Assessment of Digital PCR as a Primary Reference Measurement Procedure to Support Advances in Precision Medicine

Clinical Chemistry 64:9
1296–1307 (2018)

Special Report

Assessment of Digital PCR as a Primary Reference Measurement Procedure to Support Advances in Precision Medicine

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BACKGROUND: Genetic testing of tumor tissue and circulating cell-free DNA for somatic variants guides patient treatment of many cancers. Such measurements will be fundamental in the future support of precision medicine. However, there are currently no primary reference measurement procedures available for nucleic acid quantification that would support translation of tests for circulating tumor DNA into routine use.

METHODS: We assessed the accuracy of digital PCR (dPCR) for copy number quantification of a frequently occurring single-nucleotide variant in colorectal cancer (*KRAS* c.35G>A, p.Gly12Asp, from hereon termed G12D) by evaluating potential sources of uncertainty that influence dPCR measurement.

RESULTS: Concentration values for samples of *KRAS* G12D and wild-type plasmid templates varied by <1.2-fold when measured using 5 different assays with varying detection chemistry (hydrolysis, scorpion probes, and intercalating dyes) and <1.3-fold with 4 commercial dPCR platforms. Measurement trueness of a selected dPCR assay and platform was validated by comparison with an orthogonal method (inductively coupled plasma mass spectrometry). The candidate dPCR reference measurement procedure showed linear quantification over a wide range of copies per reaction and high repeatability and interlaboratory reproducibility (CV, 2%–8% and 5%–10%, respectively).

CONCLUSIONS: This work validates dPCR as an SI-traceable reference measurement procedure based on enumeration and demonstrates how it can be applied for assignment of copy number concentration and fractional abundance values to DNA reference materials in an aqueous solution. High-accuracy measurements using dPCR will support the implementation and traceable standardization of molecular diagnostic procedures needed for advancements in precision medicine.

With the advent of precision (also known as personalized and stratified) medicine, treatment decisions for patients with cancer are increasingly being made based on their germ line and tumor sequence variation. Tumor genotyping is required to reveal nucleotide sequence variants that will confer benefit from therapies *(1)*. Two clinical examples include screening for variants in exons 18 to 21 of the *EGFR*[13] gene in non–small cell lung cancer and exon 2 of the *KRAS* gene in colorectal cancer before treatment with EGFR-targeted therapies *(1)*. The application of molecular diagnostic methods for these examples and others in clinical oncology is challenging because of biological factors such as tumor heterogeneity and the potential presence of multiple tumor sites/metastases. In addition, technical factors such as the nature of the genetic targets, clinical samples, and the diversity of tech-

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Received December 12, 2017; accepted May 24, 2018.
Previously published online at DOI: 10.1373/clinchem.2017.285478

[13] Human Genes: *EGFR,* epidermal growth factor receptor; *KRAS,* KRAS proto-oncogene, GTPase.

nologies in use further complicate such measurements. Genetic targets are often single-nucleotide variants (SNVs)[14] differing by only 1 bp from the wild-type (*wt*) sequence. Such small differences make specific detection using molecular methods challenging when other variants, such as the *wt*, are also present. As the *wt* sequence usually predominates in clinical samples because of the presence of non–tumor-derived material, it may be detected instead of the sequence of interest. Plasma samples can contain cell-free DNA (cfDNA) concentrations as low as approximately 1000 copies/mL plasma, and circulating tumor DNA (ctDNA) represents a small (<10%) fraction of the total *(2)*. Current tissue-testing approaches typically use diverse methods including real-time quantitative PCR, Sanger sequencing, or pyrosequencing *(3)*. A wide range of next-generation sequencing platforms are also beginning to be applied to measurements of ctDNA *(4)*. These techniques may differ in performance variables such as limit of detection (LOD). In previous external quality assessment studies, errors in *KRAS* genotyping were reported in about 20% of laboratories. A common cause of errors was inadequate definition of method sensitivity *(5)*.

In clinical chemistry testing, benchmarking of test and laboratory performance is often provided using a series of reference measurement procedures (also termed higher-order reference methods) beginning with the primary reference measurement procedure *(6, 7)*, which is traceable to the "Système International" (SI). A calibration hierarchy with full metrological traceability to the SI is described in ISO Guide 17511, along with variations for which primary reference measurement procedures and/or materials are unable to meet such criteria. Primary reference measurement procedures represent the most accurate approaches and are used to quantify (value assign) primary calibrators, also termed higher-order reference materials. These materials are then used by reference laboratories and in vitro diagnostic (IVD) device manufacturers to calibrate the measurement procedure they will in turn use to quantify their working calibrators. Working calibrators are then used by clinical laboratories to quantify the analyte in patient samples. Using this calibration series, from primary reference material and measurement procedure to patient test result, accurate and reproducible clinical testing is enabled internationally.

In molecular testing, such as genetic analysis in cancer, only a limited number of reference materials are available *(8–10)*, and because no reference measurement procedures exist *(11)*, the traceability of patient results is limited. Furthermore, clinical decisions may become dependent not only on the qualitative presence or absence of a DNA variant but also on the quantification of a DNA variant *(4)*, which will increase the technical challenge. As technologies for measuring ctDNA become more widely established, establishment of a reference measurement system will maximize the impact on patient care by enabling traceable and accurate clinical measurements.

Digital PCR (dPCR) is a technique that counts individual DNA molecules without the need for a calibrator *(12, 13)* and is capable of high sensitivity in the detection of low frequency variants in a *wt* background *(14)*. Key interlaboratory studies recently confirmed (*a*) the high accuracy of dPCR for absolute quantification of DNA copy number concentration by comparison with analysis by flow cytometry and isotope dilution-mass spectrometry *(15)* and (*b*) its reproducibility for SNV quantification *(16)*.

The reported accuracy means dPCR has the potential to be an SI-traceable primary reference measurement procedure for DNA copy number concentration based on counting, with count being recognized as a dimensionless SI unit *(17)*. To comply with ISO 17511, the accuracy of the potential primary reference measurement procedure must be described for the intended use. In the context of dPCR, completeness of the reaction count (i.e. amplification of all target molecules present) and the selectivity (analytical specificity) of the assay to the target sequence must be demonstrated. Here we evaluated a range of factors (Fig. 1) that might affect these aspects of analytical performance and the uncertainty of a result including detection chemistry and platform. This was followed by validation of the trueness of dPCR measurements by comparison with an orthogonal method, inductively coupled plasma mass spectrometry (ICP-MS), which is calibrated using reference materials certified for their phosphorus mass fraction. Finally, the performance characteristics of a candidate dPCR method using a single assay and platform were defined according to ISO Guide 15193 requirements for reference measurement procedures and the validity of measurement uncertainty budgets compared through a comparison study involving 8 laboratories.

Materials and Methods

Unless otherwise stated, all experiments were performed at LGC (UK). Seven additional laboratories from Slovenia, Turkey, Italy, US, Republic of Korea, Belgium, and France also participated in the study. All kits and instruments were used following the manufacturers' instructions. A complete list of the experimental information is provided in the Data Supplement that accompanies the

[14] Nonstandard abbreviations: SNV, single-nucleotide variant; *wt*, wild-type; cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; LOD, limit of detection; IVD, in vitro diagnostic; dPCR, digital PCR; ICP-MS, inductively coupled plasma mass spectrometry; gDNA, genomic DNA; FPR, false-positive rate; FA, fractional abundance; LOQ, limit of quantification.

