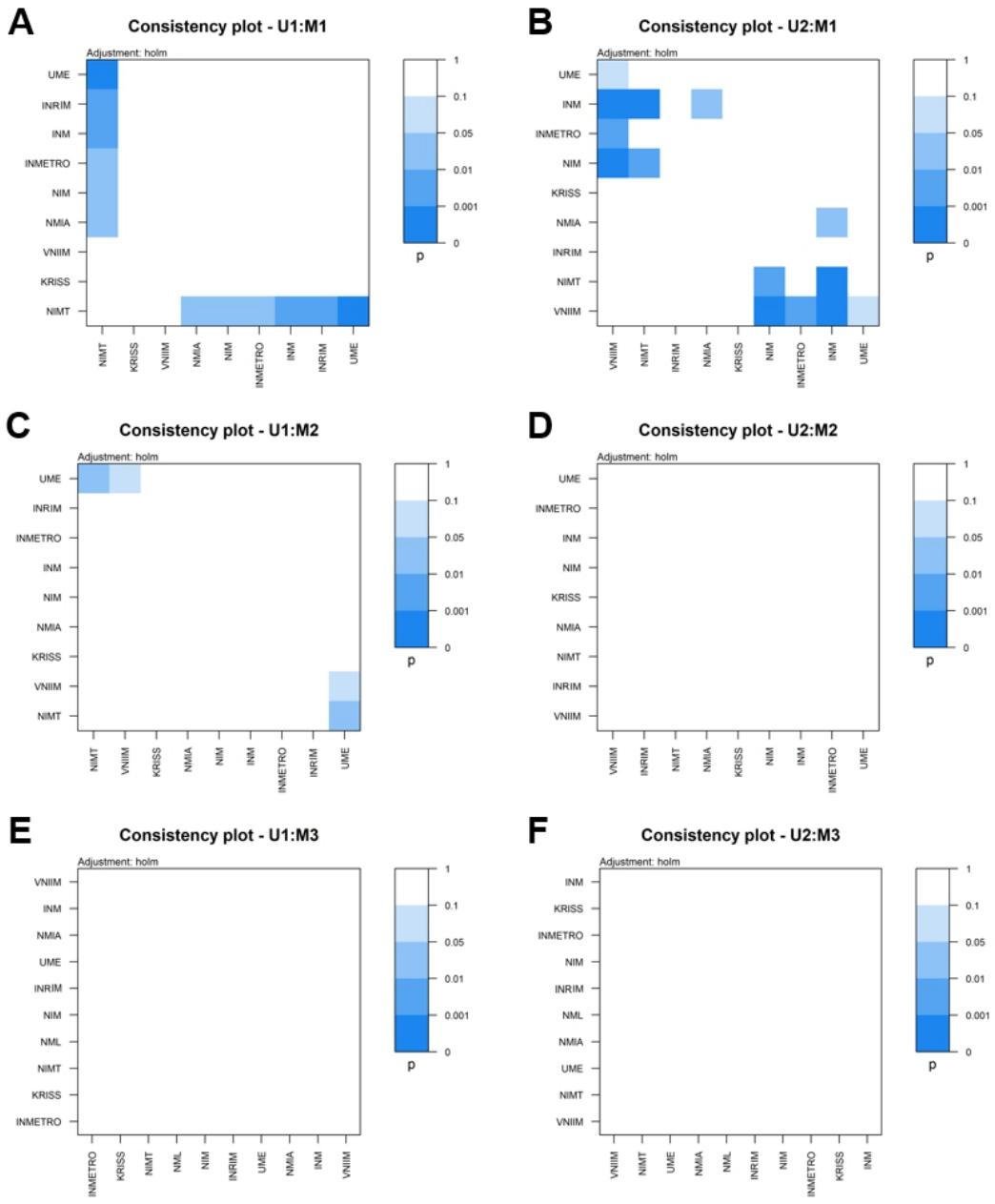

Figure 7: Consistency plots of participants' results for CCQM-K176. Plots are shown for Measurands 1-3 by row and for Sample 1 (U1) (left) and Sample 2 (U2) (right). The statistical significance of the difference in reported values between individual pairs of laboratories (in ascending order of reported value) are colored according to *p*-value (see key). The *p*-values are corrected for multiple comparisons using a Benjamini-Hochberg (BH) adjustment [14].

Six options for KCRV estimation were trialed to examine model dependency and understand different effects from the data: (i) simple arithmetic mean/SEM; (ii) the median with MAD_E

based uncertainty (calculated as the median absolute deviation multiplied by $\sqrt{\pi/2n}$); estimates utilising laboratories' reported uncertainties with an additional variance element: (iii) the original DerSimonian-Laird (DSL) estimator [15] and (iv) a restricted maximum likelihood (REML) estimator; and estimators weighted according to laboratories' reported uncertainties (v) the Graybill-Deal variance-weighted mean and (vi) a variance-weighted robust estimate using Huber's influence function ("Huber").

Initial calculations showed that uncertainties for most estimators were of similar magnitude to the homogeneity uncertainties provided in the study protocol. The homogeneity uncertainties were accordingly combined with the calculated estimator uncertainties for Measurands 1-3; for Measurands 4 and 5, no homogeneity information was available. Degrees of freedom for the different estimators were generally based on the number of participants; degrees of freedom for available homogeneity RSDs were taken as 9, based on the number of units studied. Effective degrees of freedom for the resulting combined uncertainties were determined using the Welch-Satterthwaite method [16]; coverage factors and expanded uncertainties used Student's *t* for the resulting effective degrees of freedom. The results for the six candidate RV estimators are illustrated in **Figure 8** (CCQM-K176) and listed in **Table 12** (CCQM-K176).

Inspection of the different estimates shows some important features. The reference value estimates generally agree closely within the expanded uncertainties. Uncertainties associated with Sample 1, Measurands 1-3 are similar across estimators, in part because of the inclusion of an allowance for homogeneity. For datasets showing evidence of overdispersion (Measurands 1 and 2 for both Samples), the calculated between-laboratory terms (additional variance) are very similar between DSL and REML and not accounted for by the available homogeneity information. For datasets which are mutually consistent (Measurand 3 and 5), the additional variance term is ~ 0, therefore the DSL, REML and weighted mean estimators are very similar. The median seems unusually variable compared to other estimators, including the outlier-resistant Huber estimator. Generally, the median appears less affected by the higher reported values for Sample 1 Measurands 1-3 and Sample 2, Measurands 1 and 2, leading to a noticeably lower reference value. In addition, the uncertainty for the median (based on MAD_e) is quite variable in these smaller data sets.

Given the absence of outlying values, fair to good agreement among results, evidence of modest overdispersion in several cases, and the slightly erratic behaviour of the median across this data set, the two estimators that use participant uncertainties while allowing for overdispersion – DSL and REML – appear most suitable on balance. Of the two, the REML estimator is marginally preferable on theoretical grounds; the DSL estimator used here can sometimes underestimate the between-laboratory term [16]; in this data set, the REML estimator also tends to provide a slightly more conservative (larger) uncertainty. Based on this, the REML estimator is suggested as the reference value estimator for CCQM-K176.

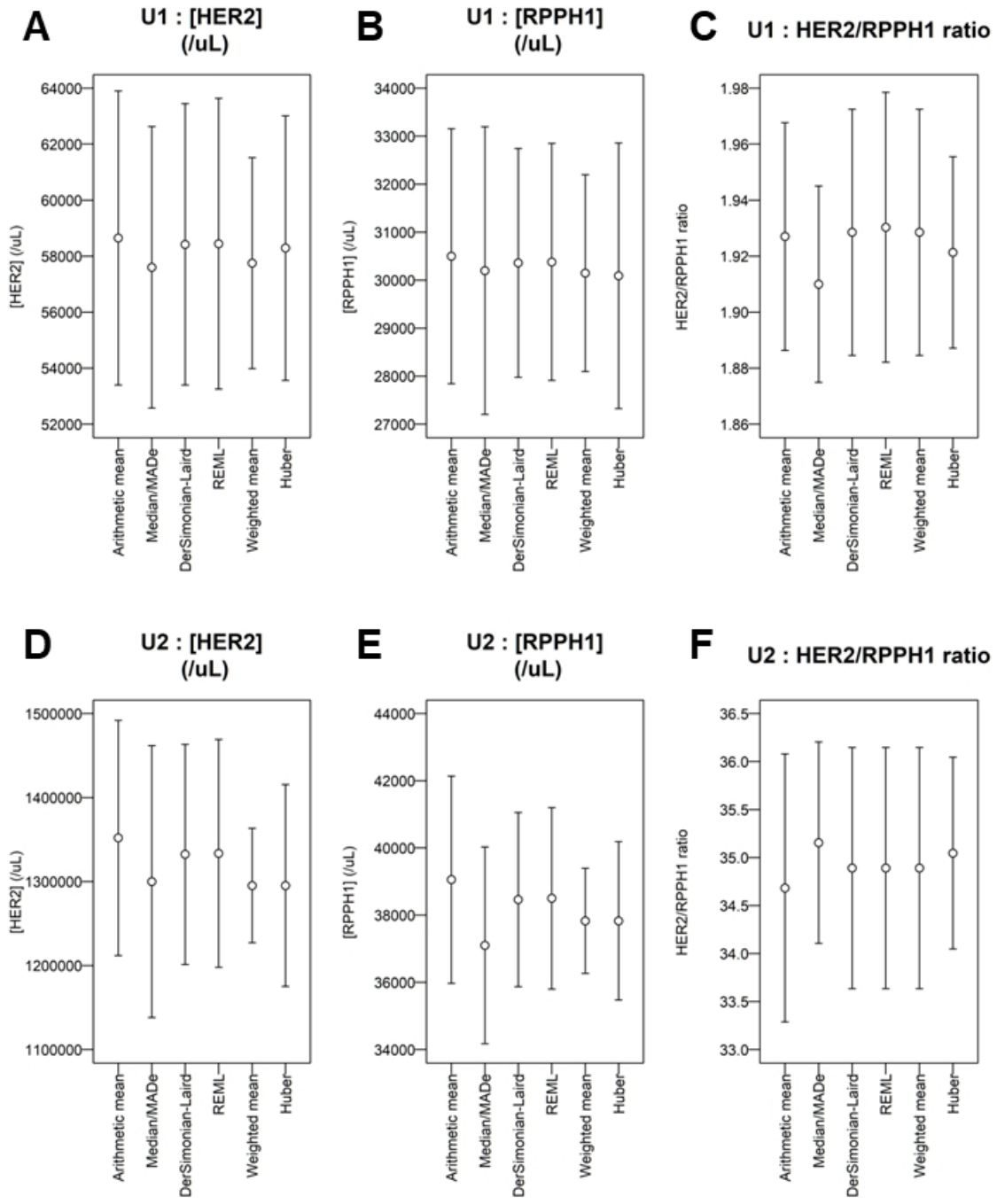

Figure 8: CCQM-K176 candidate KCRVs. Alternative KCRV estimators and their expanded uncertainties are shown for Sample 1 (U1) (A-C) and Sample 2 (U2) (D-F) Measurands 1-3.

Table 12: CCQM-K176 candidate Key Comparison Reference Values estimators[Note 1]

Sample, Measurand (units)	Estimator	KCRV	u	u_{hom}	u_c (Note 2)	DF_{eff} (Note 3)	k	U	U (k=2)	Between laboratory SD (%CV)
Sample 1, Measurand 1 (μL^{-1})	Arithmetic mean	58650	1900	1600	2500	16.1	2.12	5300	5000	
	Median/MADe	57600	1800	1600	2400	16.3	2.12	5000	4800	
	DSL	58420	1800	1600	2400	16.5	2.11	5000	4800	4620 (7.91%)
	REML	58440	1900	1600	2500	16.1	2.12	5200	4900	4949 (8.47%)
	Weighted mean	57750	790	1600	1700	13.2	2.16	3800	3500	
	Huber [Note 4]	58290	1600	1600	2200	16.9	2.11	4700	4500	
Sample 1, Measurand 2 (μL^{-1})	Arithmetic mean	30500	920	850	1300	16.7	2.11	2700	2500	
	Median/MADe	30200	1100	850	1400	15.3	2.13	3000	2800	
	DSL	30360	740	850	1100	16.9	2.11	2400	2300	1652 (5.44%)
	REML	30380	800	850	1200	17	2.11	2500	2300	1873 (6.17%)
	Weighted mean	30150	440	840	950	13.4	2.15	2000	1900	
	Huber	30090	1000	840	1300	16.2	2.12	2800	2600	
Sample 1, Measurand 3 (no units)	Arithmetic mean	1.927	0.015	0.012	0.019	17.1	2.11	0.041	0.039	
	Median/MADe	1.91	0.012	0.012	0.017	18	2.1	0.035	0.033	
	DSL	1.929	0.017	0.012	0.021	16.2	2.12	0.044	0.042	0 (0.0%)
	REML	1.93	0.019	0.012	0.023	15.1	2.13	0.048	0.045	0.0192 (0.99%)
	Weighted mean	1.929	0.017	0.012	0.021	16.2	2.12	0.044	0.042	
	Huber	1.921	0.011	0.012	0.016	17.9	2.1	0.034	0.033	

Table 12 (continued): CCQM-K176 candidate Key Comparison Reference Values estimators[Note 1]

Sample, Measur and (units)	Estimator	KCRV	u	u_{hom}	u_c (Note 2)	DF_{eff} (Note 3)	K	U	U (k=2)	Between laboratory SD (%CV)
Sample 2, Measurand 1 (μL^{-1})	Arithmetic mean	1352000	58000	27000	64000	11.4	2.19	140000	130000	
	Median/MADe	1300000	68000	26000	73000	10.3	2.22	160000	150000	
	DSL	1332000	54000	27000	60000	11.8	2.18	130000	120000	140453 (10.5%)
	REML	1333000	56000	27000	62000	11.5	2.19	140000	120000	147677 (11.2%)
	Weighted mean	1295000	19000	26000	32000	16	2.12	68000	64000	
	Huber	1295000	49000	26000	55000	12.3	2.17	120000	110000	
Sample 2, Measurand 2 (μL^{-1})	Arithmetic mean	39050	1300	470	1400	10	2.23	3100	2800	
	Median/MADe	37100	1200	450	1300	10.1	2.23	2900	2600	
	DSL	38460	1100	460	1200	10.9	2.2	2600	2400	2441 (6.35%)
	REML	38500	1100	460	1200	10.6	2.21	2700	2400	2614 (6.79%)
	Weighted mean	37830	580	450	740	15.6	2.12	1600	1500	
	Huber	37830	970	450	1100	11.4	2.19	2400	2200	

Table 12 (continued): CCQM-K176 candidate Key Comparison Reference Values estimators[Note 1]

Sample, Measurand (units)	Estimator	KCRV	u	u_{hom}	u_c (Note 2)	DF_{eff} (Note 3)	K	U	U (k=2)	Between laboratory SD (%CV)
Sample 2, Measurand 3 (no units)	Arithmetic mean	34.68	0.52	0.42	0.66	17.2	2.11	1.4	1.3	
	Median/MADe	35.16	0.25	0.42	0.49	14.7	2.13	1	0.98	
	DSL	34.89	0.43	0.42	0.6	18	2.1	1.3	1.2	0 (0.0%)
	REML	34.89	0.43	0.42	0.6	18	2.1	1.3	1.2	7.48e-05 (0.00021%)
	Weighted mean	34.89	0.43	0.42	0.6	18	2.1	1.3	1.2	
	Huber	35.05	0.19	0.42	0.46	12.4	2.17	1	0.92	

Note 1. This list is not exhaustive.

Note 2. Combined standard uncertainty $\sqrt{u_{\text{kcrv}}^2 + u_{\text{hom}}^2}$

Note 3. Effective degrees of freedom and corresponding coverage factor k calculated from degrees of freedom associated with KCRV uncertainty and homogeneity uncertainty. Degrees of freedom for the different estimators were based on the number of participants (n = 9 for Measurands 1-2 and n = 10 for Measurand 3); degrees of freedom for homogeneity uncertainties were 9, based on the number of units studied (n = 10).

Note 4. Robust estimate using reported uncertainties together with Huber weighting function. Recommended for use with generally credible uncertainties with a small number of discrepant observations.

