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New Candidate Reference Measurement Procedures for *MET* CNV Detection and Quantification Using Digital PCR

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Abstract

Background Copy number variation (CNV) of the *MET* gene is a clinically relevant alteration associated with tumorigenesis, disease progression, and therapy resistance in several cancers, particularly non-small cell lung cancer and colorectal cancer. Elevated *MET* copy number has prognostic value and can predict response to MET inhibitors, underscoring the clinical need for accurate quantification of *MET* CNV. However, current diagnostic platforms, such as FISH, qPCR, and NGS, suffer from limited reproducibility, lack of sensitivity, and poor metrological traceability. All these factors lead to poor standardization (e.g. in the reporting units and reference intervals), creating a barrier to inter-laboratory comparability and clinical harmonization.

Digital PCR (dPCR) has emerged as a powerful alternative, offering absolute quantification, high sensitivity, and robustness. These features make dPCR particularly suitable for the development of Reference Measurement Procedures (RMPs), essential to establish SI (units) traceability and support the production of certified reference materials.

Results Here, we report the design, optimization, and validation of a duplex droplet dPCR (ddPCR) assay targeting MET and the diploid reference gene RPPH1. Using synthetic constructs, diploid and MET-amplified cell lines, and reference materials, we systematically optimized assay conditions and evaluated analytical performance. The duplex ddPCR showed excellent linearity (R^2=0.988), low intra- and inter-run variability (CV≈3.8–3.9%), and reliable quantification across a dynamic range of copy numbers. Measurement uncertainty was rigorously estimated, aligning with metrological standards. Importantly, baseline measurements in diploid cell lines yielded values consistent with the expected two-copy state, confirming accuracy under physiological conditions.

Conclusion This work introduces a rigorously validated candidate RMP for MET CNV quantification. By enabling traceable and reproducible measurements, the method addresses a critical gap in CNV standardization and provides a foundation for certified reference material development. The adoption of this RMP has the potential to harmonize

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molecular diagnostics across laboratories, improve the comparability of clinical trial results, and strengthen the integration of CNV testing into precision oncology, ultimately enhancing patient outcomes.

Keywords *MET* CNV, dPCR, Reference measurement procedure, Traceability, Measurement uncertainty, Precision medicine, Standard operating procedure

Background

Copy number variation (CNV) of the *MET* gene, located on chromosome 7q31, is a clinically relevant genomic alteration that contributes to oncogenesis, disease progression, and resistance to targeted therapies in several malignancies, particularly non-small cell lung cancer and colorectal cancer [1, 2]. *MET* amplification has been associated with poor prognosis, and elevated *MET* copy number (CN) may predict response to MET inhibitors such as crizotinib and capmatinib [1–3]. For this reason, accurate and reliable quantification of *MET* CNV is of growing importance in precision oncology, where molecular data directly inform clinical decisions.

Currently, a variety of analytical platforms are used in routine diagnostic to assess CNV, including fluorescence in situ hybridization (FISH), quantitative PCR (qPCR), and next-generation sequencing (NGS) [3–5]. However, these methods often suffer from important limitations, such as lack of sensitivity, need for calibration standards, poor reproducibility, or technical complexity [4, 6–9]. In addition, they frequently lack metrological traceability, a prerequisite for result comparability across laboratories and platforms. The absence of standardized and traceable procedures for *MET* CNV assessment is therefore a critical bottleneck for harmonization and quality assurance in clinical and translational research [10–13].

In this context, digital PCR (dPCR) has emerged as a promising technology for CNV analysis, offering absolute quantification, high sensitivity, and robustness against sample quality issues such as fragmentation or low abundance DNA [14, 15]. Unlike qPCR, dPCR does not rely on standard curves and is more tolerant to reaction efficiency variations, making it particularly suited for quantifying rare events or CN changes [14–17]. These features make dPCR an ideal platform for developing Reference Measurement Procedures (RMPs), as recently highlighted in interlaboratory comparison studies and metrology-focused initiatives [18].

Despite its potential, no validated RMP for *MET* CNV is currently available. This gap limits the reproducibility and comparability of *MET* CNV results, hinders the development of certified reference materials, and complicates the integration of CNV testing into quality-controlled diagnostic pipelines. To address this need, we report the design, optimization, and initial validation of a candidate RMP for *MET* CNV quantification based on duplex droplet dPCR (ddPCR). The assay targets exon 14 of *MET* and the *RPPH1* reference gene (located on chromosome 14q11.2). Our goal is to provide a traceable and standardized approach for *MET* CNV quantification, suitable for both clinical and research use, and able to support broader harmonization in molecular diagnostics.