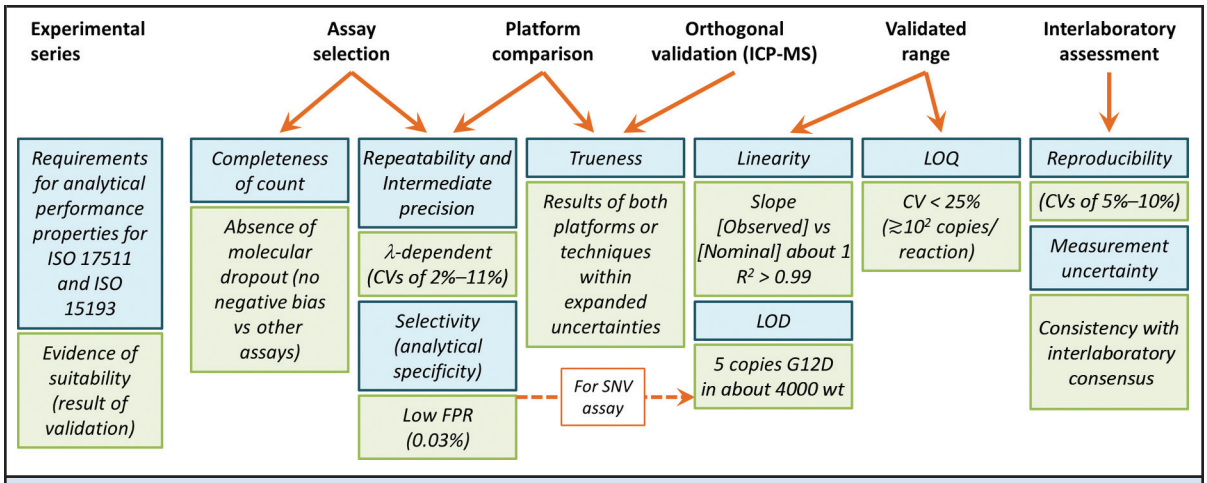

Fig. 1. Approach for the development and validation of a primary dPCR reference measurement procedure.

Schematic showing how the experimental series and results of this study meet the requirements of ISO 17511 and 15193. The experimental series is given in black text above the boxes, and orange arrows indicate which aspect of analytical performance is evaluated in each Results section. The blue boxes detail the performance criteria that must be validated to comply with ISO 17511 and 15193. The green boxes give the results of the analytical performance evaluation for the candidate *KRAS* G12D reference measurement procedure described in this study. The horizontal dashed arrow indicates that for an SNV assay, LOD is dependent on the FPR. The CV is a measure of precision. The LOQ is the lowest concentration measured with a CV <25%.

online version of this article at http://www.clinchem.org/content/vol64/issue9, in accordance with the Minimum Information for the Publication of Digital PCR Experiments guidelines *(18)*.

PRODUCTION OF LINEARIZED AND FRAGMENTED *KRAS* MATERIAL

Two previously described plasmids *(16)* prepared by Eurofins were used in this study: 4343 bp pUC57_hs_KRAS_G12D and pUC57_hs_KRAS_wt containing a portion of the human *KRAS* gene [*KRAS* c.35G>A, p.Gly12Asp, mutation G12D (Cosmic ID, COSM521)] or *wt* sequence, respectively (NCBI accession, NG_007524.1, bases 9788 to 11411). Restriction digestion reactions generated linear (4343 bp) and fragmented (373 bp and 186 bp) molecules. Manufacturer's concentration estimates based on UV spectrophotometry (Biophotometer, Eppendorf) were used to estimate approximate 'nominal' copy number concentrations in dilutions of plasmid stock solutions. Template panels containing these molecules were prepared in carrier [25 ng/μL sonicated salmon sperm genomic DNA (gDNA) (Ambion)] and are described in the relevant experiments below.

dPCR ASSAY SELECTION

Five different *KRAS* assays (primers/probes and chemistries) were compared. Three were duplex assays capable of discriminating between G12D and *wt* sequences, of which 2 used hydrolysis probes: G12D/WT assay using TaqMan™ MGB hydrolysis probes described previously *(19)* and a commercial prevalidated assay (PrimePCR™ ddPCR™ mutation detection assay, Bio-Rad), and a third using a Scorpion hybridization probe designed during the study. Two intercalating dye assays, EvaGreen 80 bp and EvaGreen 164 bp, used 2 amplicon sizes. Linearized plasmids were diluted to concentrations of approximately 10^2 and 10^4 copies/reaction to compare performance of the assays with 100% G12D or *wt* templates, or mixes of both.

dPCR PLATFORM COMPARISON

Five dPCR platforms were compared using the open G12D/WT assay *(19)*. Two platforms partitioned the reaction by water-in-oil droplets—the QX100™/QX200™ Droplet Digital™ PCR System (Bio-Rad) and RainDrop® System (RainDance™ Technologies)—and 3 platforms partitioned the reaction into prefabricated single-use consumables: the BioMark™ System for Genetic Analysis (Fluidigm) qdPCR 37K IFCs, the QuantStudio® 3D Digital PCR System (QS3D; Thermo Fisher Scientific) and the Constellation® Digital PCR system (Formulatrix®). The same template panel for the dPCR assay comparison was prepared for the platform comparison.

ORTHOGONAL METHOD VALIDATION (ICP-MS ANALYSIS)

For the parallel dPCR and ICP-MS analysis, a solution of linearized *KRAS* G12D plasmid (about 250 ng/μL) was

analyzed by ICP-MS, and a dilution of the same solution was analyzed using the QX200 (approximately 2.4×10^4 copies/reaction) and BioMark platforms (8.0×10^2 copies/reaction) with the G12D/WT assay *(19)*. For ICP-MS, 15 μL of linearized *KRAS* G12D plasmid (n = 3) and a corresponding procedural control (for restriction digestion) (n = 3) were submitted to microwave acid digestion using the Ethos EZ microwave system (Milestone) in parallel with a positive control with a certified P content, reference material NIST P3139a, and an experimental blank (negative control). Total phosphorus quantification of the digests was performed with an Agilent 8800 Triple Quadrupole ICP-MS in tandem mass spectrometry mode using a calibration curve generated from the P3139a reference material. This experiment was repeated with fresh aliquots on a separate day.

VALIDATED RANGE

Two template dilution series were analyzed with the G12D/WT assay *(19)*: (*a*) 100% linearized G12D plasmid from about 1.5×10^5 to approximately 5×10^3 copies/reaction and (*b*) with G12D plasmid from about 10^4 to 5 copies/reaction in constant background of *wt* (approximately 10^4 copies/reaction) using linearized and fragmented templates. The first dilution series above was analyzed with the QX200 and the second with the QX100 (NIB).

INTERLABORATORY ASSESSMENT

Test materials as described previously *(16)* were measured using the G12D/WT assay *(19)* by 8 laboratories using the QX100/QX200, QS3D, or RainDrop dPCR platforms. Participants were provided with positive and negative control materials containing *wt KRAS* plasmid with/without G12D, respectively, and analyzed 3 vials of each test material. For QX100/QX200 users, considered sources of uncertainty were variation between units of each material, intermediate precision, repeatability, and partition volume [for concentration values only, using the partition volume of 0.834 nL and uncertainty reported by Corbisier et al. *(20)*] so that all laboratories' uncertainties reflected the same sources of variation in technical performance and data analysis.

DATA ANALYSIS

Exported files from dPCR experiments were imported into MS Excel 2010, with further analysis carried out using GraphPad Prism 6 or the R statistical programming environment. Details of the data analysis and statistical methods used are described in the Information file included with the online Data Supplement.