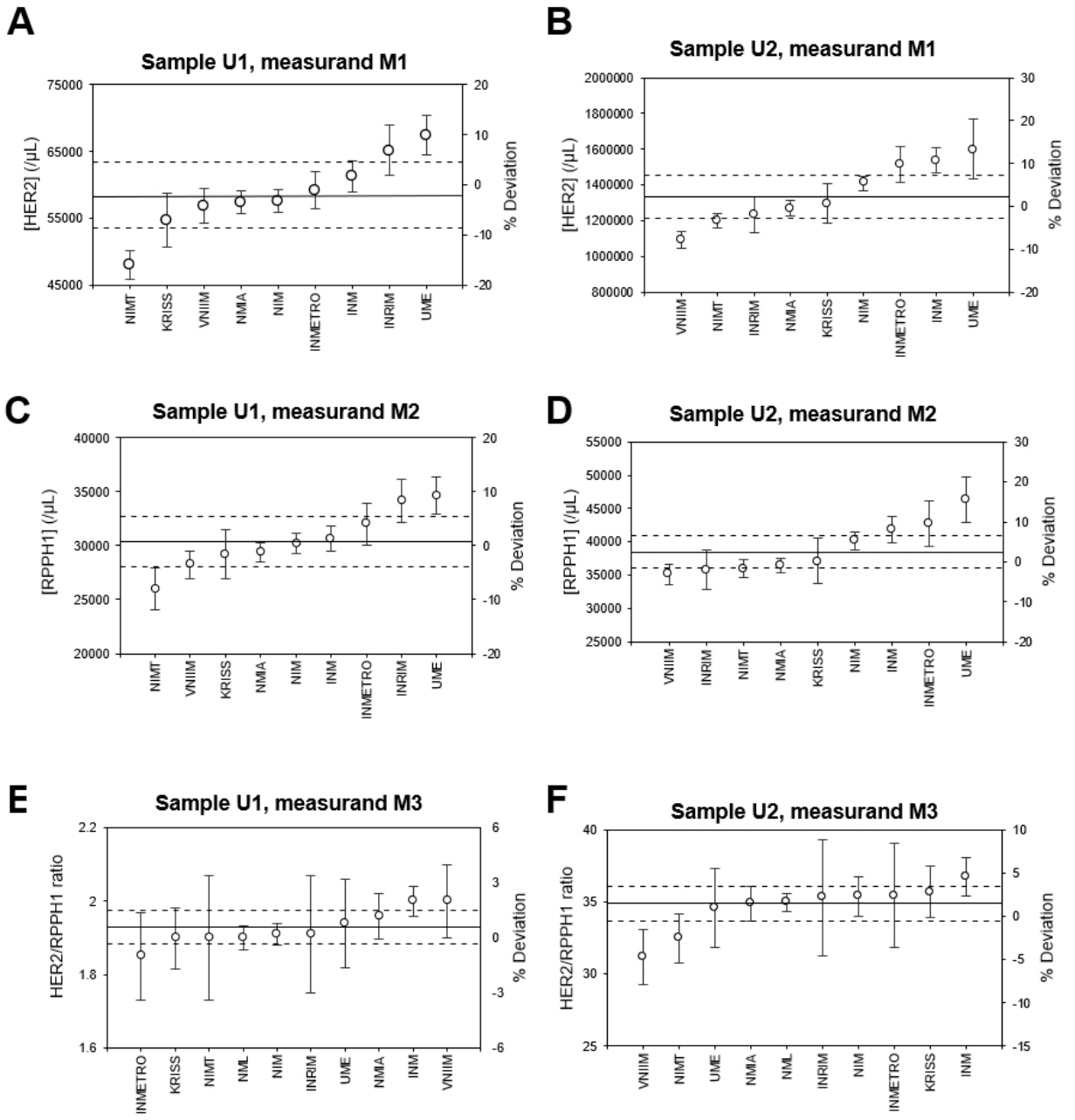

Figure 9: CCQM-K176 study results with KCRV.

Participants reported values and expanded uncertainties (circles/error bars) are shown for Sample 1 (U1) (A-C) and Sample 2 (U2) (D-F) Measurands 1-3 in order of increasing value, together with the determined KCRV based on REML and its expanded uncertainty (black horizontal solid and dashed lines respectively).

The recommended estimator of the REML was agreed by participants following circulation of the Draft A report and presentation at the April 2023 NAWG meeting. **Table 13** shows the final KCRV values and uncertainties.

Table 13: CCQM-K176 final KCRV and uncertainties

Sample	Sample 1 (μL^{-1})				Sample 2 (μL^{-1})			
Measurand	x	u	U (k=2)	U_{rel} (k=2) (%)	x	u	U (k=2)	U_{rel} (k=2) (%)
M1	58440	2500	4900	8.4	1333000	62000	120000	9.0
M2	30380	1200	2300	7.6	38500	1200	2400	6.2
M3	1.93	0.03	0.05	2.3	34.9	0.6	1.2	3.4

DEGREES OF EQUIVALENCE (DoE)

The absolute degrees of equivalence for the participants in CCQM-K176 are estimated as the signed difference between the combined value and the KCRV: $d_i = x_i$ – KCRV.

Based on the selection of the recommended KCRV based on REML, the uncertainty in the degree of equivalence for each laboratory is calculated as follows (Ref. [11] Appendix Section 3.3) for the scenario of a laboratory's result (x_i) being used to calculate the KCRV (**Equation 1**):

Equation 1: Uncertainty in the degree of equivalence for additional variance-based model for KCRV

$$u^2(d_i) = u^2(x_i) + u^2(q) - u^2(\bar{x}_{KCRV}) \quad (1)$$

where $u^2(x_i)$ is the laboratory's reported standard uncertainty, $u^2(q)$ is the uncertainty in the additional variance (between laboratory variance) term (**Table 12**) and $u^2(\bar{x}_{KCRV})$ is the standard uncertainty in the KCRV (**Table 13**). Note that the uncertainty in the KCRV is *subtracted* from the other uncertainty terms.

To enable comparison with the degrees of equivalence estimates from other studies, it is convenient to express the d_i and $U_{k=2}(d_i)$ as percentages relative to the KCRV: $\%d_i = 100 \cdot d_i/\text{KCRV}$ and $U_{k=2}(\%d_i) = 100 \cdot U_{k=2}(d_i)/\text{KCRV}$. **Table 14** and **15** below lists the numeric values of d_i, $U_{k=2}(d_i)$,$\%d_i$, and $U_{k=2}(\%d_i)$ for all participants in CCQM-K176 (Measurands 1, 2 and 3).

Table 14: Degrees of Equivalence for Sample 1, Measurand 1, 2 and 3

	Sample 1, Measurand 1, μL^{-1}				Sample 1, Measurand 2, μL^{-1}			
	d_i	$U_{k=2}(d_i)$	$\%d_i$	$U_{k=2}(\%d_i)$	d_i	$U_{k=2}(d)$	$\%d_i$	$U_{k=2}(\%d_i)$
NIMT*	-10440	9609	-17.9	16.4	-4380	4766	-14.4	15.7
KRISS	-3740	11703	-6.4	20.0	-1180	5425	-3.9	17.9
VNIIM	-1544	9991	-2.6	17.1	-2153	3808	-7.1	12.5
NMIA	-940	9194	-1.6	15.7	-980	3404	-3.2	11.2
NIM	-840	9194	-1.4	15.7	-180	3393	-0.6	11.2
INMETRO	824	10155	1.4	17.4	1636	4793	5.4	15.8
INM	2820	9720	4.8	16.6	272	3664	0.9	12.1
INRIM	6765	11393	11.6	19.5	3763	4883	12.4	16.1
UME	8970	10411	15.3	17.8	4274	4532	14.1	14.9

	Sample 1, Measurand 3, no units			
	d_i	$U_{k=2}(d_i)$	$\%d_i$	$U_{k=2}(\%d_i)$
INMETRO	-0.08	0.24	-4.1	12.3
KRISS	-0.03	0.16	-1.6	8.5
NIMT	-0.03	0.34	-1.6	17.6
NML	-0.03	0.06	-1.6	3.0
NIM	-0.02	0.05	-1.0	2.8
INRIM	-0.02	0.32	-1.0	16.5
UME	0.01	0.24	0.5	12.4
NMIA	0.03	0.12	1.6	6.3
INM	0.07	0.08	3.6	3.9
VNIIM	0.07	0.20	3.6	10.3

*$|d_i| > U_{k=2}(d_i)$

Table 15: Degrees of Equivalence for Sample 2, Measurand 1, Measurand 2 and Measurand 3

	Sample 2, Measurand 1, μL^{-1}				Sample 2, Measurand 2, μL^{-1}			
	d_i	$U_{k=2}(d_i)$	$\%d_i$	$U_{k=2}(\%d_i)$	d_i	$U_{k=2}(d_i)$	$\%d$	$U_{k=2}(\%d_i)$
VNIIM	-238560	284713	-17.9	21.4	-3399	5592	-8.8	14.5
NIMT	-133000	280916	-10.0	21.1	-2500	5423	-6.5	14.1
INRIM	-99279	338158	-7.4	25.4	-2710	7572	-7.0	19.7
NMIA	-62000	280325	-4.7	21.0	-2100	5139	-5.5	13.3
KRISS	-33000	346782	-2.5	26.0	-1400	8235	-3.6	21.4
NIM	77000	279181	5.8	20.9	1600	5323	4.2	13.8
INMETRO	183291	334698	13.8	25.1	4247	8144	11.0	21.2
INM	206486	300854	15.5	22.6	3400	6117	8.8	15.9
UME	269085	424772	20.2	31.9	7851	8289	20.4	21.5

	Sample 2, Measurand 3, no units			
	d_i	$U_{k=2}(d_i)$	$\%d_i$	$U_{k=2}(\%d_i)$
VNIIM*	-3.69	3.61	-10.6	10.3
NIMT	-2.39	3.18	-6.9	9.1
UME	-0.29	5.43	-0.8	15.6
NMIA	0.01	2.08	0.0	6.0
NML	0.11	0.37	0.3	1.1
INRIM	0.42	8.05	1.2	23.1
NIM	0.51	2.53	1.5	7.3
INMETRO	0.58	7.17	1.7	20.5
KRISS	0.81	3.39	2.3	9.7
INM	1.86	2.42	5.3	6.9

*$|d_i| > U_{k=2}(d_i)$

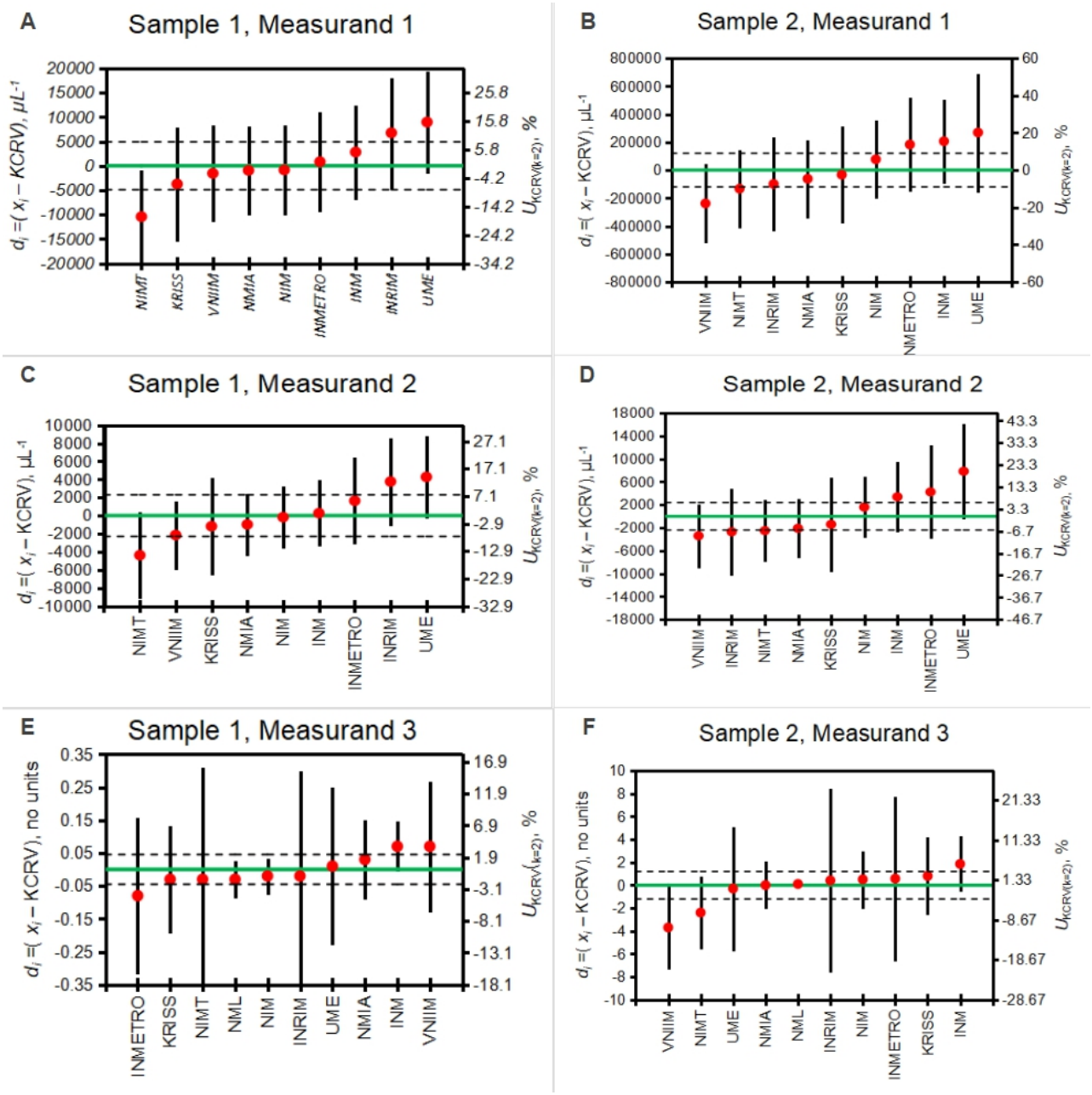

Figure 10: Degrees of Equivalence Associated with the KCRVs

All results are sorted by increasing DoE *(y)*. The y-axis to the left edge of each panel displays the absolute DoE, d_i, in units. The y-axis to the right edge of each graph displays the relative expanded uncertainty of the KCRV. Dots represent the d_i, bars their approximate 95 % expanded uncertainties, $U_{k=2}(d_i)$. The thick green horizontal line denotes perfect agreement with the candidate KCRV. The dash line represents the expanded uncertainty of the KCRV.

USE OF CCQM-K176 IN SUPPORT OF CALIBRATION AND MEASUREMENT CAPABILITY (CMC) CLAIMS

How Far the Light Shines

CCQM-K176 evaluated the interlaboratory reproducibility and consistency of measurements of both copy number ratio and absolute copy number concentration of DNA targets, therefore the study supports participants' measurement capabilities for both measurands, within the measurement ranges and degrees of equivalence demonstrated within the study.

The samples used in the CCQM-K176 were purified gDNA in buffered/aqueous solution. The measurement performance demonstrated in CCQM-K176 would be expected to be maintained with alternative purified DNA samples such as plasmid DNA or double-stranded oligonucleotide, assuming that the integrity of the targeted sequences/genes/gene regions is assured (for example, by gel electrophoresis or Sanger sequencing). However, the current study cannot support participants' competency to perform DNA measurements in biological materials which require DNA extraction (e.g. cell lines or clinical samples).

The CCQM-K176 samples consisted of high MW gDNA with a minimum fragment size exceeding 4 kbp and peak size > 60 kbp (**Figure J4**). Amplicon size was not found to be a source of bias in the current study (**Figure 11**), unlike in CCQM-P184, where the wild-type DNA in Study Material 2 consisted of fragmented gDNA (peak size between 100-300 bp). Measurements of wild-type EGFR concentration in CCQM-P184 Study Material 2 were associated with reduced copy number concentration results where assays with longer amplicons were used [17]. Therefore, the current study CCQM-K176 results also cannot be used to claim CMCs in relation to fragmented gDNA.

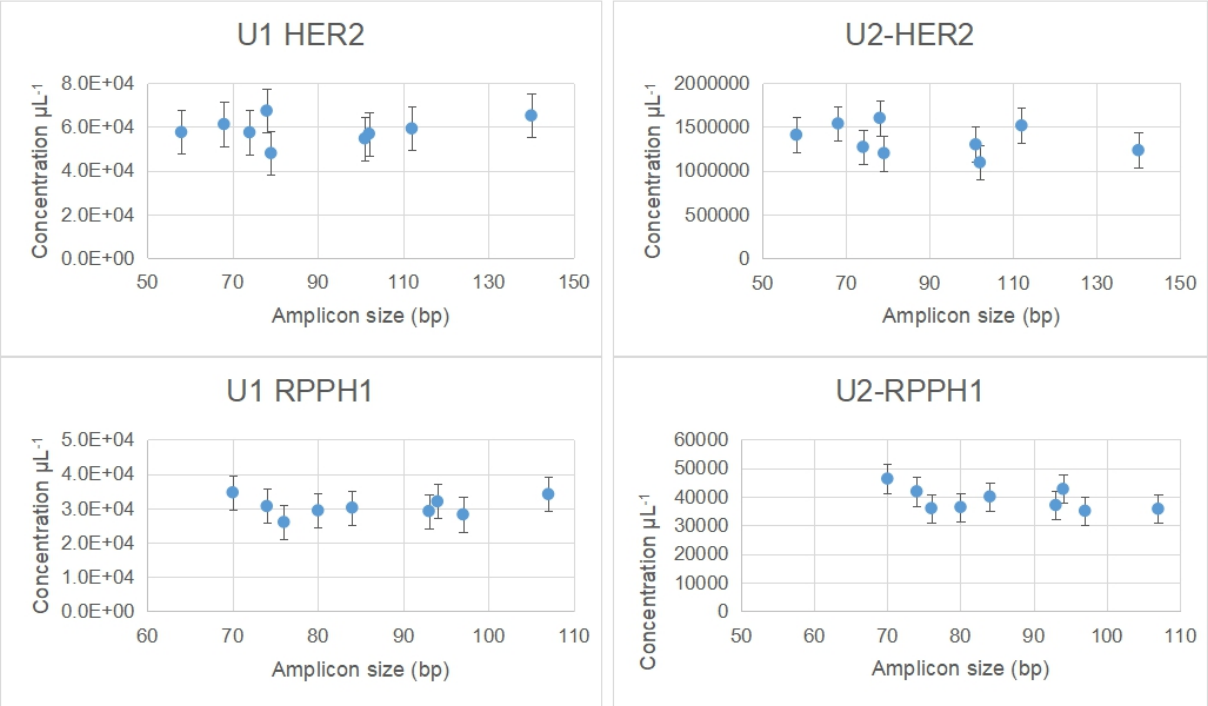

Figure 11. Participants' results as a function of amplicon size for HER2 and RPPH1 in two samples. Error bars are standard uncertainties.

The sequence length of the two target genes in CCQM-K176 were 719 bp (*RPPH1*) and 40 566 bp (*HER2*). For broader scope claims, the size of the gene or sequence which the measurements relate to should be considered. For example, measurements of copy number ratio or concentration of chromosomal-scale sequence where the target regions are several megabases in size exceeds the scope of measurement capabilities demonstrated in the current study.

Core Competency Statements and CMC support

The basic CMC claim for CCQM-K176 is "Measurement of *HER2/RPPH1* copy number ratio range from 1.0 to 40 in genomic DNA in buffered/aqueous solution". The study will also support participants' claims for measurement of *HER2* and *RPPH1* copy number concentration in gDNA in buffered/aqueous solution in the range from ~10^4 to 10^6 μL^{-1}. The copy number concentration range takes into account reported values for both the reference *RPPH1* gene and *HER2* gene sequences. Draft CMC proforma information is given in **Appendix K**. This study can support value assignment of primary reference standards and value assignment of PT samples where *HER2* is being quantified.