Methods

Primers and Probes Design

Primers and hydrolysis probes were designed to specifically amplify a region within exon 14 of *MET* gene, a clinically relevant hotspot frequently implicated in oncogenic alterations. As a reference gene, *RPPH1* was selected due to its stable diploid CN [19–21], making it a suitable calibrator for CNV analysis. Primers/Probe assays design was performed using Primer3Plus (https://www.primer3plus.com/index.html), following criteria optimized for dPCR applications. In silico validation was performed using NCBI Primer-BLAST to confirm target specificity and to ensure no off-target binding within the human genome. Additionally, genomic polymorphisms and restriction sites in the amplicon regions were avoided to ensure consistent assay performance. Primers and probe for the *MET* assay were synthesized by Eurofins Genomics, while those for the *RPPH1* assay were purchased from Metabion. The *RPPH1* assay had been previously optimized and validated within the context of the CCQM-K176 comparison study [22]. The sequences of the primers and probes targeting *MET* exon 14 and *RPPH1* gene are listed in Table 1.

Control Templates Preparation

Synthetic DNA constructs were used as control templates for assays optimization and performance evaluation. Two custom plasmids (pEX-A128 backbone, Eurofins Genomics) were engineered to carry *MET* exon 14 and *RPPH1* fragments corresponding to the regions targeted by our assays. The plasmids were synthesized and sequence-verified by Eurofins Genomics. The plasmids were resuspended upon arrival according to the manufacturer's instructions and quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) prior to dilution. Working solutions were prepared in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at − 20 °C in aliquots to avoid repeated freeze–thaw cycles. The plasmid maps, along with the exact sequences of the cloned regions, are provided in Fig. 1; Table 2, respectively.

Table 1 *MET* and *RPPH1* primer and probe sequences

Target	Oligo/probe name	Sequence (5′-3′)	Accession *n.*
MET	*MET* Fwd	TCGCTACGATGCAAGAGTACACA	NC_000007.14
	MET Rev	GGCTTACACTTCGGGCACTTA	
	MET Probe	[FAM]TCCTCATTTGGATAGGCT[MGBEQ]	
RPPH1	*RPPH1* Fwd	ACGTCATCAACCCGCTCCAAG	NC_000014.9
	RPPH1 Rev	CCACTCCCCTGTCCCTCACA	
	RPPH1 Probe	[HEX]GTGTCACTAGGCGGGAACACC[BQ]	

Table 2 *MET* and *RPPH1* sequences of plasmid inserts

Gene	Sequence (5′-3′)	Length [bp]
MET	ATCTGGGCAGTGAATTAGTTCGCTACGATGCAAGAG-TACACACTCCTCATTTGGATAGGCTTGTAAGTGCCC-GAAGTGTAAGCCCAACTACAGAAATGGTTTCAAAT-GAATCTGTAGACTACCGAGCTACTTTTCCAGAAG	141
RPPH1	TTCCCAAATCCAAAGACATTTCACGTTTATGGT-GATTTCCCAGAACACATAGCGACATGCAAATATTG-CAGGGCGCCACTCCCCTGTCCCTCACAGCCATCTTCCT-GCCAGGGCGCACGCGCGCTGGGTGTTCCCGCCTAGT-GACACTGGGCCCGCGATTCCTTGGAGCGGGTTGAT-GACGTCAGCGTTCGAATTCCATGGCGGCGCGGCGG	212

Test Samples

Genomic DNA samples were extracted from 4 human cell lines, selected as representative models of the normal diploid *MET* gene status: P-EP/SVTERT28-2 and P-MSC/TERT308 (both kindly provided by Evercyte GmbH), MG-63 and THP-1 (purchased from ATCC). In addition, 2 human cell lines with *MET* amplification were used as positive control: EBC1 and GTL16 [23, 24], both obtained from the Cell Culture Facility of the Candiolo Cancer Institute, FPO-IRCCS, where they had been authenticated and characterized. All cell lines were cultured under recommended conditions. At confluence, cells were harvested, and genomic DNA (gDNA) was extracted using the QIAamp DNA Mini Kit (Qiagen) following the manufacturer's protocol. gDNA concentration and purity were assessed using a NanoQuant Plate (Tecan). gDNA was resuspended in TE buffer and stored at – 20 °C until use.

gDNA from diploid cell lines was used (i) to assess the baseline CN value of *MET* relative to the reference gene *RPPH1* under physiological diploid conditions and (ii) as background DNA matrices for spiking experiments, in which known amounts of the *MET*-containing plasmid were added to simulate increased *MET* CN. gDNA from *MET*-amplified cell lines was used to preliminarily validate the assay.