Results

dPCR ASSAY SELECTION

The quantitative performance of 3 *KRAS* SNV-discriminating assays (duplex assays using hydrolysis or Scorpion probes) and 2 nondiscriminating assays (EvaGreen) was compared using the QX200 in terms of completeness of count and analytical specificity (Fig. 1). To evaluate completeness of count, the concentration of 100% *KRAS* mutant or *wt* templates was measured by their intended assay or probe to ensure that assay specificity was not a confounding factor. A negative bias in the count of DNA molecules corresponded to a DNA template being present but failing to amplify and be detected [termed molecular dropout *(18, 21)*] and was indicated when relative underquantification of a DNA target was observed compared with other assays. To ensure that template fragment size was not a confounding factor in this evaluation, a simpler DNA template of linearized plasmid DNA with defined fragment size was chosen rather than more complex gDNA *(21)*. For the SNV-discriminating assays, analytical specificity as a potential source of positive bias was evaluated with mixed templates, and the false-positive rate (FPR) was measured for non-target template (Fig. 2). The template panel consisted of DNA concentrations relevant to low levels observed in ctDNA (approximately 10^2 copies/reaction) (Fig. 2A) or higher abundances more akin to a tissue biopsy or applicable to a reference material (approximately 10^4 copies/reaction) (Fig. 2B).

Quantification of the 100% G12D samples demonstrated good agreement between the 5 assays with a <1.2-fold difference between the highest and lowest mean values in the approximately 10^2 copy sample (Fig. 2A) and approximately 10^4 copy sample (Fig. 2B); *wt* template quantification gave similar results (see Fig. 1 in the online Data Supplement). For all G12D and *wt*-only samples, the EvaGreen assay with the longer 164-bp amplicon produced 8% to 13% lower concentration values than the 80-bp amplicon assay, supporting increased molecular dropout associated with the longer amplicon.

Quantification of G12D in mixed mutant and *wt* *KRAS* samples using open and commercial hydrolysis probe assays demonstrated <1.05-fold difference in the estimated concentration independent of the concentration of *wt* template. The Scorpion assay measurements of approximately 10^2 copies of G12D in the presence of about 10^4 *wt* copies were 1.5-fold higher than the hydrolysis probe assays ($P < 0.001$) (Fig. 2A); this effect was not observed for the converse mixed sample of approximately 10^2 *wt* copies in a background of about 10^4 G12D copies (see Fig. 1A in the online Data Supplement). However, a negative trend was observed for the Scorpion assay with input quantities of approximately 10^4 G12D copies/reaction, with mean concentration values 1.1- to

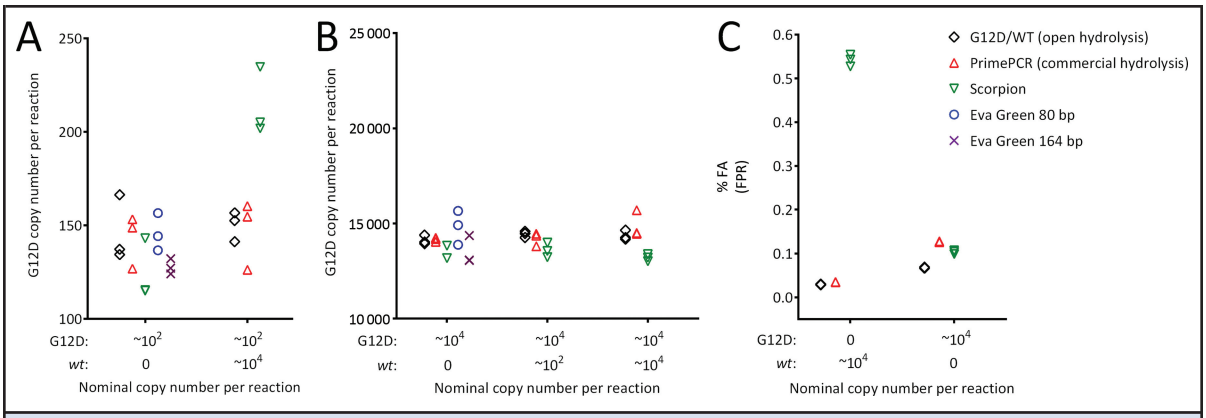

Fig. 2. dPCR assay selection and evaluation of molecular dropout and analytical specificity.

Quantification of samples containing *KRAS* G12D molecules measured at 2 nominal concentrations 2-logs apart at (A) approximately 10^2 and (B) approximately 10^4 copies per reaction using 5 dPCR assays (each shown as different symbol and color data points). *KRAS* G12D molecules were measured with and without a background of 10^4 *wt* molecules. (C), FPR for G12D and *wt* assays. Sample composition is shown on the *x* axis. Data points show the mean value of each of the 3 experiments, each of which was performed with triplicate reactions.

1.2-fold lower than the hydrolysis probes (Fig. 2B here and Fig. 1B in the online Data Supplement), suggesting possible molecular dropout in template amplification.

For the 3 discriminating assays, the FPR of the G12D and *wt* assays was evaluated using 10^4 copies/reaction of 100% *wt* template or G12D template, respectively (Fig. 2C). The G12D FPR was estimated as fractional abundance (FA) of 0.03% for the 2 hydrolysis probe assays, indicating good specificity for quantification of G12D in a background of *wt* template; however, the Scorpion assay had a substantially higher FPR (0.54%), confirming poor analytical specificity as the cause of the unacceptable positive bias described above (Fig. 2A). A *wt* FPR of approximately 0.1% was calculated for all 3 discriminating assays (Fig. 2C); however, this FPR is less critical clinically, as it represents the unlikely scenario of close to 100% of the *KRAS* molecules being mutant and would have no impact on treatment decisions.

For all combinations of dPCR assay and template concentration, repeatability and intermediate precision, expressed as the percentage CV, were evaluated for G12D and *wt* templates (see Table 1 in the online Data Supplement). Except for the failed Scorpion assay, precision of all assays expressed as % CV was ≤14% and ≤7% (both repeatability and intermediate precision) for the approximately 10^2 and 10^4 copy input quantities, respectively. The open G12D/WT hydrolysis probe assay demonstrated repeatability CV values of 8% and 2% for 10^2 and 10^4 copy input quantities, together with corresponding intermediate precision of 11% and 3%. In view of the best precision, optimal amplification profile (no evidence for molecular dropout), and analytical specificity (low FPR), this assay was chosen for further evaluation as a candidate reference measurement procedure.

dPCR PLATFORM COMPARISON

The magnitude of variation in measured *KRAS* concentration when using different dPCR platforms was investigated to identify possible biases affecting the trueness of the measured result. The same template panel applied to the assay cross-comparison was used with 5 commercially available dPCR platforms.

A 1.2- to 1.3-fold difference in mean concentration of G12D and *wt* in all samples was observed across 4 platforms, with the exception of the Constellation platform, for which a positive bias was observed (up to 1.5-fold higher than minimum results) (Fig. 3, A and B, here and Fig. 2 in the online Data Supplement). Assay specificity measured as FPR was similar in the other 4 platforms (Fig. 3C) to that characterized with the QX200 (Fig. 2C).