As this is the first KC study where DNA copy number ratio >1 and copy number concentration have been measured, additional evidence is required to extend the basic CMC to additional gene or sequence targets. In accordance with CIPM MRA-G-13 requirements for technical evidence to support CMCs, the supporting evidence may include publications, accredited methods and intra-

RMO studies and should be of sufficient metrological rigour/detail. If this evidence is available, it would be possible to claim broad scope CMC claims: "Measurement of copy number ratio (copy number variant (CNV)) of a defined target sequence relative to a reference sequence in the range from 1.0 to 40 in genomic DNA in buffered/aqueous solution" and "Measurement of gene/sequence copy number concentration in genomic DNA in buffered/aqueous solution in the range from ~10^4 to 10^6 μL^{-1}." **Appendix K** gives an example of presentation of a possible prototype "broader-scope" CMC that could be claimed based on successful participation in CCQM-K176.

For both copy number concentration (Measurands 1-2) and copy number ratio (Measurand 3) measurement, all participants showed acceptable degrees of equivalence with the KCRV, with the exception of NIMT for Measurand 1 in Sample 1. However, for those with DoE unsatisfied, they can claim CMC by increasing their uncertainty [18].

Furthermore, as the KCRVs were calculated for all measurands/samples using an additional variance ("dark uncertainty") estimator (REML), the uncertainty in the degree of equivalence includes this additional variance term (Equation 1), which is appreciable for Measurands 1-2 (between 6.2% and 11.1% when expressed relative to the KCRV) (Table 16). In some cases, laboratories' reported uncertainties alone may not be sufficient to allow agreement with the KCRV (**Figure 9**). Therefore, it is recommended that laboratories take this into consideration when determining the uncertainty range which is claimed for CMCs.

In both cases of inconsistent DoE or insufficient reported uncertainty, the minimum CMC claim uncertainty should be calculated by the following equation (**Equation 2**), and the uncertainty budget revised to account for this with a meaningful uncertainty component.

Equation 2: Calculation of increased expanded uncertainty for the purpose of CMC claim

$$U_{CMC} = U(x_i)' = k \times \sqrt{\left(\frac{d_i}{2}\right)^2 + u^2(\bar{x}_{KCRV})} \qquad (2)$$

The minimum uncertainties for CMC claims based on DoE are calculated in the **Tables 16-17** and cases are indicated where laboratories' reported uncertainties (*U(xi)*) are insufficient (*U(xi)' > U(xi)*). Note, for laboratories with a consistent DoE and sufficient reported uncertainties, minimum claimed uncertainties should be based on those reported in the study.

Table 16: Minimum uncertainty of CMC claim for Measurand 1-3 in sample 1 for all participants

Laboratory	**Measurand 1**			**Measurand 2**			**Laboratory**	**Measurand 3**		
	U(xi)'	*rel U(xi)'*	*U(xi)' > U(xi)*	*U(xi)'*	*rel U(xi)'*	*U(xi)' > U(xi)*		*U(xi)'*	*rel U(xi)'*	*U(xi)' > U(xi)*

NIMT*	11576	25%	Y	4994	20%	Y	NIMT	0.055	2.9%	N
							NML	0.087	4.6%	N
KRISS	6244	12%	N	2674	9.2%	N	KRISS	0.055	2.9%	N
VNIIM	5233	9.2%	Y	3224	12%	Y	VNIIM	0.084	4.2%	N
NMIA	5011	8.7%	Y	2566	8.7%	Y	NMIA	0.054	2.8%	N
NIM	5070	8.8%	Y	2407	8.0%	Y	NIM	0.050	2.6%	N
INMETRO	5126	8.6%	N	3285	11%	N	INMETRO	0.092	5.0%	N
INM	6314	11%	Y	2657	8.7%	Y	INM	0.092	4.6%	Y
INRM	8412	13%	Y	4463	14%	Y	INRM	0.050	2.6%	N
UME	10269	16%	Y	4902	15%	Y	UME	0.047	2.4%	N

*$| d_i | > U_{k=2}(d_i)$

Table 17: Minimum uncertainty of CMC for claim Measurand 1-3 in sample 2 for all participants

Laboratory	**Measurand 1**			**Measurand 2**			**Laboratory**	**Measurand 3**		
	U(xi)'	*rel U(xi)'*	*U(xi)' > U(xi)*	*U(xi)'*	*rel U(xi)'*	*U(xi)' > U(xi)*		*U(xi)'*	*rel U(xi)'*	*U(xi)' > U(xi)*
NIMT	181838	16%	Y	3466	9.6%	Y	NIMT	2.7	8.2%	N
							NML	1.9	5.5%	N
KRISS	128316	9.9%	N	2778	7.5%	N	KRISS	1.4	4.1%	N
VNIIM	268862	25%	Y	4161	12%	Y	VNIIM	3.9	13%	N
NMIA	138636	11%	Y	3189	8.8%	Y	NMIA	1.2	3.5%	N
NIM	145962	11%	Y	2884	7.2%	Y	NIM	1.3	3.7%	N
INMETRO	228377	16%	Y	6271	15%	N	INMETRO	1.3	3.8%	N
INM	264944	18%	Y	4578	11%	Y	INM	2.4	6.6%	N
INRM	158847	13%	N	3620	11%	N	INRM	1.3	3.6%	N
UME	296282	19%	N	8210	18%	Y	UME	1.2	3.6%	N

CONCLUSIONS

Study CCQM-K176 tested the comparability of copy number concentration and copy number ratio measurements of *HER2* and a reference gene, in two gDNA samples with low and high HER2 amplification. Results from participating NAWG members showed high reproducibility, resulting in KCRV relative expanded uncertainties of <5% and <10% for ratio and concentration measurands respectively. Ratio-based measurements showed the highest consistency between laboratories, whereas greater additional interlaboratory variance was observed for concentration measurements, which may be due to factors such as differences in DNA sample pre-treatment or differences in dPCR partition volume. Degrees of equivalence ranged from <1% to 20% for copy number concentration and from <1% to 11% for copy number ratio, demonstrating levels of accuracy which is fit-for-purpose in supporting value assignment activities in the field of cancer biomarker genetic testing.

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APPENDIX A: Sequence information

HER2 Sequence

Gene ID 2064 (https://www.ncbi.nlm.nih.gov/gene/2064)

Homo sapiens chromosome 17, GRCh38.p13 Primary Assembly

NC_000017.11: 39688094-39728660

ERBB2/*HER2*-EXON4

>NC_000017.11: 39694639-39695571 Homo sapiens chromosome 17, GRCh38.p13 Primary Assembly

aatcaagactttgtgattgccatagttgtatgcttctcaaaggttcctcgtctcctcttccttggaccaaaagtcagaggcaagaatgccctcatt
cataccccagtggtctatacctccagcagcaagtcgagtgagcaagtgatgtcctgaaaggcccagtggatcagtggaatgaagcgggca
ggaagacttagtgctcctgaaacaaggaatccagaatccaggagaaggatggctcagtggggctttcaagggacaagtatgggggttgaa
ggggtcactgtccctataccaaatccgaaaatattgtgaccaggaaccattctgtccaactcttctatttcaggtggcaaagcaaagctatattc
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cgcaggacagaagagtgctttttgtttcagcagagcagcctggggagagataaaagctactcctggggcctgggcctgcattcctgagatg
tgggtaagaggggcccagggtcagagtgtctggcaagcttggctctgccccttgctgtcctggagactagggctaatcctgggctcaggg
agtggcctccccatggttaggatacaagtgctcatcaagggccacccctaggaaggaccaattttcctatcagaagcttctaagttatcctcct
ttggcccaaagggacacctcaagcctactctgaggaactctttccaatgaactaattcctacagtcacttccccagcaacctgtgcctcagcct
caaggcactgtggg

ERBB2/*HER2*-EXON16

>NC_000017.11:39715445-39716387 Homo sapiens chromosome 17, GRCh38.p13 Primary Assembly

ggttacctatacatctcagcatggccggacagcctgcctgacctcagcgtcttccagaacctgcaagtaatccggggacgaattctgcacaa
gtgagcactgagaaagagggggcctgatggggaggagtcccagggaggagtccctgtgggaagctttgggcctgagggagtactcctg
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gggcccacccagtgtgtcaactgcagccagt

ERBB2/*HER2*-EXON24

>NC_000017.11:39724733-39725827 Homo sapiens chromosome 17, GRCh38.p13 Primary Assembly

acgtgatggctggtgtgggctccccatatgtctcccgccttctgggcatctgcctgacatccacggtgcagctggtgacacagcttatgccct
atggctgcctcttagaccatgtccgggaaaaccgcggacgcctgggctcccaggacctgctgaactggtgtatgcagattgccaaggtatg
cacctgggctctttgcaggtctctccggagcaaacccctatgtccacaaggggctaggatggggactcttgctgggcatgtggccaggccc
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ctcggaacgtgctggtcaagagtcccaaccatgtcaaaattacagacttcgggctggctcggctgctggacattgacgagacagagtacca
tgcagatgggggcaaggttaggtgaaggaccaaggagcagaggaggctgggtggagtggtgtctagcccatgggagaactctgagtgg
ccacctccccacaacacacagttggaggacttcctcttctgccctcccaggtgcccatcaagtggatggcgctggagtccattctccgccgg
cggttcacccaccagagtgatgtgtggagttatggtgtgtgatgggggtgttgggaggggtgggtgaggagccatggctggagggagg
atgagagctgggatggggagaattacggggccacctcagcatgtgaagggagggaaggggctgcctgtgccccaccttgcagggtctgt
gcacttcccaggattagggaaagaccgggtagggtctgtctcctggcatcacatctccccctgctacctgccatgatgctagactcctgagc
agaacctctggctcagtacactaaagctccctctggccctcccactcctgaccctgtctctgccttaggtgtgactgtgtgggagctgatgact
tttggggccaaaccttacgatgggatcccagcccgggagatccctgacctgctggaaaaggggggagcggctgccccagccccccatctg
caccat

***RPPH1* Sequence**

>NC_000014.9:20343050-20343764 Homo sapiens chromosome 14, GRCh38.p13 Primary Assembly

ggggtcacagtaggtggcatcgttcctttctgactgcccgccccccgcatgccgtcccgcgatattgagctccgaacctctcgccctgccgc
cgccggtgctccgtcgccgccgcgccgccatggaattcgaacgctgacgtcatcaacccgctccaaggaatcgcgggcccagtgtcact
aggcgggaacacccagcgcgcgtgcgccctggcaggaagatggctgtgagggacaggggagtggcgccctgcaatatttgcatgtcgc
tatgtgttctgggaaatcaccataaacgtgaaatgtctttggatttgggaatcttataagttctgtg/atgagaccactc/ttttcccATAGGG
CGGAGGGAAGCTCATCAGTGGGGCCACGAGCTGAGTGCGTCCTGTCACTCCACTCC
CATGTCCCTTGGGAAGGTCTGAGACTAGGGCCAGAGGCGGCCCTAACAGGGCTCTC
CCTGAGCTTCGGGGAGGTGAGTTCCCAGAGAACGGGGCTCCGCGCGAGGTCAGACT
GGGCAGGAGATGCCGTGGACCCCGCCCTTCGGGGAGGGGCCCGGCGGATGCCTCCT
TTGCCGGAGCTTGGAACAGACTCACGGCCAGCGAAGTGAGTTCAATGGCTGAGGTG
AGGTACCCCGCAGGGGACCTCATAACCCAATTCAGACTACTCTCCTCCGCCCATTtttg
gaaaaaaaaaaaaaaa

Key:
Yellow: *HER2* positive cell line only
Cyan: common to both cell lines

The *HER2* positive cell line is homozygous for the two highlighted SNVs (g/a and c/t). The *HER2* negative cell line contains the reference allele at the first position and is homozygous for the 2nd SNV (c/t) only.

Use this section to describe the target sequence(s) or variants in further detail.

APPENDIX B: Coordinating laboratory methodology

Homogeneity testing

From the batch of 200 vials of each test sample, 10 vials were randomly picked for homogeneity testing by dPCR (QX200 system), three replicate measurements for each vial, focusing on Measurands 1-3 (*HER2*, *RPPH1*-related quantities). After the sample is melted at room temperature, it was stored in a metal bath at 60 ° C, 2 min, 600 rpm. After *Eco*RI digestion, dilute Sample 1 and Sample 2 with TE 0.1 for 6 and 109 times respectively, and then carry out digital PCR. The same dPCR assays and conditions were used as for NIM participant analysis (Appendix H).

The final confirmed droplet digital PCR reaction system is: 2×ddPCR mastermix (BioRad, Shanghai) 10 μL, primer (5 mM) 1.2 μL (final concentration at 300 nM), probe (10 mM) 0.6 μL (final concentration at 300 nM), DNA template (about 5000 copies/μL) 4 μL, ddH2O complement 20 μL. PCR procedure: 95 ℃ 10 min, 94 ℃ 30 s, 60 ℃ 1 min, 40 cycles, 98 ℃ 10 min. Then the thermal cycled plate was analysed on QX200 droplet reader (BioRad, CA, USA). Data analysis was performed with the QuantaSoft software (version 1.7.4, Bio-Rad).

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-vial differences in the measurand. The value of the relevant F-test ratios was smaller than the F critical at 0.05 confidence level, which indicated that the inhomogeneity of ratio in study material was insignificant. The F-test value of the absolute copy number was bigger than the F critical at 0.05 confidence level, however the between-vial (homogeneity) uncertainty (u_{bb}) calculated based on the analysis, were smaller than 3%, indicating the inhomogeneity was mainly caused by the imprecision of the dPCR method.

Stability Assessment of Study Material

Short-term stability was tested on the study material at 4 °C and 20 °C for 0, 4, 9 and 14 days. Three vials were analysed at each time point, and three replicate measurements for each vial. This study was designed to test the material stability under transportation conditions. Samples were processed by the method in homogeneity. The samples were demonstrated to be stable at 4°C and 20°C for 14 days.

For evaluation of long-term stability, the *HER2* CNV samples were tested at 0, 1, 2, 3, 6 and 12 months stored at -70ºC. Three vials were taken at each time point and analysed using the same dPCR method as used in the short-term stability study. The trend-analysis was used to assess the stability according to ISO Guide 35. The statistical results indicated that no significant trend at 95% confidence level was detected. Hence, the instability of the material was insignificant at the study temperature over the study period.

APPENDIX C: Protocol

CCQM-K176/P218

Breast cancer biomarker *HER2* copy number variation (CNV) measurement

Key Comparison

Coordinating Laboratories: NIM, NML

Study Protocol v1.0

26 January, 2021

Introduction

Testing of the core competencies of laboratories that deliver measurement services of copy number variant (CNV) analytes and CNV measurement has not been covered previously. Agreement was received in the October 2019 meeting of the NAWG in Torino to conduct the *HER2* CNV measurement as a key comparison. This study is proposed as a follow up study to CCQM-P184 Copy number concentration and fractional abundance of a mutation (SNV or INDEL) mixed with wild-type DNA. In CCQM-P184, all participants measured single nucleotide (*BRAF* gene) and small deletion (*EGFR* gene) variants in a background of wild type genomic DNA, so CMC can be claimed for copy number concentration and copy number ratio measurements of small variants (gene regions) by digital PCR. Thus, this proposed study aims to extend participants' capability from SNV and INDEL measurement to structural variant measurement, focusing on gene copy number variation.

Breast cancer is the most common malignant tumor in women all over the world. Human epidermal growth factor receptor 2 (*HER2*)-positive breast cancer accounts for about 20% ~ 30% of invasive breast cancer, and this type of tumor is characterized by high invasiveness, high risk of recurrence, rapid progression and poor prognosis [1]. The *HER2* gene (official gene name, *ERBB2* https://www.ncbi.nlm.nih.gov/gene/2064) is a proto-oncogene located on the long arm of human chromosome 17, coding a transmembrane glycoprotein with receptor tyrosine kinase activity. Over expression or amplification of the *HER2* gene will lead to over-transmission of signals, which will stimulate the growth and metastasis of cancer cells [2]. The determination of *HER2* gene expression in tumor cells of breast cancer patients is beneficial to the choice of treatment, through targeted therapies such as trastuzumab, and prognosis. Digital PCR as an absolute copy number measurement method, is ideally fit for detection of *HER2* amplification. In

addition, dPCR has the benefit of providing quantitative results, rather than relying on the expert skill of a seasoned pathologist for determination.

The aim of the key comparison is to measure 1) *HER2* copy number concentration; 2) the copy number concentration of a single copy reference gene (ribonuclease P RNA component H1 (*RPPH1*); 3) the *HER2*/ *RPPH1* copy number ratio, using two defined sequences representative of the two genes (*HER2* and *RPPH1*) in mixed genomic DNA samples, and 4) provide evidence for CMC claims by participating laboratories when measuring purified genomic DNA. In addition, a reference region (*CEP17*) commonly measured in clinical tests and (like *HER2*) also present on chromosone 17 but previously found to be subject to genomic instability [3], will be optionally measured to inform the applicability of potential reference measurement procedures to the clinical measurand. A parallel pilot study (CCQM-P218) will also be conducted with the same material for interested parties.

This study and GMO studies (K86 serial) support absolute and relative measurement of DNA gene copy number and copy number ratio. However, the difference is the range of copy number ratio is =>1 in this study and normally is 0.1-100% (0.001-1) in GMO study.

Study Materials

There will be two study materials (Sample 1 "S1" and Sample 2 "S2") with different *HER2* CNV levels prepared by mixing normal and *HER2* positive genomic DNA. The matrix is TE buffer. *HER2* 'positive' genomic DNA (*HER2*-amplified) was extracted from a cell line, HCC1954, which was purchased from ATCC. *HER2* 'negative' genomic DNA (normal *HER2* gene copy number of 2 per diploid cell) was extracted from a cell line, PLCL7, which is from a healthy donor. The indicative range for the *HER2*/*RPPH1* of the analyte is 1.0~40, in a concentration of 90~130 ng/μL. The homogenized DNA mixture was aliquoted into 100 bottles, with content of 100 μL each. Packing was in 1.5 mL microtube. Long term storage of the material at NIM is at -70ºC.