Primer Efficiency Testing by qPCR

qPCR was performed to assess the amplification efficiency of the *MET* and *RPPH1* primer pairs and to evaluate the specificity of the amplicons via melting curve analysis. Reactions were carried out on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using iTaq Universal SYBR Green Supermix (Bio-Rad). Thermal cycling conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 30 s. A final melt curve analysis was performed by increasing the temperature from 65 °C to 95 °C in 0.5 °C increments every 5 s, to confirm the specificity of the amplification products. Primer efficiency was determined using a standard curve generated from the 5-fold serial dilutions of the template gDNA (P-MSC/TERT308). Data were analyzed by CFX Maestro software (Bio-Rad).

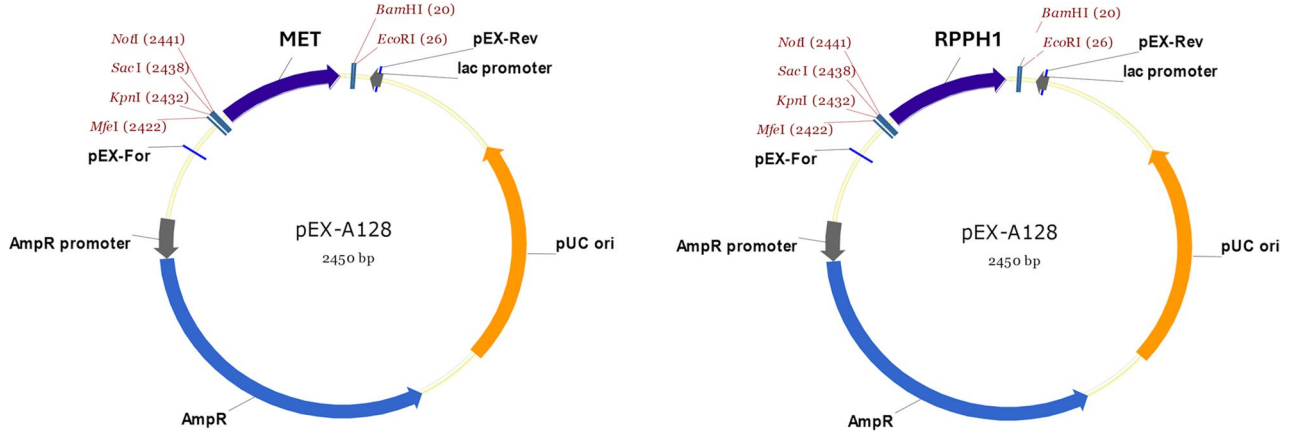

Fig. 1 *MET* and *RPPH1* plasmid maps

Annealing Temperature Optimization by ddPCR

Annealing temperature (T_a) optimization for the *MET* and *RPPH1* primer/probe assays was performed using gradient ddPCR on a QX200 Droplet Digital PCR System (Bio-Rad). Reactions were carried out using the ddPCR Supermix for Probes (no dUTP, Bio-Rad) according to the manufacturer's instructions. Each 20 µL reaction included 10 µL of Supermix, 900 nM of each primer, 250 nM of the corresponding hydrolysis probe, and 0.01 ng of DNA template (synthetic plasmids). Droplets were generated using a Manual Droplet Generator (Bio-Rad), and the thermal cycling protocol was performed on a T100 Thermal Cycler (Bio-Rad) with a gradient annealing step ranging from 55 °C to 65 °C. The cycling conditions were as follows: 95 °C for 10 min (enzyme activation), followed by 40 cycles of denaturation at 94 °C for 30 s and annealing/extension at the gradient temperatures for 60 s, with a final enzyme deactivation step at 98 °C for 10 min. A ramp rate of 2 °C/second was used for all steps.

Fluorescence amplitude data were analyzed using the QX Manager software 2.0 (Bio-Rad). The optimal T_a was selected based on clear cluster separation, maximum fluorescence amplitude, and minimal droplets rain. This temperature was subsequently used for all ddPCR experiments.