Evaluation of platform precision (see Table 2 in the online Data Supplement) showed that all platforms displayed intermediate precision and repeatability of <10% CV for G12D quantification of approximately 10^4 copy number samples; the best intermediate precision of 3% was observed for QX200. This was also the case for quantification of *KRAS wt* samples containing 10^4 copies, except for the Constellation. For reactions containing approximately 10^2 copies of G12D and *wt* template, only platforms with >1000 partitions (QX200, QS3D, and RainDrop) demonstrated intermediate precision and repeatability CV of <20%. These quantities were comparable with those found in plasma *(22)*, showing that in-

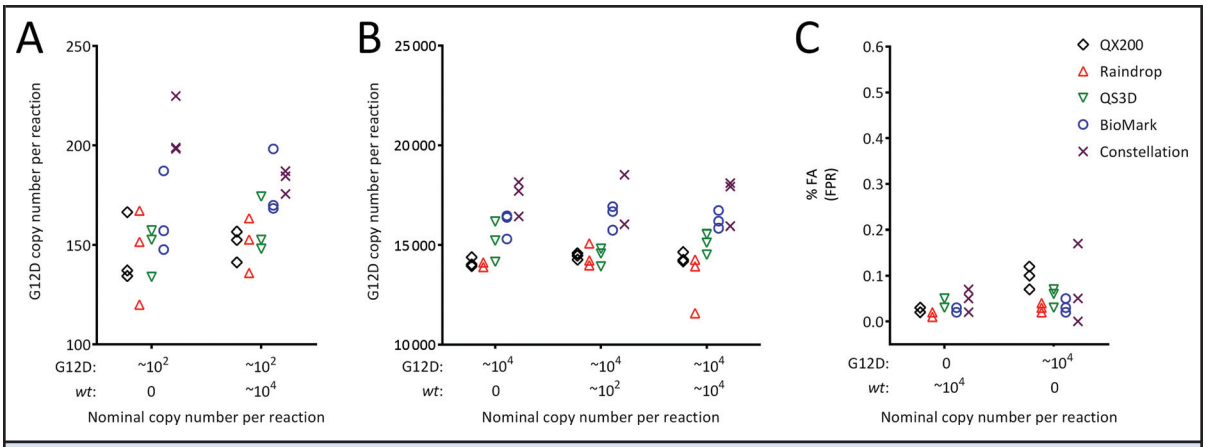

Fig. 3. Digital PCR platform comparison.

Quantification of samples containing *KRAS* G12D molecules at (A) approximately 10^2 or (B) approximately 10^4 concentration using 5 dPCR platforms. These are the same samples as those described in Fig. 2. *KRAS* G12D molecules ere measured with and without a background of *wt* approximately 10^4 molecules. FPR for G12D and *wt* assays (C). Sample composition is shown on the *x* axis. Data points show the mean value of each of the 3 experiments. Replicate reactions within an experiment were n = 3 (QX200, BioMark, and Constellation); n = 1 (QS3D and RainDrop).

struments with >1000 partitions would be the most suitable platforms for quantification of cfDNA.

ORTHOGONAL METHOD VALIDATION OF DPCR WITH ICP-MS

To validate the trueness of the concentration values measured using the candidate G12D/WT assay, dPCR measurements were compared with an orthogonal method (ICP-MS) that determined the amount of phosphate in the plasmid and could be used to highlight any systematic errors in dPCR. Measurements of replicate aliquots of the same solution of linearized *KRAS* G12D plasmid were performed with dPCR and ICP-MS; samples were measured with dilutions appropriate to the working range of the 2 techniques. dPCR measurements using 2 platforms (QX200, BioMark) were calculated as copy number concentrations and converted to mass concentration values (ng/μL) to enable comparison with the results using ICP-MS (Table 1). The concentrations measured by both dPCR platforms and ICP-MS were in agreement based on their expanded measurement uncertainties, with magnitude of differences in mean values similar to that observed in the cross-platform comparison (1.2-fold) (Fig. 3).

VALIDATED RANGE OF THE CANDIDATE REFERENCE MEASUREMENT PROCEDURE

Having investigated the potential sources of bias by varying assay and instrument and interrogated trueness through comparison with an orthogonal method, the validated range of the candidate reference measurement procedure (G12D/WT assay) was defined using a single instrument (QX200) on which linearity, limit of quantification (LOQ), and LOD were characterized, in line with the requirements of ISO Guide 15193.

Table 1. Quantification of *KRAS* G12D plasmid quantification by dPCR and ICP-MS.[a]

Method of quantification	Platform	Copy number concentration, copies/μL	Mass concentration, ng/μL	Mass concentration expanded uncertainty, ng/μL	Lower–upper range, ng/μL	Fold difference vs QX200
dPCR	QX200	4.43E + 10	197	24	173–221	
dPCR	BioMark	5.22E + 10	233	41	192–274	1.18
ICP-MS	Triple quad 8800	NA	238	18	220–256	1.21

[a] Copy number concentration of the same plasmid solution measured by ICP-MS was quantified by dPCR following gravimetric dilution as described in the Materials and Methods section. For conversion of copy number concentration to mass concentration, a conversion factor of 2.24×10^8 copies/ng was applied. Expanded measurement uncertainties (coverage factor $k = 2.0$) rounded outward to 2 significant figures and mass concentrations given to the nearest nanogram per microliter. Calculations of expanded uncertainties are described in the Information file of the online Data Supplement. NA, not applicable.

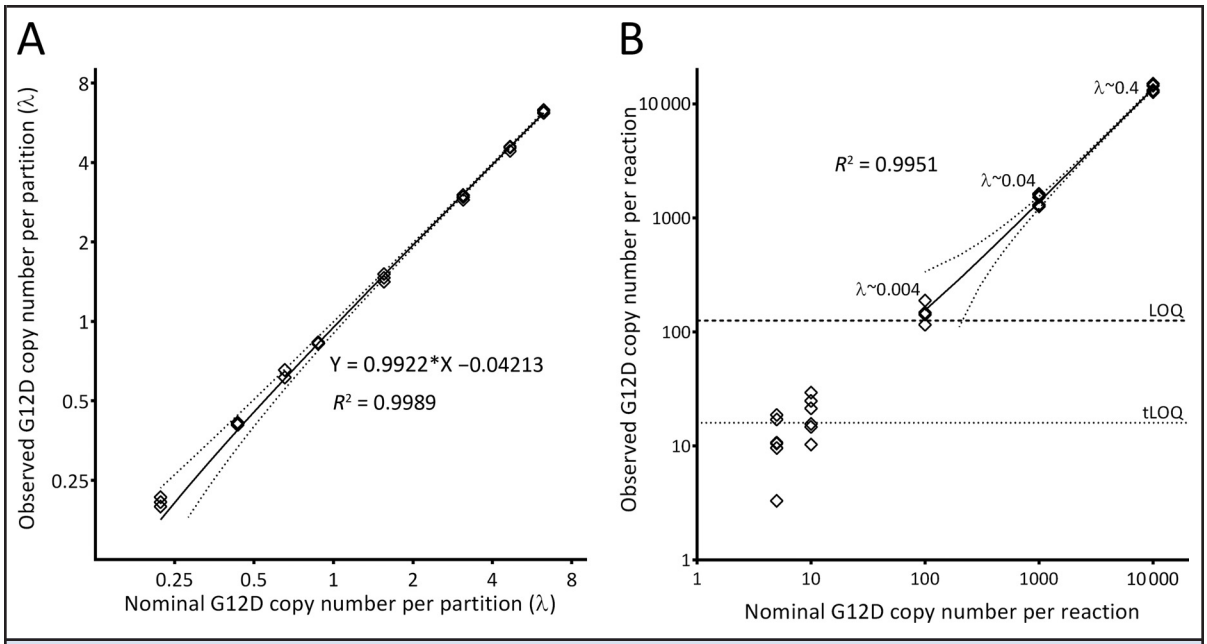

Fig. 4. Validated range of candidate reference measurement procedure.