For *RPPH1* sequence, two homozygous SNVs (g/a and c/t) occurred in the *HER2* positive cell line, with the second SNV (c/t) also homozygous in *HER2* negative cell line (see Appendix 4).

For *CEP 17* sequence, two homozygous SNVs (T/C, G/A) and two heterozygous positions (Y：C or T, R：A or G) were observed in *HER2* positive cell line, but only one homozygous SNV (T/C) and three heterozygous positions in the *HER2* negative cell lines (see Appendix 5).

Measurand

Measurand 1: copy number concentration of *HER2*

Measurand 2: copy number concentration of *RPPH1*

Measurand 3: ratio of *HER2* to *RPPH1*

Measurand 4: copy number concentration of *CEP17* (optional)

Measurand 5: ratio of *HER2* to *CEP17* (optional)

Methods

The study will require assay design, quantitative detection of the analyte in the buffer. Participants are anticipated to perform measurements by digital PCR (microfluidic dPCR, chip dPCR or droplet dPCR); however, other techniques such as next generation sequencing (NGS) may be used for the Measurand 3.

Homogeneity

All samples were kept at the storage condition of -70ºC by NIM. 10 bottles of samples were taken randomly, and analysis of triplicate sub-samples was carried out using ddPCR method. Results are shown in Figure 1.

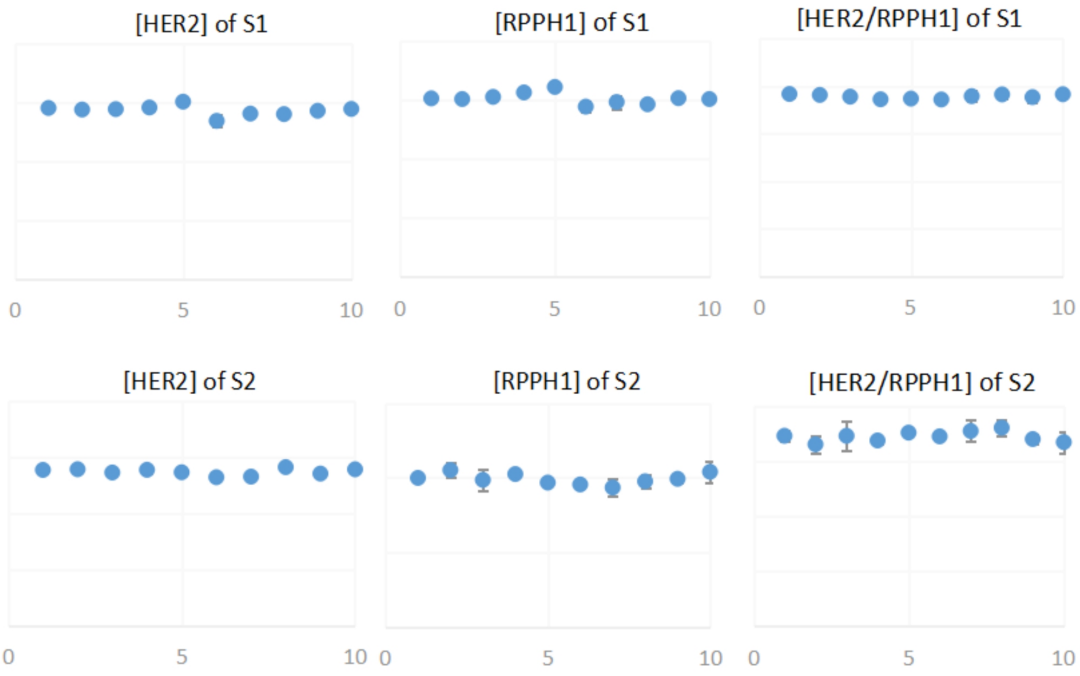


Figure 1 Homogeneity of the two unknown samples. Results are shown for 10 bottles (x-axis) with values for each bottle as mean±SD (y-axis, scale blinded).

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-packet differences in the copy concentration of the measurand (Table 1). The value of the relevant *F*-test ratios is smaller than

the F critical at 0.05 confidence level, which indicates that the inhomogeneity of the study material was insignificant.

Table 1 Summary of uncertainty evaluation for homogeneity

u_{bb}	Sample 1	Sample 2
HER2	2.7%	2.0%
RPPH1	2.8%	1.2%
HER2/RPPH1	0.62%	1.2%

Stability

Short term stability was tested on the study material at 4ºC and 20 ºC for 0, 7, 9 and 14 days. Three vials were analysed at each time point. This study was designed to test the material stability under transportation conditions. The result is shown in Figure 2. The sample can be stable at 4ºC and 20 ºC for 2 weeks. For long term stability, the *HER2* CNV samples were tested at 0, 1, 2, 3, 6 and 12 months at -70ºC. Three vials were taken at each time point and analysed using ddPCR method. The result of long-term stability are shown in Figure 3. The trend-analysis was used to assess the stability according to ISO Guide 35. The statistical results indicated that no significant trend at 95% confidence level was detected. Hence, the instability of the material was insignificant at the study temperature over the study period.

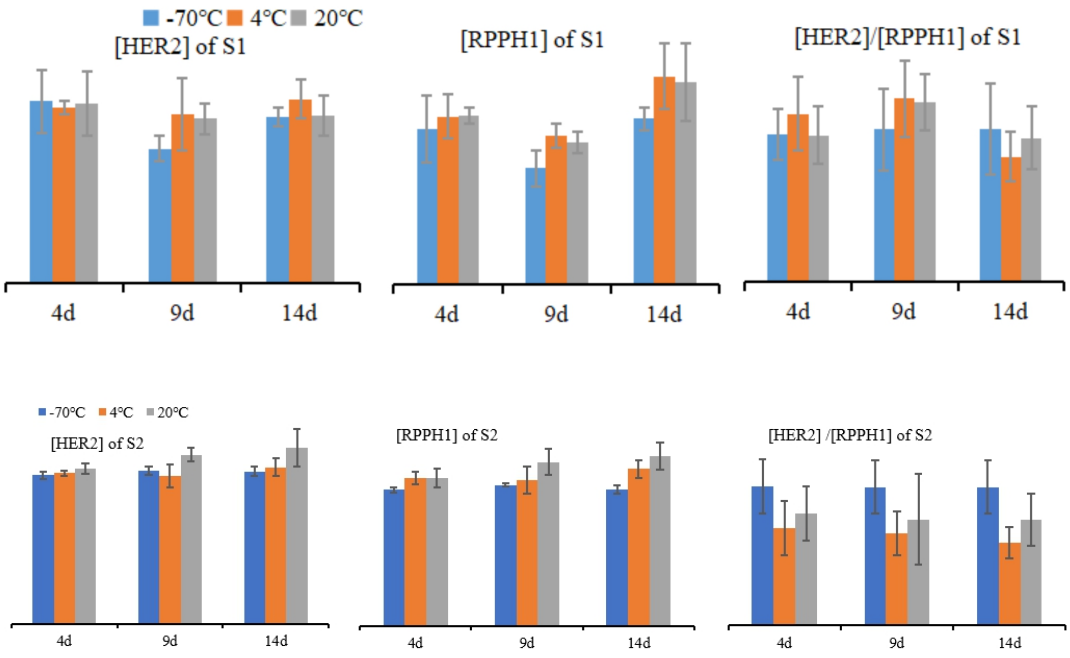


Figure 2. Short-term stability of the two unknown samples (S1 and S2). Results are shown in mean+/-SD (y-axis, scale blinded).

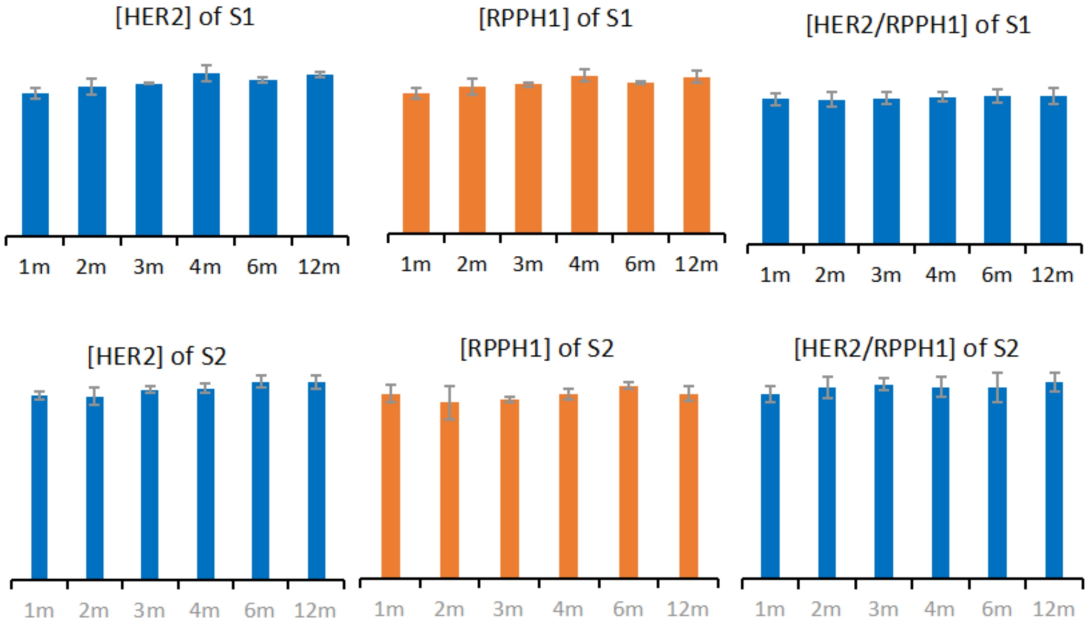


Figure 3. Long-term stability of study material. Results are shown in mean±SD (y-axis, scale blinded).

Study Guidelines

Each participant will receive 2 samples and each sample with 3 vials and, each containing 100 µL. One vial is intended for method development and the other two are to be used for determination of the final result. Samples should be stored at -70ºC. A minimum sample intake of 5 µL is recommended. An *Eco*RI (purchased from Takara) restriction enzyme digestion is needed before the qualification. The recommended digestion protocol is in Table 2. Participants may use their preferred laboratory procedures.

Table 2 Restriction digestion protocol

Reagent	Volume/Reaction
10×H buffer	2 µL
*Eco*RI	1 µL
ddH_2O	7 µL
DNA	10 µL
Total	20 µL

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 copy/µL (Measurands 1, 2 and 4) or no units (Measurands 3 and 5; copy number ratio) 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, dPCR mastermix, dPCR platform, quantification approach), 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 all NMIs/DIs participating in the key comparison that have used appropriately validated methods with demonstrated metrological traceability. The draft A report will provide candidate estimates of the KCRV and its uncertainty for review and discussion by the NAWG.

How Far Does the Light Shine?

Successful participation in this key comparison "**Breast cancer biomarker *HER2* copy number variation (CNV) measurement**" will demonstrate participant's capabilities in determining the structural variant with CNV range from 1.0 to 40 at a relative high concentration of intact (non-fragmented) eukaryotic genomic DNA. The study will also support participants' claim for measurement of gene copy concentration (and/or genomic regions of <41 Kb) in eukaryotic intact genomic DNA in a concentration of 90~130 ng/μL, corresponding to a gene copy number concentration of ~10^4-10^6 gene copies/μL (taking into account both reported reference gene and *HER2* values).

This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) value assignment of calibration solutions; (3) quantification of *HER2* CNV using dPCR.

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 K176/P218

Event	Period
Preparation of sample	Dec 2018
Homogeneity testing	Feb 2019
Stability testing	From Feb 2019
Invitation of participants	26 Jan 2021
Deadline for registration	15 Feb 2021
Dispatch of samples	April-July 2021
Deadline for submission of results	30 Sep 2021
Discussion of report at the CCQM NAWG	Oct 2021

Contact information:

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

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

Ms. Xia Wang, NIM, wangxia@nim.ac.cn

References

[1] Zhao W, Bian L, Jiang Z. Research status of HER-2 gene mutation in breast cancer. Chinese journal of mammary disease (electronic edition),2016,10(06):362-365.

[2] Moasser MM. The oncogene *HER2*: its signaling and transforming functions and its role in human cancer pathogenesis. Oncogene. 2007 Oct 4;26(45):6469-87.

[3] Zhu Y, Lu D, Lira ME, Xu Q, Du Y, Xiong J, Mao M, Chung HC, Zheng G. Droplet digital polymerase chain reaction detection of *HER2* amplification in formalin fixed paraffin embedded breast and gastric carcinoma samples. Exp Mol Pathol. 2016 Apr;100(2):287-93.

Appendix 1: Invitation letter (Including Registration Form)

Appendix 2: Reply Forms [*reproduced in Study Report Appendices D-G*]

Appendix 3: *HER2* sequence [*reproduced in Study Report Appendix A*]

Appendix 4: *RPPH1* sequence [*reproduced in Study Report Appendix A*]

Protocol Appendix 1 Invitation letter

CCQM Key comparison/pilot study (CCQM-K176/P218)

"Breast cancer biomarker *HER2* copy number variation (CNV) measurement"

Dear CCQM colleagues,

Breast cancer is the most common malignant tumor in women all over the world. *HER2*-positive breast cancer accounts for about 20% ~ 30% of invasive breast cancer, and this type of tumor is characterized by high invasiveness, high risk of recurrence, rapid progression and poor prognosis [2]. *HER2* gene is a proto-oncogene located on the long arm of human chromosome 17, coding human epidermal growth factor receptor 2 (*HER2*) transmembrane glycoprotein with receptor tyrosine kinase activity. Overexpression or amplification of the *HER2* gene will lead to over transmission of signals, which will stimulate the growth and metastasis of cancer cells [4]. The determination of *HER2* gene expression in tumor cells of breast cancer patients is beneficial to the choice of treatment and prognosis. Digital PCR as an absolute copy number measurement method is ideally fit for detection of *HER2* amplification. In addition, ddPCR has the benefit of providing quantitative results, rather than relying on the expert skill of a seasoned pathologist for determination.

We would like to invite you to participate in the CCQM key comparison (CCQM K176) "Breast cancer biomarker *HER2* copy number variation (CNV) measurement". In this study, the copy number concentration of *HER2* and a reference gene, *RPPH1*, as well as the ratio of *HER2* and *RPPH1*, in genomic DNA extracted from cell lines need to be determined. *CEP17* copy number concentration and *HER2*/*CEP17* copy number ratio measurements are optional.

The materials that will be sent to the participants will consist of a set of

Two unknown samples each containing different copy number ratio of *HER2* and *RPPH1* (and *HER2*/*CEP17*).

All NMIs and designated laboratories are invited to participate in the study. If your organization is going to participate in the study, please reply to us (lianhuadong@126.com, donglh@nim.ac.cn) with the Form 1 in Appendix 2 by 15th Feb 2021.

Timeline of the study will be as follows:

1. Distribution of draft testing protocol and call for participation: **26 Jan, 2021**

2. Sign up for participation: **15 Feb, 2021**

3. Shipping of test materials: **April-July, 2021**

4. Return of the measurement results: **20 Sep, 2021**

5. Initial discussion: **Oct, 2021**

If you have any comments or questions, please send email to us (lianhuadong@126.com and donglh@nim.ac.cn).

Best wishes,

Dr. Lianhua Dong

Center for Advanced Measurement Science

National Institute of Metrology (NIM), P. R. China

No.18 Bei San Huan Dong Lu, Beijing, 100029, China

Tel: 86-10-64524959

APPENDIX D: Registration Form

Form 1: Registration and Confirmation of sample distribution address

Institute	
Contact person	
Email address	
Address for sample reception	
Phone number	
Any preference on the AWB and invoice	

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

Vial	Measurand 1	Measurand 2	Measurand 3 (no unit)	Measurand 4 (optional)	Measurand 5 (optional)
x copies/µL					
u copies/µL					
k					
U copies/µL					
U(x)/x %					

3.2 Result of Sample 2

Vial	Measurand 1	Measurand 2	Measurand 3 (no unit)	Measurand 4 (optional)	Measurand 5 (optional)
x copies/µL					
u copies/µL					
k					
U copies/µL					
U(x)/x %					

APPENDIX G: Experimental details form

Form 4: Experimental details
Assays

Lab	Assay ID	Primer/probe	Oligonucleotide sequences (5′ → 3′)	Final (uM)	Amplicon size (bp)	Supplier & purification	PCR thermocycling	Technica 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 "Analytical Information" worksheets. 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: Summary of sample pre-treatment conditions for CCQM-K176