Singleplex vs. Duplex Assay Validation

To evaluate potential cross-reactivity and confirm assay compatibility, ddPCR reactions were performed both in singleplex and duplex formats. The *MET* probe was labeled with FAM, and the *RPPH1* probe with HEX. Singleplex and duplex reactions were tested under identical conditions to evaluate potential interference, such as signal suppression, increased rain, or altered cluster patterns. All reactions were performed using the QX200 Droplet Digital PCR System (Bio-Rad) with the ddPCR Supermix for Probes (no dUTP), and ~ 1.3e-3 pg of plasmids DNA as template. The thermal cycling protocol was as previously described, using the T_a of 63 °C, selected based on gradient optimization results.

Linearity Assessment of the Duplex ddPCR Assay

The linearity of the duplex ddPCR assay for *MET* CN quantification was evaluated using a curve of the *MET*-containing plasmid spiked into a background of gDNA. The background gDNA was obtained by pooling equimolar amounts of gDNA extracted from 4 cell lines with confirmed diploid *MET* status (P-EP/SVTERT28-2, P-MSC/TERT308, MG-63, and THP-1), in order to mimic a more realistic genomic context. A series of 8 conditions was tested: 1 baseline sample containing only the background gDNA, and 7 samples containing increasing amounts of the *MET* plasmid. Each reaction contained 10 ng of total DNA, with the proportion of plasmid adjusted to generate a dynamic range of *MET* CN increases.

ddPCR reactions were performed in duplex format under previously optimized conditions. Each point was tested in triplicate and each replicate was prepared independently to avoid underestimation of variation.

Linearity was assessed by regression analysis of measured versus expected *MET* CN values, using the coefficient of determination (R^2) to evaluate goodness of fit and residual plots to assess the adequacy of the linear model.

Input DNA Concentration Optimization

To identify the optimal input DNA concentration for accurate and reproducible quantification of *MET* CN using the duplex ddPCR assay, a series of reactions was performed using decreasing amounts of gDNA extracted from the pooled diploid cell lines (P-EP/SVTERT28-2, P-MSC/TERT308, MG-63, and THP-1). DNA input amounts ranged from 100 ng to 0.1 ng per 20 µL reaction (100 ng, 50 ng, 20 ng, 10 ng, 5 ng, 1 ng, 0.5 ng, and 0.1 ng). All reactions were performed in duplex format using the QX200 ddPCR system and the previously optimized T_a of 63 °C. Each sample was tested in triplicate.

The performance of each DNA input level was evaluated based on droplet cluster separation, fluorescence amplitude, and expected *MET* CN.

Assessment of the Impact of gDNA Digestion

To evaluate the potential impact of gDNA digestion on *MET* CN quantification, experiments were conducted using wild-type (WT) gDNA (a pool derived from P-EP/SVTERT28-2, P-MSC/TERT308, MG-63, and THP-1 cell lines) spiked with defined amounts of *MET*-containing plasmid and *MET*-amplified cell lines. The same DNA was tested under 2 conditions: undigested and digested. For the digested condition, DNA was treated with the EcoRI restriction enzyme (Thermo Fisher Scientific). The enzymatic digestion was performed by adding the EcoRI in the PCR mix, following manufacturer's recommendations, and the samples were then processed using the optimized duplex ddPCR protocol.

Baseline Copy Number Assessment in Diploid Cell Lines

To evaluate the baseline *MET* CN and confirm the suitability of the selected reference gene (*RPPH1*), gDNA from 4 independent human cell lines with no reported *MET* CNV (P-EP/SVTERT28-2, P-MSC/TERT308, MG-63, and THP-1) and a WT reference material (RM) were analyzed using the previously optimized multiplex ddPCR assay. All ddPCR reactions were carried out in duplex format using 10 ng of input DNA. The *MET* CN was calculated for each sample, and the resulting CN

values were used to determine the baseline reference range for diploid samples.

Assessment of repeatability, reproducibility, and Measurement Uncertainty

To assess the analytical performance of the duplex ddPCR assay in terms of repeatability, reproducibility, and measurement uncertainty (MU), a set of 4 RM was analyzed: Seraseq ctDNA Mutation Mix v4 AF0.1%, AF0.5%, AF5%, and WT (#0710–3097, #0710–3099, # 0710–3100, and # 0710–3101, SeraCare Life Sciences, LGC Clinical Diagnostics). These materials represent 3 different variant allele frequencies (VAFs) and include a WT control, thus enabling the evaluation of assay precision across a dynamic range of target concentrations. Each material was subdivided into 4 independent aliquots to ensure homogeneity and avoid freeze-thaw cycles. The ddPCR experiments were performed over 4 non-consecutive days by the same operator. In each run, all materials were analyzed in triplicate using the optimized duplex assay.