Evaluation of linearity of samples containing *KRAS* G12D molecules over the (A) extended λ range ($0.2 < \lambda < 6.3$) and (B) low λ range ($\lambda < 0.4$) in a background of 10^4 *wt* molecules per reaction. All data points generated using the G12D/WT open hydrolysis probe assay with (A) the QX200 instrument (LGC) and (B) QX100 (NIB). Linear correlations are shown as a solid line with the associated 95% uncertainty (dotted lines). For (A), data points are shown with a λ scale and show the mean value of each of the 3 experiments performed with triplicate reactions. The linear equation and R^2 are shown. Good linearity demonstrates across the range indicates that partition volume variance was minimal. For (B), individual data points representing the copies per reaction from 2 experiments for which triplicate reactions were made (n = 6), partition occupancy was <10%, and the equivalent λ values are given when relevant. Dashed horizontal line indicates the observed LOQ; dotted horizontal line indicates the theoretical LOQ (tLOQ) (Poisson error only).

Linear range was tested by varying the mean copy number per dPCR partition referred to as lambda (λ) *(18)*. The precision of dPCR is known to vary according to λ because of its reliance on Poisson statistics to account for partitions with multiple occupancy *(23)*. In addition, it has been hypothesized that non-uniformity in partition volume within a reaction could lead to underquantification of copy number concentration with loss of linearity toward the upper end of the linear range *(24, 25)*. Therefore, in the first of 2 experiments, we tested linearity using a dilution series of 100% G12D template from λ approximately 6.3 to 0.2 (Fig. 4A). Good linearity (slope = 0.9922; R^2 = 0.9989) was observed across all the dilution series, indicating that partition volume variance was minimal and not a significant source of bias.

In the second experiment, we characterized performance characteristics approaching the lower limit of the method. In addition to the linearized plasmid (4343 bp), 2 equimolar restriction digestions of the same plasmid stock were prepared yielding *KRAS* G12D-containing fragment sizes of 373 bp or 186 bp. The fragments, which are more representative of sizes found in cfDNA *(26)*, were prepared in a background of *wt* DNA to simulate a clinical extract. A linear relationship between input and dPCR count was evident between approximately 10^4 and 10^2 copies/reaction (Fig. 4B), and dPCR measurements of all 3 templates showed no significant differences in quantity ($P = 0.33$) or linearity (see Fig. 3 in the online Data Supplement). Reactions containing a mean of ≥130 copies (to 2 significant figures) fulfilled the criterion for an LOQ with a CV of <25% (Fig. 4B here and Fig. 3 in the online Data Supplement). The theoretical LOQ based on Poisson error alone was calculated to be 16 copies/reaction *(27)* (Fig. 4B); therefore, refinement of the LOQ within this range could be achieved with further template dilutions. Samples with approximately 5 and 10 G12D copies/reaction had observed CVs >35% (see Table 3 in the online Data Supplement) and were not significantly different from each other ($P = 0.053$), consistent with these concentrations being below the LOQ.

The LOD of the candidate approach was calculated by statistical modeling of the distribution of false-positive droplets in the blank (100% *wt*) sample and counts in a

theoretical true-positive distribution, as described previously for SNV detection assays *(28, 29)*. For the linearized and fragmented 100% *wt* controls, the maximum G12D FPR (FA of 0.033%) was used to calculate the limit of blank (0.078%), for which the probability of a false-positive result (α-error) was set as 5% (see Table 4 in the online Data Supplement). A second true-positive distribution was modeled for which the probability of a false-negative result at the limit of blank was 5% (β-error), and the LOD calculated as an FA of 0.123% (equivalent to 5 G12D copies in a reaction containing approximately 4×10^3 *wt* copies) (see Table 4 in the online Data Supplement).

ASSESSMENT OF THE CANDIDATE REFERENCE MEASUREMENT PROCEDURE BY INTERLABORATORY COMPARISON

The performance of dPCR and its associated measurement uncertainty was further tested under reproducibility conditions *(30)* with 8 different laboratories participating. Variation of between 1.1- and 1.3-fold in G12D and *wt* concentrations, as well as G12D FA, was demonstrated for all 8 laboratories and 3 platforms (Fig. 5 here and Table 5 of the online Data Supplement).

Six of the participating laboratories analyzed the samples with the QX100/QX200, enabling further performance criteria for the candidate reference measurement procedure to be analyzed (see Table 6 of the online Data Supplement). Reproducibility was calculated with interlaboratory CVs varying between 5% and 10%. Median intralaboratory expanded measurement uncertainties were between 2% and 10%, apart from laboratory 2, which reported uncertainties of between 13% and 25%. Inspection of the associated 2D scatter plots indicated that this larger uncertainty may be attributable to less clearly demarcated positive and negative droplet clusters (see Information file and Fig. M5 of the online Data Supplement). This could make threshold setting and classification of positive and negative partitions variable between replicates.

Further statistical analysis revealed that individual laboratories' reported measurement uncertainties did not fully explain the observed interlaboratory variation in reported concentration or FA values ($P < 0.01$), with the exception of G12D concentration for sample C, which showed consistency between laboratories (see Tables 7 and 8 in the online Data Supplement). Technical reasons for the outlying lower G12D concentration (sample D) and FA (both samples) values reported by laboratory 1 could be the setting of a high FAM− (mutant probe) threshold. This could result in misclassification of double positive droplets (see Information file in the online Data Supplement), which typically have reduced fluorescence when measuring SNV mixtures *(31)*, and which has been previously described as a source of bias *(16)*. The lower *wt* and G12D concentrations reported by laboratory 6 for sample D may be attributable to deviations from the protocol, including the use of a lower annealing temperature, which may have caused molecular dropout.

Consensus values and uncertainties were calculated from the 6 QX100/QX200 laboratories' results (see Table 6 in the online Data Supplement). Comparison of the interlaboratory consensus values with our earlier interlaboratory study *(16)*, which used the alternative hydrolysis probe assay (as per assay comparison; Fig. 2) further demonstrated the robustness of dPCR measurements of SNVs, with the median values reported by Whale et al. *(16)* being within ≤1.05-fold of the consensus values and all results being within the upper and lower limits of the measurement uncertainty range.

Discussion

dPCR enables quantification of DNA copy number concentration without the need for external calibration. When the method performs with sufficient accuracy, this offers the possibility of it being used as a primary reference measurement procedure for SI-traceable quantification of primary reference materials through molecular enumeration (dimensionless SI unit) *(17)*. In clinical chemistry testing, reference (calibrator) materials are often used to standardize results between both clinical laboratories and IVD manufacturers. This standardization supports decision-making in the application of clinical thresholds, when routinely used, and in the development of newer approaches.

In current clinical genetic testing, analyses of tumor gDNA are normally qualitative. However, establishment of the LOD, which is a quantitative metric, remains important because it defines the analytical sensitivity of techniques for samples of mixed tumor and normal tissue *(3)*. A shift in clinical reporting from qualitative genotype to the relative abundance of a sequence is also becoming increasingly apparent. For example, the CE-IVD Cobas® *EGFR* Mutation Test (version 2) (Roche Molecular Systems) for plasma *EGFR* mutation testing in lung cancer includes a semi-quantitative index indicating percent mutation in plasma cfDNA *(32)*. The capability of next-generation platforms (that include both dPCR and sequencing-based technologies) for quantification also offers the potential to monitor therapeutic response *(4)*. As such approaches are increasingly used by clinical testing laboratories, the regulatory frameworks that support existing testing will need to apply increasingly complex criteria *(33, 34)*. Yet such measurements offer considerable challenges when considering reproducibility and standardization between laboratories. Reference materials are an established means by which traceable measurements can be made in viral load testing and for treating patients with chronic myeloid leukemia *(35, 36)*. Fur-