<table>
<tr><th>Laboratory ID</th><th>Sample mixing/pre-treatment (e.g. vortexing/heating)</th><th>Restriction digestion conditions (enzymes name/source/buffer/units, DNA input (ng), reaction volume (µL), pre-dPCR or in situ)</th><th>Other pre-treatment</th></tr>
<tr><td>1</td><td>Vortex/Spin down</td><td>EcoRI-HF (NEB, Cat. No.: R3101S)
Digestion conditions: 37 °C, 30 min; 65 °C, 10 min
<table>
<tr><th>Component</th><th>Volume (µ L)</th></tr>
<tr><td>10*H buffer</td><td>2</td></tr>
<tr><td>EcoRI</td><td>1</td></tr>
<tr><td>ddH_2O</td><td>12</td></tr>
<tr><td>DNA</td><td>5</td></tr>
<tr><td>total</td><td>20</td></tr>
</table></td><td>After digestion, SM1 was diluted 10-fold and SM2 100 -fold with water (Autoclaved)</td></tr>
<tr><td>2</td><td>Manual vortexing of the sample (~1600 rpm, 10 ± 5s) followed by short centrifugation</td><td>EcoRI-HF (NEB, Cat. No.: R3101T) + CutSmart Buffer (NEB, Cat. No.: B7204S)
Reaction mix (final volume = 20 µL):
Buffer (10x): 2 µL
Enzyme: 15 Units
DNA; 10 µL (undiluted, equivalent to 900 – 1300 ng of DNA)
Nuclease-free water
Digestion conditions: 37 °C, 30 min; 65 °C, 10 min.</td><td>After digestion, SM1 was diluted 10-fold and SM2 90-fold volumetrically in nuclease-free water (Ambion, Cat. No.: AM9939)</td></tr>
<tr><td>3</td><td>brief vortexing and centrifugation</td><td>EcoRI (NEB)
Digestion conditions: 37 °C, 30 min, 65 °C, 10 min
<table>
<tr><th>Component</th><th>Volume (µ L)</th></tr>
<tr><td>10*H buffer</td><td>2</td></tr>
<tr><td>EcoRI</td><td>1</td></tr>
<tr><td>ddH_2O</td><td>12</td></tr>
<tr><td>DNA</td><td>5</td></tr>
<tr><td>total</td><td>20</td></tr>
</table></td><td>Digestion reaction dilutes materials 1:4. Material 2 was additionally diluted 1/40 after digest.</td></tr>
<tr><td>4</td><td>brief vortexing and spin-down centrifugation</td><td>FastDigest EcoR I (Thermo Scientific, Cat# FD0274)
EcoRI digestion of study materials was performed by addition of enzyme directly in ddPCR reaction mixture from the beginning. Every 20 µL of reaction mix contained 0,1 µL of enzyme.</td><td>Material 1 was preliminary diluted 1:20, Material 2 was diluted 1:20 for RPPH1 and CEP17 measurements and 1:400 for HER2 measurements</td></tr>
<tr><td>5</td><td>Allow to thaw, mix by vortexing for 10 seconds, then spin in pulse centrifuge</td><td>No EcoRI pre-digestion recommended in the Study Protocol was performed. Instead, estimated digestion bias</td><td>Not applicable</td></tr>
</table>

<table>
<tr>
<td>6</td>
<td>Incubate at 60 ℃, 600rpm for 2 min;
short centrifugation after vortex</td>
<td>EcoR I (Takara，Code No.1040S)
Digestion conditions: 37 °, 30 min, 65°C, 10 min
<table>
<tr><th>Component</th><th>Volume (μ L)</th></tr>
<tr><td>10*H buffer</td><td>2</td></tr>
<tr><td>EcoRI</td><td>1</td></tr>
<tr><td>ddH_2O</td><td>7</td></tr>
<tr><td>DNA</td><td>10</td></tr>
<tr><td>total</td><td>10</td></tr>
</table>
</td>
<td>After enzymatic digestion, study material 1 was diluted 6 fold,
and study material 2 was diluted 109 fold with TE0.1 buffer before the dPCR.</td>
</tr>
<tr>
<td>7</td>
<td>Brief vortexing (>5 s) and spin-down</td>
<td>EcoRI (NEB, Cat. No.: R3101S)
Digestion conditions: 37 ℃, 30 min, 65℃, 10 min
<table>
<tr><th>Component</th><th>Volume (μ L)</th></tr>
<tr><td>DNA</td><td>10</td></tr>
<tr><td>EcoRI</td><td>1</td></tr>
<tr><td>10x buffer</td><td>2</td></tr>
<tr><td>ddH_2O</td><td>7</td></tr>
<tr><td>Total volume</td><td>20</td></tr>
</table>
</td>
<td>Material 1 was preliminary diluted 1:16; Material 2 was diluted 1:10 and 1:400.</td>
</tr>
<tr>
<td>8</td>
<td>Manual sample mixing and short centrifugation</td>
<td>EcoRI digestion of the study material was performed according to the manufacturer's specifications (Ref R601A, Promega).
<table>
<tr><th>Component</th><th>Volume (μL)</th></tr>
<tr><td>Deionized water</td><td>8.85</td></tr>
<tr><td>10X Buffer</td><td>2</td></tr>
<tr><td>Acetylated BSA (10 μg/μl)</td><td>0.2</td></tr>
<tr><td>EcoRI (12 U/μl)</td><td>1.25</td></tr>
<tr><td>DNA</td><td>7.7</td></tr>
<tr><td>Total volume</td><td>20</td></tr>
</table>
The tubes were incubated at 37 °C for 4 h and 65 °C</td>
<td>After enzymatic digestion, subsampling of the digested study material NIM S01 and NIM S02 was performed, followed by a dilution of 1/2 for NIM S01, and 1/25 for NIM S02 with TE 1X buffer.</td>
</tr>
</table>

<table>
<tr><td></td><td></td><td>for 15 min.</td><td></td></tr>
<tr><td>9</td><td>After the arrival the samples were stored at -20°C.
The day before the analysis the samples were defrosted completely in ice, then briefly vortexed (about 5s), spinned down (about 10s) and replaced immediately in ice. After 2-3 minutes we added the sample to the digestion mix.</td><td>20 µL for each tube were digested, as follows (digestion with EcoRI, Thermo Scientific, cat. n.: ER0271):
<table><tr><th>Component</th><th>Volume (µL)</th></tr><tr><td>Nuclease-free water</td><td>14</td></tr><tr><td>10x Buffer EcoRI</td><td>4</td></tr><tr><td>DNA</td><td>20</td></tr><tr><td>EcoRI (10 U/µL)</td><td>2</td></tr><tr><td>Total</td><td>40</td></tr></table>
The tubes were incubated at 37°C for 1h 30’. After digestion the enzyme was inactivated at 65°C for 20 minutes.</td><td>Tubes were vortexed for 5 seconds (moving up and down each tubes during vortex), spinned and samples diluted (S1 sample was diluted 1:10, S2 diluted 1:200). Dilution were stored at -20°C.</td></tr>
<tr><td>10</td><td>Vortex-spin down</td><td>Restriction digestion
FastDigest EcoRI Catalog number: FD0274 was used.
• 2 µL 10X FastDigest® buffer,
• 1 µL FastDigest® enzyme,
• 7 µL Water, nuclease-free and
10 µL, 310 ng (Sample 1) and 260 ng (Sample 2) DNA
total volume 20 µL.
Digestied at 37 °C 5 min.
Study material 1 was diluted 10 fold,
Study material 2 was diluted 100 fold before the dPCR</td><td></td></tr>
</table>

Table H-2: Summary of Analytical Techniques for CCQM-K176

Laboratory ID	Instrument model* (manual (M) or auto (A) droplet generation)	Mastermix* (with/without dUTP)	Thermal Cycler Model (Manufacturer)	Analysis Software*	Mean observed accepted partition number per well (min-max)
1	QX200 (A)	ddPCR Supermix for Probes No dUTP	T100 (BioRad)	QuantaSoft 1.7.4.0917	19301 (18925-23028)
2	QX200 (M)	ddPCR Supermix for Probes (without dUTP)	C1000 (Bio-Rad)	QuantaSoft 1.7.4.0917	16339 (10137 - 20633)
3	QX200	Bio-Rad ddPCR Supermix (with dUTP)	Veritipro 96-well thermal Cycler	QuantaSoft 1.7.4	18976 (13670-21102)
4	QX200	ddPCR Supermix for Probes (No dUTP)	C1000 Touch (BioRad)	QuantaSoft 1.7.4	(9709 – 12811)
5	QX150	dPCR Supermix for Probes (No dUTP)	C1000 (Bio-Rad)	QuantaSoft 1.7.4	16014 (13304-17617)
6	QX200	ddPCR Supermix for Probes (with dUTP)	Veritipro 96-well thermal Cycler	QuantaSoft 1.7.4	(12613-18759)
7	QX200 (A)	ddPCR Supermix for Probes (No dUTP)	C1000 Touch (BioRad)	QuantaSoft 1.7.4	14852 (12516-16793)
8	QX200	ddPCR Supermix for Probes (with dUTP)	CFX96 Touch Real Time PCR Detection System (Bio-Rad)	QuantaSoft	15419 (11853 – 19435)
9	QX200	ddPCR Supermix for Probes (No dUTP)	TX100 (Bio-Rad)	QuantaSoft™ Analysis Pro 1.0.596	(9959-18900)
10	QX200 (M)	ddPCR Supermix for Probes: (No dUTP)	C1000 Touch (BioRad)	QuantaSoft 1.7.4.0917	12557 (8011 - 19393

*Manufacturer of all instruments, mastermix and software: Bio-Rad

Table H-3: PCR assay specifications for CCQM-K176

Laboratory ID	Assay name (Uniplex/Duplex)		Forward primer/Reverse primer/Probe Oligonucleotide sequences and modifications (5' → 3')	Final concentration Forward/ Reverse/ Probe (uM)	Amplicon (bp)	Supplier/ purification
1 （HER2/RPPH1 duplex HER2/CEP17 Duplex）	HER2 Ex16	Forward	AACACCCACCTCTGCTTCGT	0.8	79	Macrogen/HPLC
		Reverse	CAGTGTGGAGCAGAGCTTGGT	0.8		
		Probe	FAM- TGCCCTGGGACCAGCTCTTTCG-BHQ1	0.25		
	RPPH1	Forward	GCGGATGCCTCCTTTGC	0.8	76	Macrogen/HPLC
		Reverse	ACCTCACCTCAGCCATTGAACT	0.8		
		Probe	Hex-CTTGGAACAGACTCACGGCCAGCG-BHQ1	0.25		
	CEP17	Forward	AGGCAACCGCCTATTGCA	0.8	71	Macrogen/HPLC
		Reverse	ACGGCAGCAAGAGAGGAAAG	0.8		
		Probe	Hex-CACATGGGCACTGCCTGAGCACC-BHQ1	0.25		
2 (Each HER2 assay was duplexed with CEP17 or RPPH1 assays)	HER2-EXON4	Forward	CCCTGAGCAAAGAGTCACAGA	0.9	88	Biosearch / Salt free
		Reverse	TCTGTCCTGCGACTCACCTCA	0.9		Biosearch / Salt free
		Probe	FAM-CAGGACAAACGCAGTGCAGCAC-BHQ1	0.25		Biosearch / dual HPLC
	HER2-EXON16	Forward	GAGTGTGGTAAGACAGGGAGC	0.9	100	Biosearch / Salt free
		Reverse	CTGCACAAGTCCAAGAACGC	0.9		Biosearch & Salt free
		Probe	FAM-AGTGTGCGCACTCCCCATCTGC-BHQ1	0.25		Biosearch / dual HPLC
	HER2-EXON24	Forward	TGCACTTCCCAGGATTAGGG	0.9	71	Biosearch / Salt free
		Reverse	CAGGTAGCAGGGGGAGATGT	0.9		Biosearch / Salt free
		Probe	FAM-AAAGACCGGGTAGGGTCTGTCTCC-BHQ1	0.25		Biosearch / dual HPLC
	CEP17_Probe 1 (F1/B-R7)	Forward	CTAGGGGTACAGGCATCCCA	0.9	75	Biosearch / Salt free
		Reverse	CCACTGGCCTTCCCTTTCTC	0.9		Biosearch / Salt free

		Probe	HEX-AACACTGGACTGGTGCTGTGGCT-BHQ1	0.25		Biosearch / dual HPLC
	RPPH1_MGB	Forward	GCGGAGGGAAGCTCATCAG	0.9	64	Biosearch / Salt free
		Reverse	GGACATGGGAGTGGAGTGACA	0.9		Biosearch / Salt free
		Probe	HEX-CACGAGCTGAGTGCG-MGB_NFQ	0.25		Eurofins / HPLC
3	HER2		F: accaggaaccattctgtcca R: gggagtagctttgcatgtgg probe: FAM-ttcaggtggcaaagcaaagctat-BHQ-1	1, 1, 0.25	101	Genotech, HPLC
	RPPH1		F: atcaacccgctccaaggaat R: tgtccctcacagccatcttc Probe: HEX-cagtgtcactaggcgggaaca-BHQ-1	1, 1, 0.25	93	Genotech, HPLC
	CEP17		F: CATGACCCTATGCCAGTCCT R: CAGAGGAGAGAACAGGCACA Probe: HEX-CCAGAGAGGACATCTGAGGACTC-BHQ-1	1, 1, 0.25	98	Genotech, HPLC
4	RPPH1	Forward	CGGAGCTTGGAACAGACTCA	0.4	97	Syntol, Russia PAAG (primers), PAAG+HPLC (probes)
		Reverse	GGAGAGTAGTCTGAATTGGGTTATG	0.4		
		Probe	FAM-CCTCACCTCAGCCATTGAACTCAC-BHQ1	0.5		
	CEP17	Forward	AAAGCCACAGGTAAGAAGTAGG	0.4	97	
		Reverse	CTAGATCACGGCAGCAAGAG	0.4		
		Probe	FAM-CTATTGCAGCACGTGGCACATGG-BHQ1	0.5		
	H2 E4	Forward	GGTGGCAAAGCAAAGCTATATTC	0.4	102	
		Reverse	CGTTTGTCCTGGCCATTCTA	0.4		
		Probe	HEX-ACATGCAAAGCTACTCCCTGAGCA-BHQ1	0.5		
	H2 E16	Forward	CCTCTTGGCATGGCTTCTC	0.4	96	
		Reverse	TGTAGGGAGAGGAAGAGTTCTG	0.4		
		Probe	HEX-AAGGATGCCAAGGCAGGTAGGAC-BHQ1	0.5		
5	HER2 Target1	Forward Primer	GAGTCACAGATAAAACGGGG	0.9	74	Merck HPLC
		Probe	FAM- ATGGCCAGGACAAACGCAGTGCAGC- BHQ1	0.25		
		Reverse Primer	CAGGGTCTGAGTCTCTGT	0.9		
	HER2_ Target2	Forward Primer	GACGAGTGTGGTAAGACAG	0.9	99	Merck HPLC
		Probe	FAM- ATCTGCCAGCACACAGCAGTGCCCA- BHQ1	0.25		
		Reverse	ACAAGTCCAAGAACGCTG	0.9		

		Primer				
	HER2 Target3	Forward Primer	TCAAGAGTCCCAACCATGTC	0.9	84	Merck HPLC
		Probe	FAM- TGGCTCGGCTGCTGGACATTGACGA- BHQ1	0.25		
		Reverse Primer	CATCTGCATGGTACTCTGTC	0.9		
	RPPH1 Target1	Forward Primer	CTTGGAACAGACTCACGG	0.9	80	Merck HPLC
		Probe	HEX- TCAATGGCTGAGGTGAGGTACCCCGCA- BHQ1	0.25		
		Reverse Primer	GAATTGGGTTATGAGGTCCC	0.9		
	RPPH1_ABI 4403326	VIC™ (5'), TAMRA™ Quencher (3')		VIC™ (5'), TAMRA™ Quencher (3')	87	As supplied
	CEP17_Target1	Forward Primer	AACCGCCTATTGCAGC	0.9	87	Merck HPLC
		Probe	HEX- TGGGCACTGCCTGAGCACCAGCTTT- BHQ1	0.25		
		Reverse Primer	GATGCCTGTACCCCTAGAT	0.9		
	CEP17_Target2	Forward Primer	GCTGATGATCATAAAGCCACAGGTA	0.9	81	Merck HPLC
		Probe	HEX- TGCTGCAATAGGCGG- MGB- NQF	0.25		
		Reverse Primer	TGGTGCTCAGGCAGTGC	0.9		
6	HER2-F	Primer	CCAGTAGAATGGCCAGGACAA	0.3	58	BGI，PAGE
	HER2-R	Primer	TGGCTGCCAGGGTCTGA	0.3		BGI，PAGE
	HER2-P	Probe	FAM-CGCAGTGCAGCACAG-BHQ1	0.3		BGI，HPLC
	RPPH1-F	Primer	GAGGGAAGCTCATCAGTGG	0.25	84	BGI，PAGE
	RPPH1-R	Primer	CCCTAGTCTCAGACCTTCC	0.25		BGI，PAGE
	RPPH1-P	Probe	VIC-CCACGAGCTGAGTGC-BHQ1	0.1		BGI，HPLC
	CEP17-F	Primer	GCTGATGATCATAAAGCCACAGGTA	0.8	81	BGI，PAGE
	CEP17-R	Primer	TGGTGCTCAGGCAGTGC	0.8		BGI，PAGE
	CEP17-P	Probe	VIC-TGCTGCAATAGGCGG-BHQ1	0.25		BGI，HPLC
7	RPPH1 (performed in	RPPH1-probe	HEX- GCCCTCCTTTGCCGGAGCTT- BHQ1	0.9	94	Exxtend (Brazil) HPLC
		RPPH-f	GTCAGACTGGGCAGGAGATG	0.25		AlphaADN

	duplex with HER2)					(Canada), HPLC
		RPPH-r	TGGCCGTGAGTCTGTTCC	0.25		AlphaADN (Canada), HPLC
	HER2 (performed in duplex with RPPH1)	HER2-Probe	FAM- ACCCAGCTCTTTGAGGACAACTATGC- BHQ1	0.9	112	AlphaADN (Canada), HPLC
		HER2-f	CTCATCGCTCACAACCAAGT	0.25		AlphaADN (Canada), HPLC
		HER2-r	GGTCTCCATTGTCTAGCACG	0.25		AlphaADN (Canada), HPLC
8	HER2 gene	HER2- 16- F	CCTATA CATCTC AGCATG GC	0.6	68	Biosearch Technologies
		HER2- 16- R	GGATTA CTTGCA GGTTCT GG	0.6		Biosearch Technologies
		HER2- 16- P	HEX- CAGCCT GCCTGA CCTCAG CGT- BHQ	0.3		Biosearch Technolo gies
	RPPH1 gene	RPPH1- 1- F	ACAGTA GGTGGC ATCGTT	0.6	74	Biosearch Technologies
		RPPH1- 1- R	GAGGTT CGGAG CTCAAT ATC	0.6		Biosearch Technolo gies
		RPPH1- 1- P	FAM- CCTTTC TGACTG CCCGCC CCC- BHQ	0.3		Biosearch Technologies
	ABL gene (Quality control)	ENF1003 (Direct primer)	TGGAG ATAACA CTCTAA GCATAACTAAAG GT	0.5	122	Biosearch Technologies
		ENF1043 (Reverse primer)	GATGTA GTTGCT TGGGAC CCA	0.5		Biosearch Technologies
		ENP1043 (Probe)	FAM- CCATTT TTGGTT TGGGCT T CACACC ATT- BHQ1	0.3		Biosearch Technologies
	BRC gene (Quality control)	ENF501 (Direct primer)	TCCGCT GACCAT CAATAA GGA	0.5	149	Biosearch Technologies
		ENF561 (Reverse primer)	CACTCA GACCCT GAGGCT CAA	0.5		Biosearch Technologies
		ENP541 (Probe)	HEX- CCCTTC AGCGG CCAGTA GCATCT GA- BHQ1	0.3		Biosearch Technologies
9	HER2 _ fw	Primer	ggctgctggacattgacgaga	0.9	140	Mass spectrometry