The resulting dataset comprised 12 replicate measurements per material (3 replicates × 4 runs). Repeatability (within-run precision) was calculated as the pooled standard deviation (SD) across the 4 runs, by combining the within-run variances from each triplicate measurement and weighing them by their respective degrees of freedom. Reproducibility (between-run precision) was assessed by calculating the SD of the value obtained for each material across the 4 independent runs. MU, expressed as relative expanded uncertainty (U%), was estimated by considering 3 primary sources of uncertainty that contribute to the overall uncertainty budget of the ddPCR assay: (i) the uncertainty associated with the dilution factor (D_f), (ii) the uncertainty in the partitioning statistics, expressed as the ratio of negative to total droplets (N_{neg}/N), and (iii) the uncertainty in the droplet volume (V_d). The uncertainty evaluation approach adopted in this study follows GUM [25] principles and is consistent with the recommendations of ISO 20395:2019 [26]; it is further aligned with methodologies applied and validated in international comparisons coordinated within the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM)-Nucleic Acids Working Group (NAWG). A detailed description of the uncertainty estimation model is provided in Additional File 1.

Statistical Analysis

Statistical analyses were performed using Origin 2022 software (OriginLab Corporation). One-way ANOVA with post-hoc Tukey multiple comparisons was used to assess significant differences among groups.

Results

Optimization of the ddPCR Assay Conditions

To ensure optimal performance and robustness of the ddPCR assay for *MET* CNV quantification, a comprehensive optimization strategy was undertaken.

Initially, primer efficiency and specificity were evaluated for both *MET* and the reference gene *RPPH1* using SYBR Green-based qPCR. The *MET* assay showed an amplification efficiency of 103.6% with an R^2 of 0.981, while the *RPPH1* assay exhibited an efficiency of 110.6% with an R^2 of 0.997, both within the acceptable range for qPCR-based validation (Fig. 2a). Melting curve analysis confirmed single, well-defined peaks for each target, with no signs of primer-dimer formation or non-specific amplification, supporting the specificity of the designed assays (Fig. 2b).

The T_a was then optimized using a ddPCR temperature gradient from 55 °C to 65 °C. Although a temperature range between 61 °C and 65 °C yields satisfactory results, 63 °C returned the clearest separation between positive and negative droplets and the most consistent fluorescence amplitude, and was thus selected as the optimal condition for subsequent assays (Fig. 2c).

Singleplex and duplex configurations were compared to assess potential interference between primers and probes. No significant differences in droplet separation, signal amplitude, or CN determination were observed between the approaches, validating the use of a duplex assay with *MET* (FAM-labeled) and *RPPH1* (HEX-labeled) in the same reaction (Fig. 2d).

To determine the optimal input DNA concentration, a dilution series ranging from 0.1 ng to 100 ng of WT gDNA was tested. Quantification performance was consistent within a range of 5 ng to 20 ng, while lower concentrations resulted in increased variability, and higher concentrations reduced droplet generation efficiency (Additional File 2).

Finally, the potential impact of gDNA digestion on ddPCR performance was evaluated. The results showed negligible differences in either droplet quality or quantification between digested and undigested samples, indicating that restriction digestion was not needed for this assay configuration (Fig. 2e).

Linearity and Baseline Value Evaluation

To assess the quantitative performance of the *MET* ddPCR assay, we evaluated its linearity across a dynamic range of *MET* CN inputs. A pooled WT gDNA, obtained from cell lines characterized by diploid *MET* status, was used as a background matrix. Increasing amounts of *MET*-containing plasmid were spiked into this background matrix to generate a 7-point series simulating different *MET* CN scenarios. The assay demonstrated strong linearity across the tested range (~2–7 CN), with