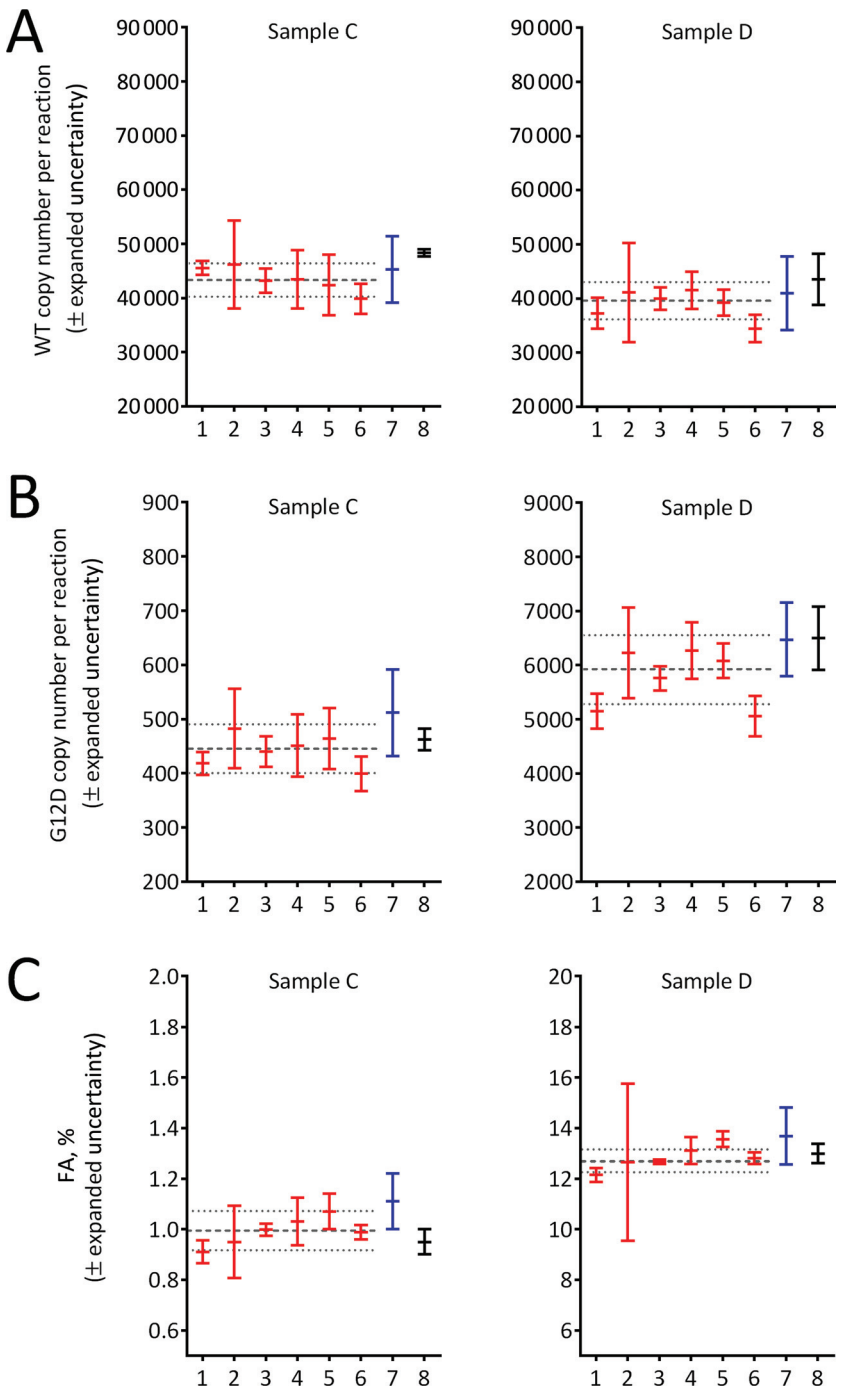

Fig. 5. Interlaboratory assessment of candidate reference measurement procedure.

Interlaboratory results for the (A) *KRAS wt* concentration, (B) G12D concentration, and (C) % G12D FA. The anonymized laboratory number is given on the *x* axis. Values (midpoints) and expanded measurement uncertainties (error bars) are colored based on dPCR platform: QX100/QX200 (red, laboratories 1–6), QS3D (blue, laboratory 7), and RainDrop (black, laboratory 8). For the QX100/QX200 platform, 3 experiments were performed with triplicate measurements of 3 units of each sample (n = 27). For the QS3D, 3 experiments were performed with single measurements of 3 units of each sample (n = 9). For the RainDrop, single measurements were performed on 3 units of each sample (n = 3) only. To enable comparability, the copies per reaction from the QS3D and RainDrop platform were normalized to align with QX100/QX200 results.

thermore, WHO recently approved the International Reference Panel for genomic KRAS codons 12 and 13 mutations reflecting the need to support this challenge *(37)*. However, to date, although there are increasing examples of the use of reference materials to support molecular measurements, reference measurement procedures have not been described.

dPCR offers the possibility to complement these efforts by acting as a primary reference measurement procedure traceable to a count of the number DNA molecules per given unit volume. dPCR has been used for value assignment of plasmid reference materials for monitoring of therapeutic response in chronic myeloid leukemia *(9)*. Here we build on this report and other studies that have demonstrated high reproducibility of dPCR for quantification of genome copies *(27, 38)* and develop a dPCR method following criteria as outlined in ISO Guides 17511 and 15193. These documents specify the content and presentation of reference measurement procedures, namely, measurement trueness, precision and sources of uncertainty, and validation by orthogonal analysis and interlaboratory comparison.

The key properties of an assay are its selectivity (analytical specificity) and completeness of amplification, ensuring trueness in the molecular count, which is the fundamental output of a dPCR experiment. We have shown that different assays, using a variety of reporter chemistries, can give highly reproducible copy number concentration values. Here we demonstrated that <1.2-fold difference can be observed between 5 different assays. However, our findings using the Scorpion probes demonstrate that this concurrence between assays cannot be assumed, and validation of all new primers/probes and chemistries is required for dPCR if it is to be considered as a gold standard for SNV quantification. Choice of dPCR instrument influences measurement trueness through impact of reagents on assay performance *(27)* and variable partition volume. We demonstrate that there are differences in SNV quantification with most commercially available dPCR platforms of <1.3-fold in magnitude. A number of other studies have also indicated that platform-specific variation of similar magnitude can occur and that accurate definition of instrument partition volume is paramount for accurate quantification *(12, 20, 27, 39, 40)*. The QX100/QX200 droplet system has been extensively analyzed in terms of partition volume, adding confidence to the QX200-derived concentration values *(20, 39–42)*. The 3 platforms that showed the greatest consistency within a single laboratory (QX200, RainDrop, and QS3D) also demonstrated good comparability between different laboratories. This provides indirect evidence that the respective partition volumes were sufficiently accurate and did not provide a major source of bias in these dPCR measurements.

Furthermore, dPCR results using 2 platforms were in agreement with the result from a calibrated chemical approach, ICP-MS *(43)*. As no other recognized SI-traceable method exists for directly measuring DNA molecules, we validated trueness with an orthogonal technique as in previous studies *(15, 44)*. It is important to note that the quantity intended to be measured (measurand) differs between dPCR, which measures the amplifiable, accessible target molecules *(45)*, compared with ICP-MS, which measures the phosphorus component of the DNA molecule. By using simple plasmid DNA of defined fragment length, our experimental system enables results for the 2 measurands to be as comparable as possible, giving confidence to evaluation of trueness and providing evidence for the suitability of the candidate dPCR method as a primary reference measurement procedure for *KRAS* copy number concentration.