	HER2_rv	Primer	ctgtgtgttgtggggaggtgg	0.9		Mass spectrometry
	HER2_P	Probe	FAM -tgggggcaaggttaggtgaag - BHQ1	0.25		Mass spectrometry & HPLC
	RPPH1 _ fw	Primer	acgtcatcaacccgctccaag	0.9	107	Mass spectrometry
	RPPH1_rv	Primer	ccactcccctgtccctcaca	0.9		Mass spectrometry
	RPPH1_P	Probe	HEX –gtgtcactaggcgggaacacc – BHQ1	0.25		Mass spectrometry & HPLC
10	HER2	Forward	AAGACCGGGTAGGGTCTGTC	0.9	78	Oligomer, Turkey, HPLC
		Reverse	GGTTCTGCTCAGGAGTCTAGC	0.9		
		Probe	FAM-TCTCCCCCTGCTACCTGCCA-BHQ1	0.25		Macrogen, Korea, HPLC
	RPPH1	Forward	GTCACAGTAGGTGGCATCGT	0.9	70	Oligomer, Turkey, HPLC
		Reverse	GGAGCTCAATATCGCGGGAC	0.9		
		Probe	HEX-CTTTCTGACTGCCCGCCCCC-BHQ1	0.3		Macrogen, Korea, HPLC
	CEP17	Forward	TAGGCAACCGCCTATTGCAG	0.9	86	Oligomer, Turkey, HPLC
		Reverse	TGTACCCCTAGATCACGGCA	0.9		
		Probe	HEX-GGGCACTGCCTGAGCACCAG-BHQ1	0.5		Macrogen, Korea, HPLC

Table H-4 dPCR partition volume information for CCQM-K176

Laboratory ID	Partition volume	Partition volume uncertainty†	Partition volume basis*	If 1*, type of mastermix	If 2*, reference(s)
1	0.85 nL	0.01235	2		Philippe Corbisier, *et al* Anal Bioanal Chem, 2015
2	0.749 nL	0.0385 (5.13%)	2	n/a	Dagata JA *et al.* 2016. NIST SP 260-184. Kosir, A. B., *et al*. Anal Bioanal Chem, 2017; 409 (28), 6689-97. Pinheiro, L. B., *et al*. Anal Chem. 2017;89(21):11243-11251.
3		5.47%	3		
4	0.792 nL	3.7 %	1	ddPCR supermix for probes (BioRad)	
5	0.788 nL	4.4%	1	Bio-Rad ddPCR Supermix for Probes (no dUTP)	
6	0.838 nL	0.8%	2		Dong L *et al*. Scientific Reports, 2015(5):13174
7	0.762 nL	0.030 (3.94%)	2	ddPCR Supermix for Probes (no dUTP)	Average value weighted by uncertainty using data from; Kosir, AB *et al*. Anal Bioanal Chem, 2017; 409 (28), 6689-97; Dagata JA *et al.* 2016. NIST SP 260-184; Philippe Corbisier *et al* Anal Bioanal Chem, 2015; 407(7): 1831–40; Mehle, N *et al*. Plants 2020; 9(3), 326; Emslie, KR *et al.* Anal Chem 2019; 91(6) 4124-31
8	0.772 nL	2.8% (standard)	1	ddPCR supermix for probes (dUTP)	

9	0.741 nL	0.002 (3%)	1	ddPCR supermix for probes (dUTP)	
10	0.750 nL	3 %	2		CCQM P199: Participant results Alison Devonshire Molecular & Cell Biology team CCQM NAWG 3-4 October 19, INRIM, Torino, Dagata *et al*. 2016 (NIST) Pinheiro *et al*. 2017 (NMIA) Kosir *et al*. 2017 (NIB) Kosir *et al*. 2017 (INRIM)

† If included in MU budget

*Basis for partition volume value: 1 = in-house measurement; 2 = Literature; 3 = Bio-Rad value; 4 = other.

#personal communication ##reagent comparison.

Table H-5: dPCR thermal cycling conditions

Lab. ID	Pre-incubation (°C)	Pre-incubation (min)	PCR initial step temp (°C)	PCR initial step time (min)	PCR cycling temp 1 (°C)	PCR cycling time 1 (s)	PCR cycling temp 2 (°C)	PCR cycling time 2 (s)	Cycle number	PCR final incubation (Hold)	Ramp rate
1	/	/	95	10	94	30	58	60	40	98°C / 10 min; hold at 4°C	2°C/s
2	n/a	n/a	95	10	94	30	60	60	40	98°C / 10 min; hold at 4°C	2°C/s
3			95	10	94	30	58	60	50	98°C / 10 min, hold at 4°C	1.5℃/s
4			95	10	94	30	57	60	60	98°C / 10 min; hold at 4°C	2°C/s
5			95	10	95	30	59	60	45	98°C / 10 min, hold at 12°C	3°C/s
6	/	/	95	10	94	30	60	60	40	98°C / 10 min, hold at 4°C	2℃/s
7			95	10	94	30	59	60	45	98°C / 10 min, hold at 16°C	2°C/s
8			95	10	94	30	60	60	40	98°C / 10 min, hold at 4°C	2°C/s
9	/	/	95	10	94	30	60	60	40	98°C / 10 min, hold at 4°C	2°C/s
10	/	/	95	5	95	10	60	30	40	95℃ 10 min, hold at 4°C	2℃/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 and presented in alphabetized acronym order.

NIMT (Laboratory 1)

$$u_{ratio} = HER2_{ratio} \times \sqrt{\left(\frac{u_{HER2}}{HER2}\right)^2 + \left(\frac{u_{RHHP}}{RHHP}\right)^2}$$

HER2 material 1	
Parameter	Std Uncertainty u(xi)
Mean	48100
Relative Uncertainty of Precision	0.0440
Relative Uncertainty of Droplet volume	0.0124
Relative Uncertainty of Volume	0.0036
Combined Uncertainty	2205.09
Expanded Uncertainty	4,410.20
U(x)/x %	9.17

RPPH1 material 1	
Parameter	Std Uncertainty u(xi)
Mean	26400
Relative Uncertainty of Precision	0.0710
Relative Uncertainty of Droplet volume	0.0124
Relative Uncertainty of Volume	0.0036
Combined Uncertainty	1904.95
Expanded Uncertainty	3,809.91

U(x)/x %	14.43

HER2 material 2	
Parameter	Std Uncertainty u(xi)
Mean	1,243,000
Relative Uncertainty of Precision	0.0314
Relative Uncertainty of Droplet volume	0.0124
Relative Uncertainty of Volume	0.0036
Combined Uncertainty	42,182.14
Expanded Uncertainty	84,364.28
U(x)/x %	6.79

RHHP material 2	
Parameter	Std Uncertainty u(xi)
Mean	36,300.00
Relative Uncertainty of Precision	0.0351
Relative Uncertainty of Droplet volume	0.0124
Relative Uncertainty of Volume	0.0036
Combined Uncertainty	1,357.10
Expanded Uncertainty	2,714.20
U(x)/x %	7.48

NML (Laboratory 2)

Specific components included in the uncertainty estimation are given in Table I-2. The equation used to calculate relative combined uncertainty for Measurand 4 is given below:

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

Degrees of freedom for combined uncertainties were estimated based on the Welch-Satterthwaite equation.

Study Material 1

Measurand No.	3	4	5	
Measurand	HER2/RPPH1	CEP17	HER2/CEP17	Type of Uncertainty
Unit	Ratio	μL^{-1}	Ratio	
Value (x)	1.9 (final *x* rounded 1 d.p.)	24544.74 (final *x* rounded to nearest 10^2)	2.08 (final *x* rounded 1 d.p.)	
Type A relative uncertainty $u_{A,rel}$	0.7184%	11.28%	0.4215%	A
Partition volume relative uncertainty $u_{Vp,rel}$	-	5.13%	-	B
Relative combined uncertainty $u_{c\,rel}$	-	12.39%	-	
(Combined) degrees of freedom	3	4.19	50	
k	3.18	2.78	2.01	
Relative expanded uncertainty U_{rel}	2.29%	34.40%	0.85%	
Unrounded Expanded uncertainty U	0.0434 (final U rounded up to 1 s.f.)	8444.62 (final U rounded up to 2 s.f.)	0.0176 (final U rounded up to 1 s.f.)	

Study Material 2

Measurand No.	3	4	5	
Measurand	HER2/RPPH1	CEP17	HER2/CEP17	Type of Uncertainty
Unit	Ratio	μL^{-1}	Ratio	
Value (x)	35.19 (final *x* rounded 0 d.p.)	58159.89 (final *x* rounded to nearest 10^3)	19.99 (final *x* rounded 1 d.p.)	
Type A relative uncertainty $u_{A,rel}$	1.05%	15.43%	0.78%	A
Partition volume relative uncertainty $u_{Vp,rel}$	-	5.13	-	B
Combined relative uncertainty $u_{c\,rel}$	-	16.26	-	
(Combined) degrees of freedom	3	3.66	2	

k	3.18	3.18	4.30	
Relative expanded uncertainty U_{rel}	3.34%	51.75%	3.36%	
Unrounded expanded uncertainty U	1.175898 (final U rounded up to 1 s.f.)	30096.56 (final U rounded up to 2 s.f.)	0.670878 (final U rounded up to 1 s.f.)	

KRISS (Laboratory 3)

For Measurand 1 and 2: intermediate precision (reproducibility) and run repeatability were included in Type A evaluation. Additionally, relative standard uncertainties were individually derived for the following factors: partition volume, manual thresholding settings, homogeneity, weighing, assay dependency. For Measurand 3, weighing and partition volume uncertainties were omitted as their effect s are expected to be cancelled out.

VNIIM (Laboratory 4)

Measurements for both study materials were performed in two different days. Each day 8 dPCR replicates were performed. The following equation was used to estimate uncertainty:

$$u_c = \sqrt{u_{\text{repeat}}{}^2 + u_{reprod}{}^2 + u_{dil}{}^2 + u_{droplet}{}^2}$$

Measurement uncertainty for Measurand 3 (*HER2/RPPH1*) and Measurand 5 (*HER2/CEP17*) was estimated by equation using calculated uncertainties for gene copy number concentrations:

$$u_c = \sqrt{u_{\text{HER2}}{}^2 + u_{reference\ gene}{}^2}$$

Sample		*HER2*	*RPPH1*	*HER2/RPPH1* (no units)	*CEP17*	*HER2/CEP17* (no units)
S1	x, μL^{-1}	56 896	28 227	2,0	27 487	2,1
	u_{repeat}, μL^{-1}	832	360	-	328	-
	u_{reprod}, μL^{-1}	544	145	-	81	-
	u_{dil}, μL^{-1}	1138	565	-	550	-
	$u_{droplet}$, μL^{-}	2105	1044	-	1017	-

	[1]					
	u, μL^{-1}	2 591	1 248	0,1	1 205	0,1
	k	2				
	U, μL^{-1}	5 181	2 497	0,3	2 409	0,3
	U, %	9,1	8,8	12,7	8,8	12,6
S2	x, μL^{-1}	1 094 440	35 101	31,2	62 865	17,4
	u_{repeat}, μL^{-1}	12960	496	-	744	-
	u_{reprod}, μL^{-1}	3741	48	-	212	-
	u_{dil}, μL^{-1}	21889	702	1	1257	0
	$u_{droplet}$, μL^{-1}	40494	1299	1	2326	1
	u, μL^{-1}	47 968	1 557	1,9	2755	1,1
	k	2				
	U, μL^{-1}	95 937	3 115	3,9	5 511	2,2
	U, %	8,8	8,9	12,5	8,8	12,4

NIM (Laboratory 6)

Due to the sample being diluted by the gravimetric method, the uncertainty of our results includes the uncertainty introduced by repeatability, uniformity, droplet volume, and gravimetric dilution. Calculate the relative standard uncertainty according to Equation (1).

$$u_{c\,rel} = \sqrt{u_{\mathrm{a\,rel}}{}^2 + u_{df\,rel}{}^2 + u_{vp\,rel}{}^2 + u_{bb\,rel}{}^2} \quad \text{Equation (1)}$$

Sample1	HER2	RPPH1	HER2/RPPH1	CEP17	HER2/CEP17	Type of Uncertainty
x	5.76E+04	3.02E+04	1.91	2.86E+04	2.02	

	copies/µL	copies/µL		copies/µL		
Repeatability uncertainty $u_{a,rel}$（%）	0.91	0.63	1.42	5.09	6.19	A
Sample dilution (gravimetric) $u_{df,rel}$（%）	0.019	0.019	0.019	0.019	0.019	B
Partition volume $u_{vp,rel}$（%）	0.8	0.8	0.8	0.8	0.8	B
Homogeneity uncertainty u_{bb}（%）	2.7	2.8	0.62	2.8	0.62	A
Standard Uncertainty $u_{c\,rel}$（%）	2.96	2.98	1.75	5.87	6.27	
Relative Uncertainty U_{rel} (k=2) (%)	5.9	6.0	3.5	12	12	
U(k=2)	3.4E+03 copies/µL	1.8E+03 copies/µL	0.07	3.4E+03 copies/µL	0.25	

Sample2	HER2	RPPH1	HER2/RPPH1	CEP17	HER2/CEP17	Type of Uncertainty
x	1.41E+06 copies/µL	4.01E+04 copies/µL	35.3	7.41E+04 copies/µL	19.2	
Repeatability uncertainty $u_{a,rel}$（%）	1.69	2.90	3.66	2.38	2.32	A
Sample dilution (gravimetric) $u_{df,rel}$（%）	0.019	0.019	0.019	0.019	0.019	B
Partition volume $u_{vp,rel}$（%）	0.8	0.8	0.8	0.8	0.8	B
Homogeneity uncertainty u_{bb}（%）	2	1.2	1.2	1.2	1.2	A
Standard Uncertainty $u_{c\,rel}$（%）	2.74	3.24	3.93	2.79	2.73	
Relative Uncertainty U_{rel} (k=2) (%)	5.5	6.5	7.9	5.6	5.5	
U(k=2)	7.7E+04 copies/µL	2.6E+03 copies/µL	2.8	4.1E+03 copies/µL	1.0	

INMETRO (Laboratory 7)

Measurements was done in three different days, with HER2 and RPPH1 reactions performed in duplex. Material 1 and Material 2 was diluted 1:16 and 1:400 respectively before *Eco*RI digestion and prepared with two technical replicates. Uncertainties took into account reproducibility, repeatability, partition volume (as an estimate from different droplet measurements described in literature), thresholds, and gravimetric preparation. The uncertainty of Measurand 3 was obtained by combining HER2 and RPPH1 uncertainties as the root of its quadratic sum.

INM Colombia (Laboratory 8)

From each study material two tubes were measured on two different days. Three digestions were performed for each tube, two subsamples were taken from each digestion tube, and every subsample were measured in triplicate in duplex mode: HER2/RPPH1. From this design, the assigned value (mean of concentration values) and the precision component (R, through the nested design) was obtained.

The measurement model for Measurand 1 and 2 is composed by the lambda parameter λ (copies per partition), the droplet volume (v), the gravimetric dilution d and the precision component (R)

$$C_i = \frac{\lambda_i}{v * d_i} * R \quad \text{Ec 1}$$

The measurement model for Measurand 3 is composed by the ratio among HER2 and RPPH1 lambda values and the precision component

$$ratio = \frac{\lambda_{HER2}}{\lambda_{RPPH1}} * R \quad \text{Ec 2}$$

Based on Ec 1 and 2 the measurement uncertainty for Measurand 1 - 2 and 3 was calculated according to Ec 3 and 4 respectively

$$u_{(C_i)} = C_i * \sqrt{\left(\frac{u(\lambda_i)}{\lambda_i}\right)^2 + \left(\frac{u(v)}{v}\right)^2 + \left(\frac{u(d_i)}{d_i}\right)^2 + \left(\frac{u(R)}{R}\right)^2} \quad \text{Ec 3}$$

$$u_{(ratio)} = ratio * \sqrt{\left(\frac{u(\lambda_{HER2})}{\lambda_{HER2}}\right)^2 + \left(\frac{u(\lambda_{RPPH1})}{\lambda_{RPPH1}}\right)^2 + \left(\frac{u(R)}{R}\right)^2} \quad \text{Ec 4}$$

Following tables shows uncertainty Budget for Measurands 1, 2 and 3.