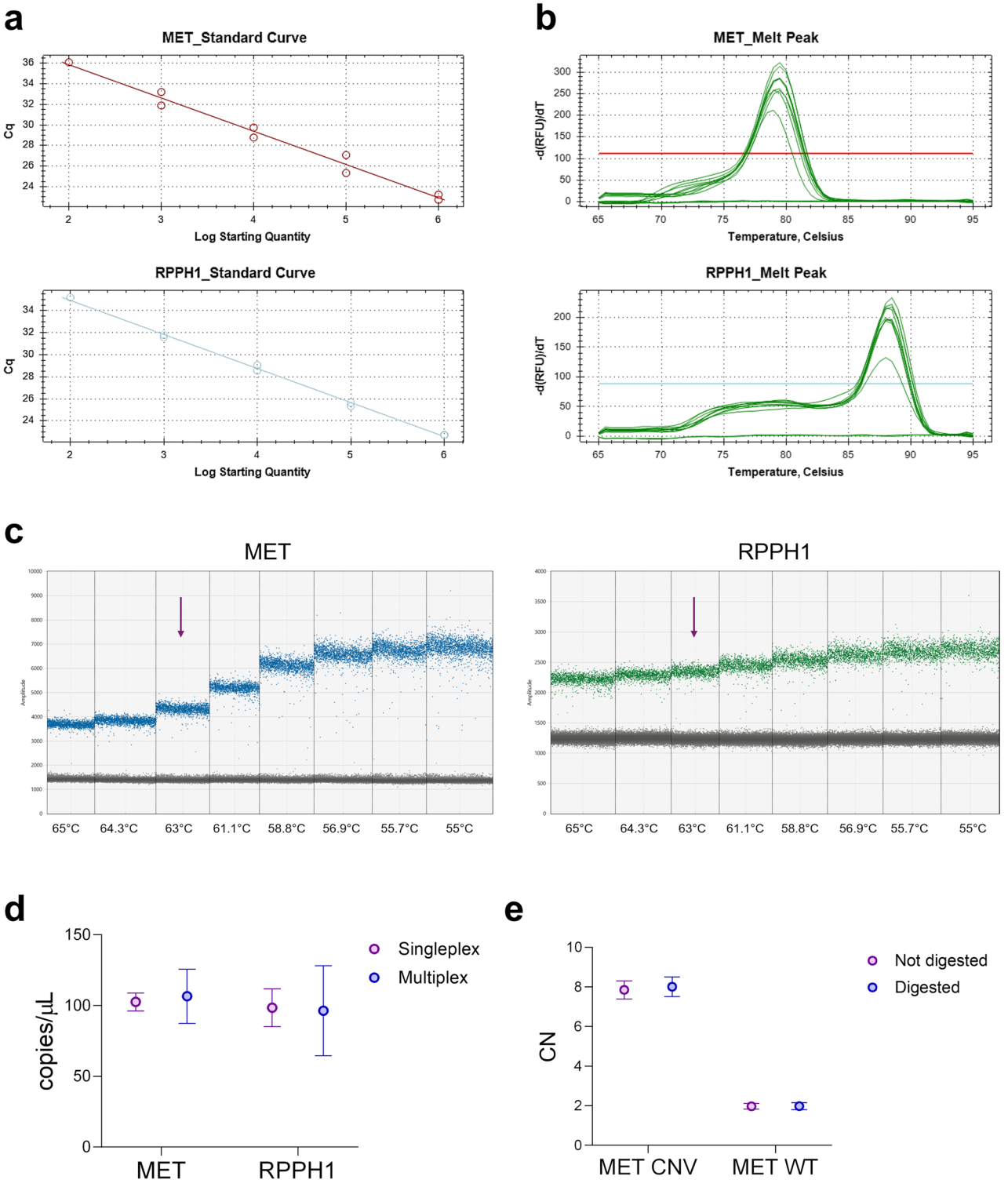

Fig. 2 Optimization of the *MET* ddPCR assay. **a** Primer efficiency curves for *MET* and *RPPH1*. **b** Melting curve analysis confirming specificity of *MET* and *RPPH1* amplicons. **c** Annealing temperature (T_a) optimization using a ddPCR gradient. The purple arrow indicates the chosen T_a. **d** Comparison of singleplex and duplex assay approaches in terms of target concentration (copies/µL on the *y*-axis). **e** Comparison between undigested and EcoRI-digested gDNA in terms of *MET* copy number (CN on the *y*-axis) in samples with (MET CNV) and without (MET WT) *MET* amplification

an R^2 of 0.988 (Fig. 3a). Linear regression analysis showed a slope close to 1 (1.04; 95% CI: 0.99–1.09) and an intercept not significantly different from zero, indicating proportional agreement between expected and measured copy number values. Residual analysis revealed small deviations between expected and measured values, with residuals remaining within ± 0.25 copies across the tested range and without evidence of systematic non-linear trends. Furthermore, the slope was significantly different from zero ($p < 0.0001$), confirming a statistically significant association between expected and measured values. Overall, these results support the suitability of the assay for quantitative copy number analysis.