The candidate reference measurement procedure (Fig. 1) using the selected openly available assay *(19)* and dPCR platform with the best characterized partition volume (QX100/QX200) was further validated in terms of its performance characteristics. The method performed robustly with a range of template sizes and demonstrated precision both within and between laboratory, which is also appropriate for the intended clinical applications of the reference measurement procedure. Our testing of linearity demonstrates how the proportionality of observed dPCR measurement output can be assessed by slope and R^2 using dilution series spanning a wide dynamic range. The dynamic range of the QX200 of 4 orders of magnitude highlights how dPCR can be applied to sample types of variable concentration and underscores the importance of validating a dPCR method over a specified range of copies per reaction (determining λ), as this parameter also influences dPCR precision *(18)*. We report an LOD of ≥0.13% G12D, which fulfills the primary objective of developing a quantitative method *(16)* and similar to that described for clinical tests for ctDNA *(46)*.

Our interlaboratory study indicates consistency of reported values between the majority of laboratories; however, in some cases, measurement uncertainties did not capture interlaboratory variation in values, highlighting the benefits of reference material value assignment by multiple laboratories *(9)*. The approximately 1.2-fold difference in the range of values reported in the interlaboratory study and the between-mean values measured by the candidate dPCR reference measurement procedure and ICP-MS support that single laboratory expanded measurement uncertainties of significantly <20% may not capture all sources of uncertainty reflecting the current state of the art for copy number quantification and indicate continued scope for improvement and parallel validation with orthogonal approaches *(15)*.

These findings also have implications for using dPCR as a primary reference measurement procedure for

the wider quantification of nucleic acid molecules. We demonstrate accurate reproducible quantification of mixtures of 2 single base variants of *KRAS*. As such mixtures are technically challenging to measure *(31)*, the findings of this study can also be extended to less complex analytes, such as plasmids composed of a single sequence as described in previous reports *(9, 15, 47)*. Although our findings support using dPCR for SI-traceable quantification of low molecular weight DNA of discrete fragment length in an aqueous solution, further work is required to assess the potential use of dPCR as a reference measurement procedure for quantification of larger gDNA. It is paramount to define the measurand in terms of amplicon and characterize the fragment size profile of the reference material or sample, as the complex nature of the template *(21)* or presence of fragments smaller than the amplicon may influence the measurement result. Development of dPCR for calibration of RNA solutions, as well as for quantification of DNA or RNA in matrix matched calibrators or real clinical samples, requires further work to describe the additional sources of uncertainty [e.g., reverse transcription *(48)* and extraction *(27)*].

Conclusion

This study demonstrates that dPCR can act as a primary reference measurement procedure to provide SI-traceable values with performance in terms of accuracy and measurement uncertainty commensurate with current clinical measurements being made within the field of precision medicine. This enables the establishment of a calibration hierarchy in accordance with ISO 17511 whereby copy number concentration and FA values can be assigned to reference materials and IVD calibrators, fulfilling the requirement for their traceability in the new EU IVD Regulation *(49)*. As well as assisting in regulatory approval of diagnostic tests, validated reference measurement procedures for DNA quantification using dPCR can support current cancer genotyping testing through external quality assessment and the translation of quantitative assays for ctDNA into routine clinical practice.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: M.P. Sassi, Istituto Nazionale di Ricerca Metrologica.
Consultant or Advisory Role: V. Taly, Raindance Technologies.
Stock Ownership: None declared.
Honoraria: V. Taly, Raindance Technologies.
Research Funding: The work described in this paper was funded by the UK government Department for Business, Energy & Industrial Strategy (BEIS) and the European Metrology Research Programme (EMRP) joint research project (SIB54) Bio-SITrace, which is jointly funded by the EMRP participating countries within EURAMET and the European Union. The work performed by NIB was performed using ddPCR™ equipment financed by the Metrology Institute of the Republic of Slovenia (MIRS) with financial support from the European Regional Development Fund. The equipment is wholly owned by the Republic of Slovenia.
Expert Testimony: None declared.
Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or final approval of manuscript.

Acknowledgments: The authors thank Professor Allen Chan (The Chinese University of Hong Kong) for his advice on the design of the *KRAS* targets. The authors also thank Laura Revel (INRIM) for support with measurement uncertainty calculation, Philip Wilson (LGC) for support with statistical analysis, and David Littlemore (LGC) for packing and shipping of samples for the interlaboratory study.

References

1. Duffy MJ, Crown J. Companion biomarkers: paving the pathway to personalized treatment for cancer. Clin Chem 2013;59:1447–56.
2. Diehl F, Li M, Dressman D, He Y, Shen D, Szabo S, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. Proc Natl Acad Sci U S A 2005;102:16368–73.
3. Westwood M, van Asselt T, Ramaekers B, Whiting P, Joore M, Armstrong N, et al. KRAS mutation testing of tumours in adults with metastatic colorectal cancer: a systematic review and cost-effectiveness analysis. Health Technol Assess 2014;18:1–132.
4. Wan JC, Massie C, Garcia-Corbacho J, Mouliere F, Brenton JD, Caldas C, et al. Liquid biopsies come of age: towards implementation of circulating tumour DNA. Nat Rev Cancer 2017;17:223–38.
5. Tembuyser L, Ligtenberg MJ, Normanno N, Delen S, van Krieken JH, Dequeker EM. Higher quality of molecular testing, an unfulfilled priority: results from external quality assessment for KRAS mutation testing in colorectal cancer. J Mol Diagn 2014;16:371–7.
6. Vesper HW, Thienpont LM. Traceability in laboratory medicine. Clin Chem 2009;55:1067–75.
7. White GH. Metrological traceability in clinical biochemistry. Ann Clin Biochem 2011;48:393–409.
8. Saldanha J, Silvy M, Beaufils N, Arlinghaus R, Barbany G, Branford S, et al. Characterization of a reference material for BCR-ABL (M-BCR) mRNA quantitation by real-time amplification assays: towards new standards for gene expression measurements. Leukemia 2007;21:1481–7.
9. White H, Deprez L, Corbisier P, Hall V, Lin F, Mazoua S, et al. A certified plasmid reference material for the standardisation of BCR-ABL1 mRNA quantification by real-time quantitative PCR. Leukemia 2015;29:369–76.
10. Lih CJ, Si H, Das B, Harrington RD, Harper KN, Sims DJ, et al. Certified DNA reference materials to compare HER2 gene amplification measurements using next-generation sequencing methods. J Mol Diagn 2016;18:753–61.
11. JCTLM database: laboratory medicine and in vitro diagnostics. http://www.bipm.org/jctlm/ (Accessed January 2017).
12. Bhat S, Herrmann J, Armishaw P, Corbisier P, Emslie KR. Single molecule detection in nanofluidic digital array enables accurate measurement of DNA copy number. Anal Bioanal Chem 2009;394:457–67.
13. Sanders R, Huggett JF, Bushell CA, Cowen S, Scott DJ, Foy CA. Evaluation of digital PCR for absolute DNA quantification. Anal Chem 2011;83:6474–84.
14. Vogelstein B, Kinzler KW. Digital PCR. Proc Natl Acad Sci U S A 1999;96:9236–41.
15. Yoo HB, Park SR, Dong L, Wang J, Sui Z, Pavsic J, et al. International comparison of enumeration-based quan-

tification of DNA copy-concentration using flow cytometric counting and digital polymerase chain reaction. Anal Chem 2016;88:12169–76.