Sample	Measurand	Copies/uL	u λ rel (%)	u v rel(%)	u d rel(%)	u R rel (%)	u est rel (%)	u est (copies/uL)
1	HER2	61260	1.17	2.94	0.51	2.02	3.79	2319
	RPPH1	30652	1.22	2.94	0.51	1.83	3.70	1135

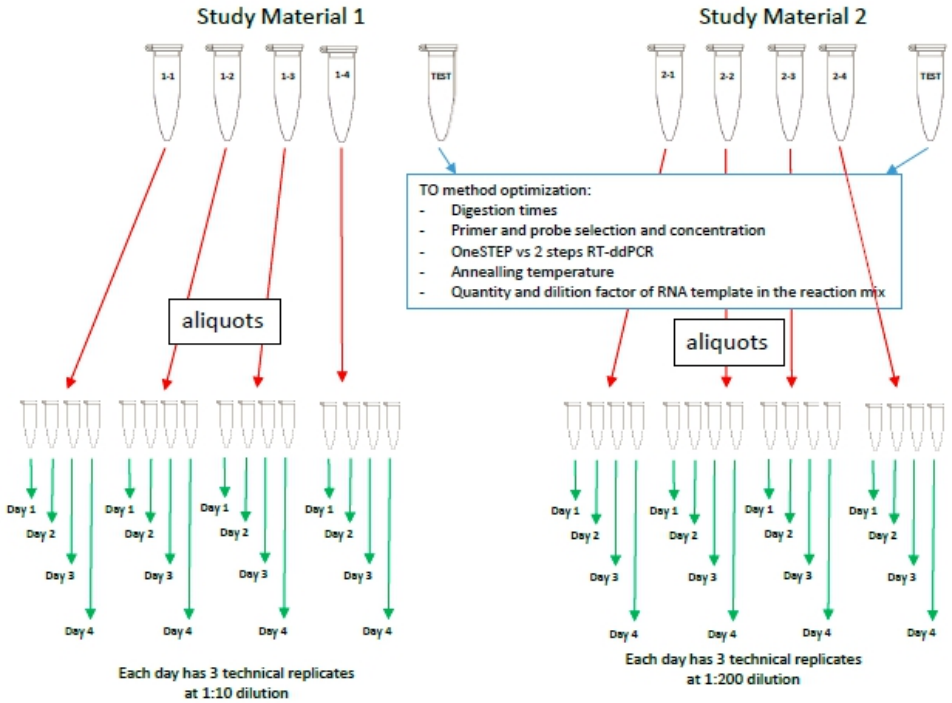
2	HER2	1539486	1.69	2.94	0.68	2.78	4.44	68292
	RPPH1	41900	2.82	2.94	0.68	2.35	4.75	1990

Sample	Measurand	Ratio	u $\lambda\, rel$ (%) HER2	u $\lambda\, rel$ (%)RPPH1	u $R\, rel$ (%)	u est rel (%)	u est (copies/uL)
1	Ratio HER2/RPPH1	2.00	1.17	1.22	1.07	2.00	0.04
2	Ratio HER2/RPPH1	36.75	1.69	2.82	1.67	3.69	1.35

INRIM (Laboratory 9)

One of the four vials received of each material were used to optimize the measurement protocol; the other three vials were considered as biological replicates. Each biological replicates were aliquoted and analysed in 5 different days with three technical replicates. Study material 1 (S1) were diluted 1:10 and study material 2 (S2) were diluted 1:200.

The experimental design is summarized below:



The mathematical model used to calculate the concentration of each study material (SM) is the following:

$$C_{gene,SM} = D_f \times (-ln\frac{N_{neg}}{N}) \div V_d \ [\text{c/µL}]$$

Where:
$C_{gene,SM}$ = copy number concentration of gene HER2 or RPPH1 in each SM [c/µL]
D_f = dilution factor
N_{neg}/N = the ratio between the number of negative droplets and those accepted
Vd = droplet volume [µL]

The contribution to the uncertainty given by D_f is evaluated as a type B contribution (correction of the volume withdrawn, after pipettes calibration) and also as a type A contribution (variability of the volume dispensed). The uncertainty of N_{neg}/N has two components: one related to the reproducibility and the second coming from the repeatability. The uncertainty of V_d has one component: the variability on the estimation of the droplet diameter measured on images acquired by using an optical microscope.

The expanded uncertainty of Measurands 1 and 2 is calculated as the root of the quadratic sum of the components, with k=2.

The ratio of HER2 to RPPH1 (Measurand 3) was first calculated for each replicate (technical and biological) and then the mean value was obtained. The measurement uncertainty of the ratio was calculated by combining the standard uncertainties of HER2 copy number concentration and the standard uncertainties of RPPH1 copy number concentration.

The mathematical model used to calculate the ratio of HER2 to RPPH1 is the following:

$$Ratio = \frac{C_{HER2}}{C_{RPPH1}}$$

The uncertainty budget for S1 Measurand 1 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	208	Pipette calibration	10.27	$-\frac{ln(\frac{N_{neg}}{N})}{V_d}$	3˙220
N_{neg} / N	0.7929	Measurement reproducibility	0.0105	$-\frac{D_f}{(\frac{N_{neg}}{N})\times V_d}$	0.0020

V_d	7.41x10^{-4}	Volume variability	2.22x10^{-5}	$\frac{D_f \times ln(\frac{N_{neg}}{N})}{V_d^2}$	-1.96x10^3
$u(CHER2) = \sqrt{(3.220)^2 + (0.0020)^2 + (-1.96x10^3)^2}$					
		C_{HER2} [c/µL]	u(C_{HER2}) [c/µL]	U(C_{HER2}) [c/µL], k=2	U(C_{HER2}) [%], k=2
		65.205	3.769	7.538	11.56

The uncertainty budget for S1 Measurand 2 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	208	Pipette calibration	10.27	$-\frac{ln(\frac{N_{neg}}{N})}{V_d}$	1.686
N_{neg} / N	0.886	Measurement reproducibility	0.006	$-\frac{D_f}{(\frac{N_{neg}}{N}) \times V_d}$	-0.001
V_d	7.41x10^{-4}	Volume variability	2.22x10^{-5}	$\frac{D_f \times ln(\frac{N_{neg}}{N})}{V_d^2}$	-1.03x10^3
$u(CRPPH1) = \sqrt{(1.686)^2 + (-0.001)^2 + (-1.03x10^3)^2}$					
		C_{RPPH1} [c/µL]	u(C_{RPPH1}) [c/µL]	U(C_{RPPH1}) [c/µL], k=2	U(C_{RPPH1}) [%], k=2
		34.143	1.973	3.947	11.56

The uncertainty budget for S1 Measurand 3 is:

Component (y)	**value**	**Uncertainty u(y)**		**Sensibility coefficient** $c_y = \frac{\partial x}{\partial y}$	**Contribution to u(ratio)** $c_y \times u(y)$
		source	Standard uncertainty		
$C_{HER2, S1}$	65.205	Measurement	3.769	$\frac{1}{C_{RPPH1}}$	0.1104
$C_{RPPH1, S1}$	34.143	Measurement	1.973	$-\frac{C_{HER2}}{(C_{RPPH1})^2}$	-0.1104
$u(ratio) = \sqrt{(0.1104)^2 + (-0.1104)^2}$					
		ratio	u(ratio)	U(ratio) k=2	U(ratio) [%], k=2
		1.91	0.156	0.312	16.3

The uncertainty budget for S2 Measurand 1 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	4265	Pipette calibration	332.51	$-\frac{ln(\frac{N_{neg}}{N})}{V_d}$	9.620

N_{neg} / N	0.807	Measurement reproducibility	0.032	$-\frac{D_f}{(\frac{N_{neg}}{N})\times V_d}$	-0.12
V_d	7.41x10^{-4}	Volume variability	2.22x10^{-5}	$\frac{D_f \times ln(\frac{N_{neg}}{N})}{V_d^2}$	-3.71x10^4
$u(CHER2) = \sqrt{(9.620)^2 + (-0.12)^2 + (-3.71x10^4)^2}$					
		C_{HER2} [c/µL]	u(C_{HER2}) [c/µL]	U(C_{HER2}) [c/µL], k=2	U(C_{HER2}) [%], k=2
		1˙233˙721	103˙069	206˙138	16.71

The uncertainty budget for S2 Measurand 2 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˙265	Pipette calibration	332.51	$-\frac{ln(\frac{N_{neg}}{N})}{V_d}$	2˙790
N_{neg} / N	0.994	Measurement reproducibility	0.002	$-\frac{D_f}{(\frac{N_{neg}}{N})\times V_d}$	-0.005
V_d	7.41x10^{-4}	Volume variability	2.22x10^{-5}	$\frac{D_f \times ln(\frac{N_{neg}}{N})}{V_d^2}$	-1.07x10^3
$u(CRPPH1) = \sqrt{(2.790)^2 + (-0.005)^2 + (-1.07x10^3)^2}$					
		C_{RPPH1} [c/µL]	u(C_{RPPH1}) [c/µL]	U(C_{RPPH1}) [c/µL], k=2	U(C_{RPPH1}) [%], k=2
		35˙790	2˙990	5˙980	16.71

The uncertainty budget for S2 Measurand 3 is:

Component (y)	value	Uncertainty u(y)		Sensibility coefficient $c_y = \frac{\partial x}{\partial y}$	Contribution to u(ratio) $c_y \times u(y)$
		source	Standard uncertainty		
$C_{HER2, S2}$	1˙233˙721	Measurement	103˙069	$\frac{1}{C_{RPPH1}}$	2.8798
$C_{RPPH1, S2}$	35˙790	Measurement	2˙990	$-\frac{C_{HER2}}{(C_{RPPH1})^2}$	-2.8798
$u(ratio) = \sqrt{(2.8798)^2 + (-2.8798)^2}$					
		ratio	u(ratio)	U(ratio) k=2	U(ratio) [%], k=2
		35.31	4.073	8.145	23.07

TUBITAK UME (Laboratory 10)

Study material 1 measurements were performed in two different days and study material 2 measurements were performed in three different days. Each day 5 PCR replicates were

performed. RPPH1 and CEP17 probes were labelled with HEX and HER2 gene probe was labelled with FAM dye with BHQ1 quenchers.

Study material 1

Measurand 1

Uncertainty components	Value *x*	Unit	Standard Uncertainty *u(x)*	Unit	Relative Uncertainty *u(x)/x*	Type of Uncertainty
Repeatability uncertainty, u_r	67410	%	2049	%	0.030	A
Intermediate Precision uncertainty, u_{ip}	67410	%	752	%	0.011	A
Partition Volume uncertainty* for HER2	100	%	3	%	0.030	B
Combined relative uncertainty					0.044	

HER2 Copy number	67410
Expanded uncertainty, *U*, (k=2)	5952
Relative Expanded uncertainty (%)	8.8

Measurand 2

Uncertainty components	Value *x*	Unit	Standard Uncertainty *u(x)*	Unit	Relative Uncertainty *u(x)/x*	Type of Uncertainty
Repeatability uncertainty, u_r	34654	%	1388	%	0.04	A
Intermediate Precision uncertainty, u_{ip}	34654	%	242	%	0.01	A
Partition Volume uncertainty* for RPPH1	100	%	3.00	%	0.03	B
Combined relative uncertainty					0.05	

RPPH1 Copy number	34654
Expanded uncertainty, *U*, (k=2)	3502
Relative Expanded uncertainty (%)	10.1

Measurand 3

Uncertainty components	Value *x*	Unit	Standard Uncertainty *u(x)*	Unit	Relative Uncertainty *u(x)/x*	Type of Uncertainty
Repeatability uncertainty, u_r	1.94	%	0.09	%	0.04	A
Intermediate Precision uncertainty, u_{ip}	1.94	%	0.02	%	0.01	A
Partition Volume uncertainty* for HER2	100	%	3.00	%	0.03	B
Partition Volume uncertainty* for RPPH1	100	%	3.00	%	0.03	B
Combined relative uncertainty					0.06	

HER2/RPPH1 Copy number ratio (%)	1.94
Expanded uncertainty, *U*, (k=2)	0.24
Relative Expanded uncertainty (%)	12.3

Study material 2

Measurand 1

Uncertainty components	Value *x*	Unit	Standard Uncertainty *u(x)*	Unit	Relative Uncertainty *u(x)/x*	Type of Uncertainty
Repeatability uncertainty, u_r	1602085	%	42326	%	0.026	A
Intermediate Precision uncertainty, u_{ip}	1602085	%	151794	%	0.095	A
Partition Volume uncertainty* for HER2	100	%	3	%	0.030	B
Combined relative uncertainty					0.103	

HER2 Copy number	1602085
Expanded uncertainty, *U*, (k=2)	329502
Relative Expanded uncertainty (%)	20.6

Measurand 2

Uncertainty components	Value *x*	Unit	Standard Uncertainty *u(x)*	Unit	Relative Uncertainty *u(x)/x*	Type of Uncertainty
Repeatability uncertainty, u_r	46351	%	1996	%	0.04	A
Intermediate Precision uncertainty, u_{ip}	46351	%	2422	%	0.05	A
Partition Volume uncertainty* for RPPH1	100	%	3.00	%	0.03	B
Combined relative uncertainty					0.07	

RPPH1 Copy number	46351
Expanded uncertainty, *U*, (k=2)	6866
Relative Expanded uncertainty (%)	14.8

Measurand 3

Uncertainty components	Value *x*	Unit	Standard Uncertainty *u(x)*	Unit	Relative Uncertainty *u(x)/x*	Type of Uncertainty
Repeatability uncertainty, u_r	34.6	%	2.1	%	0.06	A
Intermediate Precision uncertainty, u_{ip}	34.6	%	1.2	%	0.03	A
Partition Volume uncertainty* for HER2	100	%	3	%	0.03	B
Partition Volume uncertainty* for RPPH1	100	%	3	%	0.03	B
Combined relative uncertainty					0.08	

HER2/RPPH1 Copy number ratio (%)	34.6
Expanded uncertainty, *U*, (k=2)	5.6
Relative Expanded uncertainty (%)	16.0

Table I-1: Summary of measurement uncertainty sources included by CCQM-K176 participants for copy number concentration results (Measurands 1-2)

Factor (A/B*) / Laboratory ID	Method repeatability	Intermediate precision	Between vial/ Homogeneity	Restriction digestion	Sub-sampling	Sample dilution (volumetric)	Sample dilution (gravimetric)	Reaction preparation (volumetric)	Reaction preparation (gravimetric)**	Assay	Partition volume	Threshold setting	Poisson error	Reaction inhibition	Other (please state)
Example	✓ (A)	✓ (A)	✓ (B)				✓ (B)	✓ (B)		✓ (A)	✓ (B)				
1	✓ (A)					✓ (B)					✓ (B)				
2	✓ (A)	✓ (A)	✓ (A)	✓ (A)						✓ (A)					
3	✓ (A)	✓ (A)	✓ (B)				✓ (B)			✓ (B)	✓ (B)	✓ (B)			
4	✓ (A)	✓ (A)				✓ (B)		✓ (B)			✓ (B)				
5	✓ (A)						✓ (A)				✓ (B)			✓ (A)	From droplet volume bias (due to gDNA digestion) (A)
6	✓ (A)		✓ (A)				✓ (B)				✓ (B)				
7	✓ (A)	✓ (A)					✓ (A)			✓ (A)	✓ (B)	✓ (B)			
8	✓ (A)	✓ (A)		✓ (A)	✓ (A)		✓ (B)			✓ (A)	✓ (B)		✓ (B)		
9	✓ (A)	✓ (A)					✓ (A) ✓ (B)				✓ (A)				
10	✓ (A)	✓ (A)									✓ (B)				

*Type A or Type B approach to measurement uncertainty

**Uncertainty due to gravimetric reaction preparation including uncertainty of sample and mastermix density

Table I-2: Summary of measurement uncertainty sources included by CCQM-K176 participants for copy number ratio (CNV) results (Measurand 3)

Factor (A/B*) / Laboratory ID	Method repeatability	Intermediate precision	Between vial/ Homogeneity	Restriction digestion	Sub-sampling	Sample dilution (volumetric)	Sample dilution (gravimetric)	Reaction preparation (volumetric)	Reaction preparation (gravimetric)**	Assay	Partition volume	Threshold setting	Poisson error	Reaction inhibition	Other (please state)
1	✓ (A)					✓ (B)					✓ (B)				
2	✓ (A)	✓ (A)	✓ (A)	✓ (A)						✓ (A)					
3	✓ (A)	✓ (A)	✓ (B)							✓ (B)		✓ (B)			
4	✓ (A)	✓ (A)				✓ (B)		✓ (B)			✓ (B)				
5	✓ (A)						✓ (A)				✓ (A)			✓ (A)	
6	✓ (A)		✓ (A)				✓ (B)				✓ (B)				
7	✓ (A)	✓ (A)					✓ (A)			✓ (A)	✓ (B)	✓ (B)			
8	✓ (A)	✓ (A)					✓ (A)			✓ (A)			✓ (B)		
9	✓ (A)	✓ (A)					✓ (A) ✓ (B)				✓ (A)				
10	✓ (A)	✓ (A)									✓ (B)				

*Type A or Type B approach to measurement uncertainty

**Uncertainty due to gravimetric reaction preparation including uncertainty of sample and mastermix density

APPENDIX J: Additional results

NIMT's first reported result using restriction digestion with *Hind*III

Table J-1: Result of Sample 1

Vial	Measurand 1	Measurand 2	Measurand 3 (no unit)	Measurand 4(optional)	Measurand 5(optional)
x copies/µL	56140	29375	1.912387	-	-
u copies/µL	2392.287435	1280.393307	0.116573	-	-
k	2	2	2	-	-
U copies/µL	4,784.57	2,560.79	0.233147	-	-
U(x)/x %	8.52	8.72	12.19	-	-

Table J-2: Result of Sample 2

Vial	Measurand 1	Measurand 2	Measurand 3 (no unit)	Measurand 4(optional)	Measurand 5(optional)
x copies/µL	1,341,142.86	40,238.10	33.39355	-	-
u copies/µL	135,924.26	4,121.22	4.811661	-	-
k	2	2	2	-	-
U copies/µL	271,848.51	8,242.45	9.623322	-	-
U(x)/x %	20.27	20.48	28.8179	-	-

Follow-up to K176 copy number concentration results (NML)

Background

Three different sample preparation workflows (**Table J-3**) were investigated to evaluate between-experiment measurement variability observed during study participation by NML for Measurands 1, 2 and 4. Potential sources of error included insufficient mixing/vortexing and variability introduced during restriction digestion (1 digestion reaction performed per unit in each experiment). New units of Study Materials 1 and 2 were utilised in the follow-up study investigating sample preparation. The processed materials were subsequently analysed by dPCR in three independent experiments, mirroring the experimental design of the main study participation.