In parallel, the baseline CN of *MET* was evaluated in WT gDNA without plasmid spike-in. Independent DNA extractions from 4 different cell lines and 1 RM were tested across multiple experimental replicates. *MET*-amplified cell lines (GTL16 and EBC1) were used as positive control [23, 24, 27]. The average *MET* CN relative to the diploid reference gene *RPPH1* was 2.25 ± 0.30 (mean ± SD), consistent with the expected diploid status and confirming assay accuracy under physiological conditions (Fig. 3b).

Repeatability, reproducibility, and Measurement Uncertainty

The analytical performance of the duplex ddPCR assay was evaluated in terms of repeatability, reproducibility, and MU using 4 reference materials with defined CNV (CN range: 0–15). The assay demonstrated high precision across the tested dynamic range. The mean repeatability, calculated as the pooled intra-run coefficient of variation (CV) across all materials, was 3.8% (Seraseq ctDNA Mutation Mix v4 AF0.1%=4.2%; AF0.5%=3.7%; AF5%=3.3%; WT = 4.2%), indicating low variability within runs (Fig. 4a). The mean reproducibility, assessed as the CV of the inter-run mean values, was 3.9% (Seraseq ctDNA Mutation Mix v4 AF0.1%=4.2%; AF0.5%=3.7%; AF5%=3.3%; WT = 4.3%), confirming the consistency of results across different experimental days (Fig. 4b). The mean relative expanded measurement uncertainty ($k = 2$), derived from contributions of D_f, N_{neg}/N, and V_d, was estimated at 23.8% (Seraseq ctDNA Mutation Mix v4 AF0.1%=14.1%,; AF0.5%=38.0%; AF5%=23.0%; WT = 20.0%), reflecting the overall confidence in quantitative measurements produced by the assay (Fig. 4c). The *MET* CN values obtained in our analysis were consistent with the producer's declared values (Pearson's $r = 0.999$, $p = 0.014$; Fig. 4d).

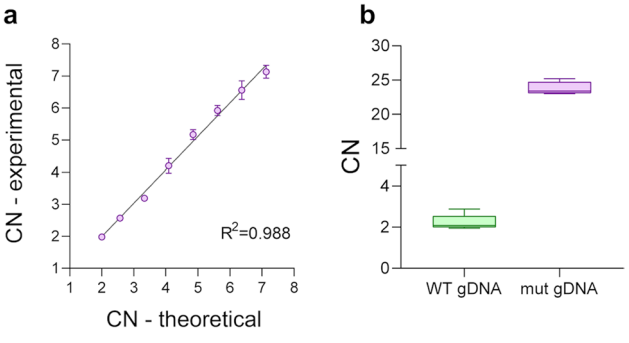

Fig. 3 Linearity and baseline assessment of the *MET* ddPCR assay. **a** Linearity curve obtained by spiking increasing amounts of *MET*-targeting plasmid into wild-type (WT) genomic DNA (gDNA). CN, copy number. **b** Baseline *MET* copy number determined in WT gDNA from 4 different diploid cell lines and the WT reference material (Seraseq ctDNA Mutation Mix WT). Cell lines *MET*-amplified (mut gDNA) were used as positive control

Discussion

In this study, we developed and validated a candidate RMP for the quantification of *MET* CNV using ddPCR. The duplex assay, targeting *MET* exon 14 and the reference gene *RPPH1*, demonstrated excellent performance in terms of linearity, repeatability, and reproducibility. These findings are consistent with prior studies highlighting the advantages of dPCR over conventional CNV detection techniques such as qPCR and FISH, particularly in terms of absolute quantification, sensitivity, and robustness [4, 14, 28, 29]. By ensuring high assay specificity, optimal annealing conditions, and stable quantification across a range of input DNA concentrations, this method addresses critical gaps in CNV standardization, one of the key challenges currently limiting harmonization across diagnostic laboratories [30].