16. Whale AS, Devonshire AS, Karlin-Neumann G, Regan J, Javier L, Cowen S, et al. International interlaboratory digital PCR study demonstrating high reproducibility for the measurement of a rare sequence variant. Anal Chem 2017;89:1724–33.
17. BIPM. SI brochure. https://www.bipm.org/en/publications/si-brochure/ (Accessed December 2017).
18. Huggett JF, Foy CA, Benes V, Emslie K, Garson JA, Haynes R, et al. The digital MIQE guidelines: minimum information for publication of quantitative digital PCR experiments. Clin Chem 2013;59:892–902.
19. Taly V, Pekin D, Benhaim L, Kotsopoulos SK, Le Corre D, Li X, et al. Multiplex picodroplet digital PCR to detect KRAS mutations in circulating DNA from the plasma of colorectal cancer patients. Clin Chem 2013;59:1722–31.
20. Corbisier P, Pinheiro L, Mazoua S, Kortekaas AM, Chung PY, Gerganova T, et al. DNA copy number concentration measured by digital and droplet digital quantitative PCR using certified reference materials. Anal Bioanal Chem 2015;407:1831–40.
21. Whale AS, Cowen S, Foy CA, Huggett JF. Methods for applying accurate digital PCR analysis on low copy DNA samples. PLoS One 2013;8:e58177.
22. Devonshire AS, Whale AS, Gutteridge A, Jones G, Cowen S, Foy CA, Huggett JF. Towards standardisation of cell-free DNA measurement in plasma: controls for extraction efficiency, fragment size bias and quantification. Anal Bioanal Chem 2014;406:6499–512.
23. Weaver S, Dube S, Mir A, Qin J, Sun G, Ramakrishnan R, et al. Taking qPCR to a higher level: analysis of CNV reveals the power of high throughput qPCR to enhance quantitative resolution. Methods 2010;50:271–6.
24. Huggett JF, Cowen S, Foy CA. Considerations for digital PCR as an accurate molecular diagnostic tool. Clin Chem 2015;61:79–88.
25. Jacobs BK, Goetghebeur E, Clement L. Impact of variance components on reliability of absolute quantification using digital PCR. BMC Bioinformatics 2014;15:283.
26. Heitzer E, Ulz P, Geigl JB. Circulating tumor DNA as a liquid biopsy for cancer. Clin Chem 2015;61:112–23.
27. Devonshire AS, Honeyborne I, Gutteridge A, Whale AS, Nixon G, Wilson P, et al. Highly reproducible absolute quantification of *Mycobacterium tuberculosis* complex by digital PCR. Anal Chem 2015;87:3706–13.
28. Milbury CA, Zhong Q, Lin J, Williams M, Olson J, Link DR, Hutchison B. Determining lower limits of detection of digital PCR assays for cancer-related gene mutations. Biomol Detect Quantif 2014;1:8–22.
29. Whale AS, Bushell C, Grant PR, Cowen S, Guttierrez-Aguirre I, O'Sullivan DM, et al. Detection of rare drug resistance mutations by digital PCR in a human influenza A virus model system and clinical samples. J Clin Microbiol 2016;54:392–400.
30. International Organization of Standardization. In vitro diagnostic medical devices–measurement of quantities in samples of biological origin–requirements for content and presentation of reference measurement procedures. ISO Guide 15193. Geneva (Switzerland); ISO: 2009.
31. Whale AS, Huggett JF, Tzonev S. Fundamentals of multiplexing with digital PCR. Biomol Detect Quantif 2016;10:15–23.
32. Cobas EGFR SQI brochure: introducing the semi-quantitative index (SQI) reporting tool. https://molecular.roche.com//app/uploads/2017/11/cobas-EGFR-SQI-Brochure.pdf (Accessed April 2018).
33. International Organization of Standardization. Medical laboratories–requirements for quality and competence. ISO Guide 15189. Geneva (Switzerland); ISO: 2012.
34. International Organization of Standardization. Medical devices– quality management systems–requirements for regulatory purposes. ISO Guide 13485. Geneva (Switzerland); ISO: 2016.
35. Fryer JF, Heath AB, Minor PD. A collaborative study to establish the 1st WHO International Standard for human cytomegalovirus for nucleic acid amplification technology. Biologicals 2016;44:242–51.
36. Scott S, Travis D, Whitby L, Bainbridge J, Cross NCP, Barnett D. Measurement of BCR-ABL1 by RT-qPCR in chronic myeloid leukaemia: findings from an International EQA Programme. Br J Haematol 2017;177:414–22.
37. WHO. Expert Committee on Biological Standardization (Geneva, 17 to 20 October 2017). Collaborative study to evaluate the proposed WHO 1st International Reference Panel for genomic KRAS codons 12 and 13 mutations. Geneva (Switzerland); WHO: 2017.
38. Hayden RT, Gu Z, Sam SS, Sun Y, Tang L, Pounds S, Caliendo AM. Comparative evaluation of three commercial quantitative cytomegalovirus standards by use of digital and real-time PCR. J Clin Microbiol 2015;53:1500–5.
39. Dong L, Meng Y, Sui Z, Wang J, Wu L, Fu B. Comparison of four digital PCR platforms for accurate quantification of DNA copy number of a certified plasmid DNA reference material. Sci Rep 2015;5:13174.
40. Kosir AB, Divieto C, Pavsic J, Pavarelli S, Dobnik D, Dreo T, et al. Droplet volume variability as a critical factor for accuracy of absolute quantification using droplet digital PCR. Anal Bioanal Chem 2017;409:6689–97.
41. Pinheiro LB, Coleman VA, Hindson CM, Herrmann J, Hindson BJ, Bhat S, Emslie KR. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. Anal Chem 2012;84:1003–11.
42. Dagata JA, Farkas N, Kramar JA. Method for measuring the volume of nominally 100 μm diameter spherical water-in-oil emulsion droplets. NIST Special Publication 260–184. Gaithersburg (MD): NIST; 2016.
43. Holden MJ, Rabb SA, Tewari YB, Winchester MR. Traceable phosphorus measurements by ICP-OES and HPLC for the quantitation of DNA. Anal Chem 2007;79:1536–41.
44. Dong L, Meng Y, Wang J, Liu Y. Evaluation of droplet digital PCR for characterizing plasmid reference material used for quantifying ammonia oxidizers and denitrifiers. Anal Bioanal Chem 2014;406:1701–12.
45. Kline MC, Romsos EL, Duewer DL. Evaluating digital PCR for the quantification of human genomic DNA: accessible amplifiable targets. Anal Chem 2016;88:2132–9.
46. Gale D, Lawson ARJ, Howarth K, Madi M, Durham B, Smalley S, et al. Development of a highly sensitive liquid biopsy platform to detect clinically-relevant cancer mutations at low allele fractions in cell-free DNA. PLoS One 2018;13:e0194630.
47. Haynes RJ, Kline MC, Toman B, Scott C, Wallace P, Butler JM, Holden MJ. Standard reference material 2366 for measurement of human cytomegalovirus DNA. J Mol Diagn 2013;15:177–85.
48. Sanders R, Mason DJ, Foy CA, Huggett JF. Evaluation of digital PCR for absolute RNA quantification. PLoS One 2013;8:e75296.
49. Regulation (EU) 2017/746 of the European Parliament and of the Council of 5 April 2017 on in vitro diagnostic medical devices. Annex I chapter II: requirements regarding performance, design and manufacture. Brussels (Belgium): European Commission; 2017.
50. Jones GM, Busby E, Garson JA, Grant PR, Nastouli E, Devonshire AS, Whale AS. Digital PCR dynamic range is approaching that of real-time quantitative PCR. Biomol Detect Quantif 2016;10:31–3.
51. International Organization of Standardization. General requirements for the competence of calibration and testing laboratories. ISO/IEC 17025. Geneva (Switzerland): ISO; 2005.
52. International Organization of Standardization. Uncertainty of measurement–part 3: guide to the expression of uncertainty in measurement (GUM: 1995). ISO/IEC Guide 98–3. Geneva (Switzerland): ISO; 2008.
53. Eurachem/CITAC guide: quantifying uncertainty in analytical measurement. 3rd Ed. London (UK): Eurachem; 2012.