Table J-3: Summary of the three sample preparation conditions evaluated in NML follow-up study

Unit	Condition	Description
1	A1	Heat at 60°C whilst shaking at 600 rpm, vortex and dilute in 1× TE
1	A2	As for A1 with addition of *Eco*RI digestion, dilute in 1X TE (**NIMC conditions**)
2	B	Vortex and dilute in yeast tRNA carrier (**NMIA conditions**)

Methods

Sample preparation

Study Material 1 (SM1) and Study Material 2 (SM2) were evaluated alongside a human genomic DNA control (Promega G304A, diluted to 100 ng/µL in nuclease-free water (Ambion; Cat. No. AM9937)). One unit of each study material was thawed from -80°C and equilibrated to ambient temperature, along with the DNA control, and then transferred to a thermomixer for the 'Condition A1' workflow (**Table J3**). The samples were incubated at 60°C with shaking at 600 rpm for 2 minutes. Following this the materials were vortexed at 1,600 rpm for 10 seconds and then pulse centrifuged at 5,000 rpm for 15 seconds. At this timepoint, a 10 µL aliquot from each of the SM1 and SM2 units and the DNA control was removed for the 'Condition A2' workflow. The Condition A1 workflow was completed by diluting the samples to a final dilution factor (DF) of 20-fold (SM1, DNA control) or 180-fold (SM2) in 1X TE buffer (Sigma BioUltra pH8.0, 93282). The diluted samples were vortexed at 1,600 rpm for 5 seconds to mix.

The 10 µL aliquot (equivalent to approximately 1 µg of DNA) of each material that was allocated for Condition A2 was digested with *EcoR*I-HF (NEB R3101T) in CutSmart Buffer (NEB B7204S) in a total reaction volume of 20 µL, containing 15 units of enzyme per reaction (Table H-1). Following incubation at 37°C for 30 mins, and heat inactivation at 65°C for 10 mins, the samples were diluted to a final DF of 20-fold (SM1, DNA control) or 180-fold (SM2) in 1X TE buffer (Sigma BioUltra pH8.0, 93282). The diluted samples were vortexed at 1,600 rpm for 5 seconds to mix.

For 'Condition B', a secondary whole unit of each Study Material was thawed from -80°C and equilibrated to ambient temperature, along with an aliquot of the DNA control. The samples were vortexed at 1,600 rpm for 10 seconds and then pulse centrifuged at 5,000 rpm for 15 seconds. The samples were diluted to a final DF of 20-fold (SM1, DNA control) or 180-fold (SM2) in yeast tRNA (AM7119) prepared at 5 ng/µL in 1X TE buffer, and vortexed at 1,600 rpm for 5 seconds to mix.

All dilutions were stored at 4°C prior to dPCR analysis.

Digital PCR

The DNA samples that had been pre-treated using Conditions A1, A2 and B were analysed by dPCR using the RPPH1 and HER2-EXON24 assays (Table H-3). 5.5 µL of each DNA template was added to a prepared volume of 22 µL, along with nuclease-free water (AM9937) and ddPCR Supermix for Probes (No dUTP) (Bio-Rad 1863024). No template controls of nuclease-free water and Yeast tRNA carrier (Ambion, Part Number AM7119) were included, all samples were analysed in triplicate and the experiment repeated on three different days. The cycling conditions are given in Table H-5. Data were analysed using QuantaSoft version 1.7.4, and a partition volume of 0.749 nL was used to calculate concentration values (Table H-4).

Electrophoretic analysis

Aliquots of Study Materials 1 and 2 were analysed using Genomic DNA reagents (Agilent, Part Number 5067-5366) and ScreenTape® (Agilent, Part Number 5067-5365) on an Agilent 4150 TapeStation. The samples were prepared and analysed according to the manufacturer's instructions.

Statistical analysis

Data analysis was performed in Graphpad Prism version 9.3.1 using mixed effects models (selecting the option two-way ANOVA). Posthoc testing was performed using Tukey's test.

Results

Figure J-1 indicates that there was no discernible difference in HER2 copy number concentration between the three different workflows or three experiments for SM1 ($p > 0.05$). A small difference ($\leq$ 5%) in reference gene copy number concentration between the three workflows was indicated ($p = 0.03$), although this may be attributed to the fact that a different unit of SM1 was used for condition B. There was found to be no difference in HER2 or reference gene copy numbers for the genomic DNA control ($p > 0.05$), suggesting that there is unlikely to be an inherent bias in copy number concentration between these three sample preparation methods for these samples.

Figure J-1B indicates differences in HER2 and reference gene copy number concentration between the three conditions ($p < 0.0001$) and experiments for SM2 ($p < 0.01$). ERBB2 concentration for condition A1 was 13% and 25% lower than conditions A2 and B respectively ($p < 0.0001$) and RPPH1 concentration (A1) was 14% and 33% lower than A2 and B respectively ($p < 0.0001$). Greater inter-experimental variability was observed for condition A1 compared to A2 (F-test $p<0.05$).

Table J-4 illustrates that the HER2/RPPH1 ratio (Measurand 3) observed in the follow-up study is generally in agreement with the K176 KCRVs for SM1 and SM2 (Table 13). The observed ratio for SM2 workflow B is lower than that submitted by NML (Table 13), which is attributed to a

proportionally greater increase in RPPH1 copy number concentration compared to HER2 copy number concentration (Figure J-1B), and may be due to unit-unit variation.

Electrophoretic plots following analysis of SM1 and SM2 on the Agilent 4150 TapeStation System are presented in **Figure J-2**. The Study Materials were found to contain intact, high molecular weight DNA at least 60,000 bp in size.

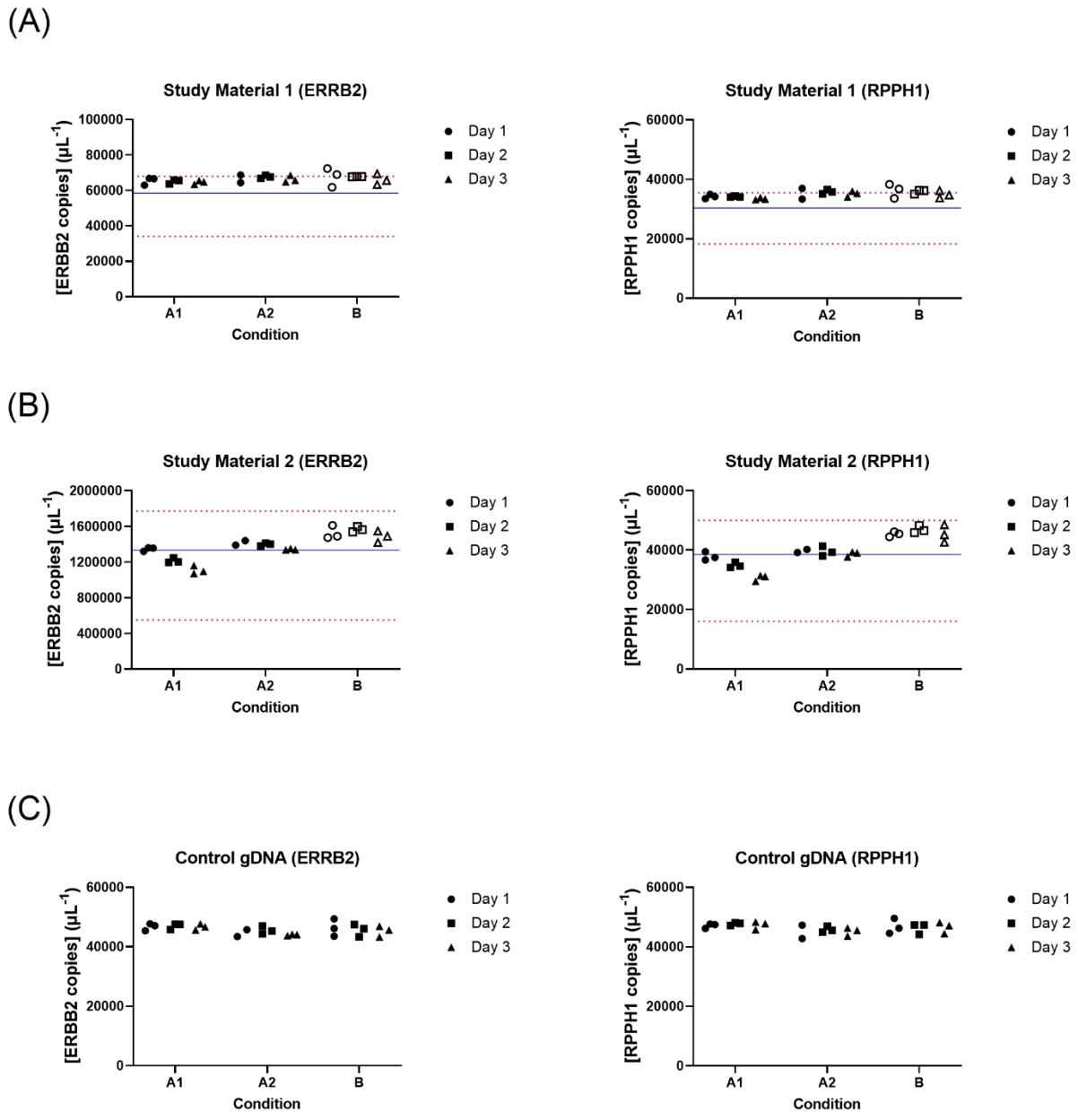


Figure J-1: Results of NML follow-up study of pre-treatment conditions. ERBB2 and RPPH1 copy number concentrations for (A) SM1, (B) SM2, (C) gDNA control following pre-treatment with three conditions (A1) heating at 60°C whilst shaking at 600 rpm, vortexing and dilution in 1× TE; (A2) with *Eco*RI digestion or (B) vortexing and dilution in yeast tRNA carrier. Follow-up results are shown in black symbols (corresponding to a single dPCR reaction). Unfilled shapes for SM1 and SM2 represent that a different unit was used for condition B. Solid blue lines represent the K176 KCRV for Measurand 1 (ERRB2) and Measurand 2 (RPPH1) for each Study Material in the respective graphs. Dashed red lines show Laboratory 2 expanded uncertainties (U) from the initial participant analysis (not reported).

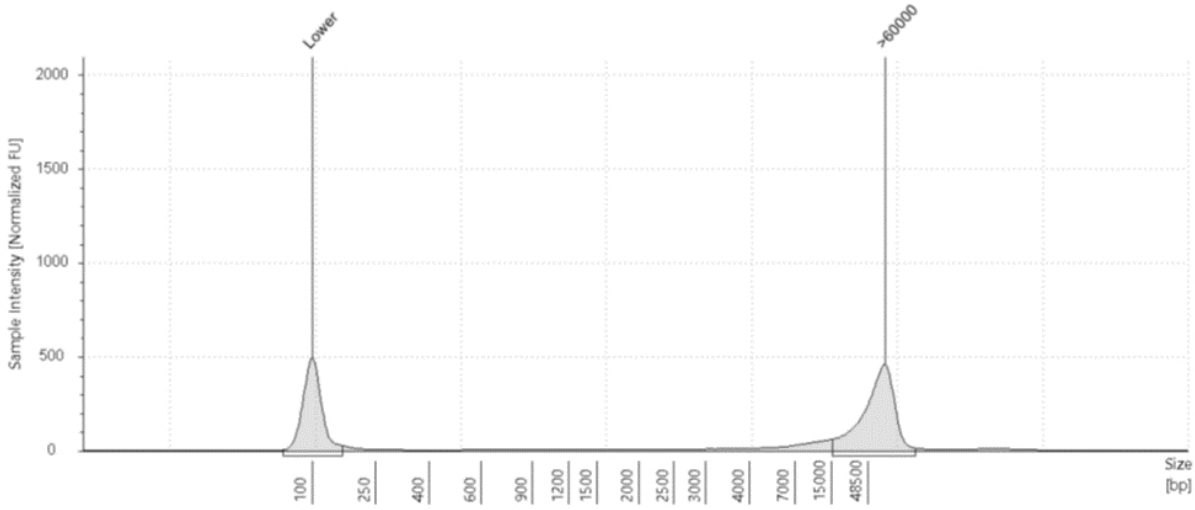
Table J-4: Summary of HER2/reference gene copy number ratio (Measurand 3) observed in

Material	Unit	Condition	Follow-up study copy number ratio	KCRV copy number ratio ± U
SM1	1 1 2	A1 A2 B	1.9 1.9 1.9	1.93 ± 0.045
SM2	1 1 2	A1 A2 B	35.4 35.2 33.2	34.89 ± 1.2
Genomic DNA control	N/A	A1 A2 B	1.0 1.0 1.0	1.0*

NML follow-up experiments

Results are compared with the K176 KCRV based on REML (Table 16). N/A – not applicable
**Expected value for genomic DNA from healthy donor cells.*

Study Material 1



Study Material 2

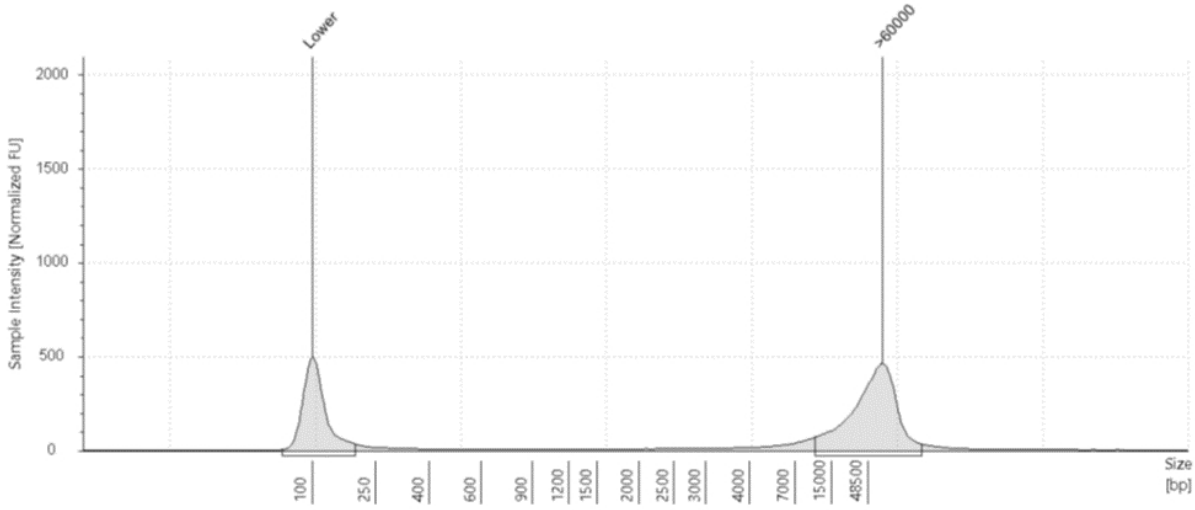


Figure J-4: Electrophoretic analysis of Study Materials 1 and 2 using the Agilent TapeStation Genomic DNA ScreenTape performed at NML

Conclusions

The NML follow-up study indicates that restriction digestion does not appear to be required for CNV measurement of ERBB2 (HER2) in the cancer cell line present in Study Materials 1 and 2 (HCC1954). This is indicated by the similarity in copy number ratios between the evaluated workflows. The anomalous DNA copy number concentration in the original NML non-submitted results for Measurands 1 and 2 are likely to have been due to insufficient mixing and/or refreezing

the sample following preparation of aliquots, as between-experiment variability was reduced in the follow-up experiments. The CCQM-K176 study materials were confirmed to contain high molecular weight (>60 kb) genomic DNA, which can be extremely viscous, and may have led to the initial mixing conditions being insufficient to ensure within-sample homogeneity. This underscores the importance of selecting appropriate sample pre-treatment conditions for accurate copy number concentration results.

Variation between the alternative pre-treatment conditions was observed for SM2 in the follow-up study and between-experiment variation was a still a significant factor, compared to only minimal differences between workflows and the absence of inter-experiment variability for SM1. This may be attributable to differences between the units analysed in the case of the between-workflow variability and may be due to the greater heterogeneity associated with cancer cell line DNA in general.

APPENDIX K: CMC proformas

Basic CMC Claims for All Participants

Claim 1

Measurement service	Organic solutions
Measurement service sub-category	3.4 Other
Matrix	Aqueous or buffered solution
Analyte	Human erb-b2 receptor tyrosine kinase 2 (*HER*2) gene DNA (Gene symbol *ERBB2*, Gene ID: 2064)
Measurand	DNA copy number concentration
Dissemination range of measurement capability	From 10^4 to 10^6 Unit: μL^{-1}
Supporting Evidence	Successfully participated in CCQM-K176

Claim 2

Measurement service	Organic solutions
Measurement service sub-category	3.4 Other
Matrix	Aqueous or buffered solution
Analyte	Human ribonuclease P RNA component H1 gene DNA (Gene symbol *RPPH1*, Gene ID: 85495)
Measurand	DNA copy number concentration
Dissemination range of measurement capability	From 10^4 to 10^6 Unit: μL^{-1}
Supporting Evidence	Successfully participated in CCQM-K176

Claim 3

Measurement service	Organic solutions
Measurement service sub-category	3.4 Other
Matrix	Aqueous or buffered solution
Analyte	Human erb-b2 receptor tyrosine kinase 2 (HER2) gene DNA (Gene symbol *ERBB2*, Gene ID: 2064)
Measurand	DNA copy number ratio
Dissemination range of measurement capability	From 1.0 to 40.0 Unit: no unit
Supporting Evidence	Successfully participated in CCQM-K176
Additional information to be published with CMC	Reference gene is human ribonuclease P RNA component H1 gene (Gene symbol *RPPH1*, Gene ID: 85495)

Prototype Broader Claims

Claim 1

Measurement service	Organic solutions
Measurement service sub-category	3.4 Other
Matrix	Aqueous or buffered solution
Analyte	Defined DNA sequence/gene
Measurand	DNA copy number concentration
Dissemination range of measurement capability	From 10^4 to 10^6 (or as demonstrated by additional evidence) Unit: μL^{-1}
Supporting Evidence	CCQM-K176 participation with additional evidence

Claim 2

Measurement service	Organic solutions
Measurement service sub-category	3.4 Other
Matrix	Aqueous or buffered solution
Analyte	Defined DNA sequence/gene
Measurand	DNA copy number ratio
Dissemination range of measurement capability	From 1.0 to 40.0 (or as demonstrated by additional evidence) Unit: no unit
Additional information to be published with CMC	Validation reference genes may be specified
Supporting Evidence	CCQM-K176 participation with additional evidence