A major strength of this work lies in its metrological approach to method development, with particular emphasis on measurement traceability, uncertainty evaluation, and value assignment in the context of a candidate RMP. While the assay design, optimization, and experimental practices are consistent with established recommendations for dPCR, including ISO 20395:2019 [26] and the digital MIQE guidelines [31], the primary focus of this study is the application of metrological principles rather than formal compliance with reporting-oriented guidelines. In addition, although this study was performed using a ddPCR platform, the assay design and the proposed candidate RMP are not intrinsically platform-dependent. The same assays can, in principle, be implemented on other dPCR systems. This platform-independent perspective is essential to support broader adoption and inter-laboratory harmonization. Nonetheless, some limitations should be acknowledged. The study primarily utilized synthetic constructs, well-characterized cell lines, and RM, which may not fully replicate the complexity of clinical specimens such as formalin-fixed paraffin-embedded (FFPE) tissues, cell free DNA (cfDNA) from plasma, or tumor types characterized by genomic instability. In particular, FFPE processing often leads to DNA fragmentation and chemical modifications

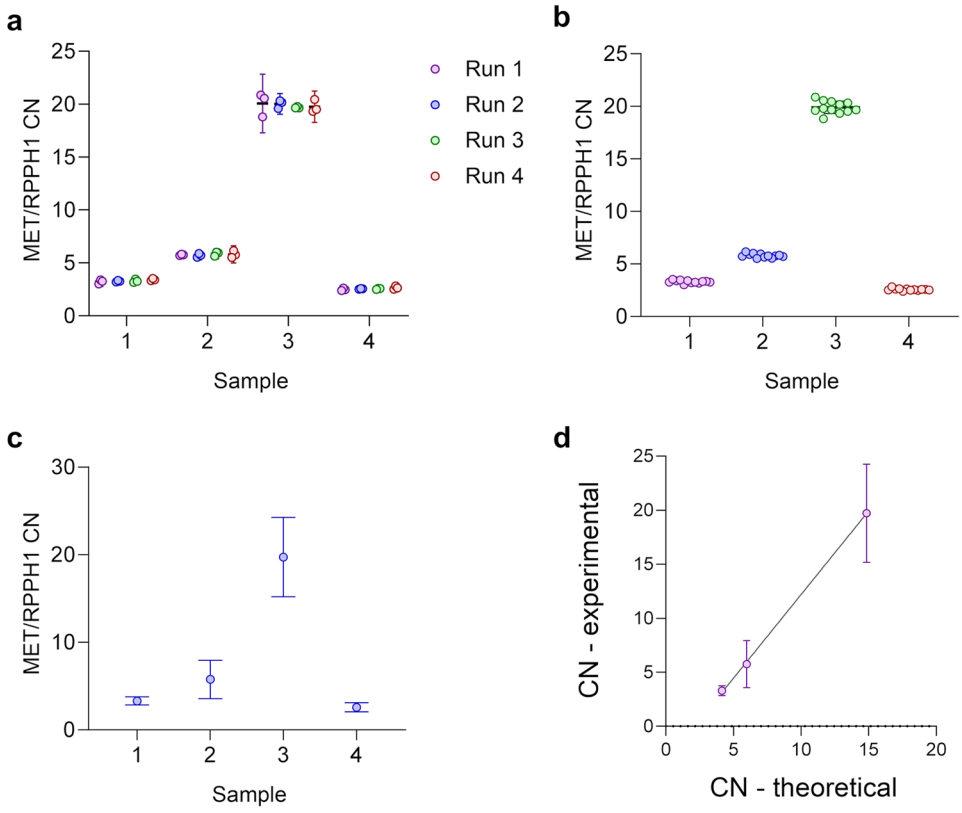

Fig. 4 Repeatability, reproducibility, and *MU* assessment of the *MET* ddPCR assay. Sample 1, Seraseq ctDNA Mutation Mix v4 AF0.1%; Sample 2, Seraseq ctDNA Mutation Mix v4 AF0.5%; Sample 3, Seraseq ctDNA Mutation Mix v4 AF5%; Sample 4, Seraseq ctDNA Mutation Mix v4 WT. **a** Repeatability (intra-run variability). **b** Reproducibility (inter-run variability). **c** Samples value assignment. Error bars indicate the expanded uncertainty ($k=2$). **d** Correlation between *MET* CN values obtained in our analysis (*y*-axis) and producer's declared values (*x*-axis). Error bars indicate the expanded uncertainty ($k=2$)

that can reduce amplification efficiency and bias CN estimates. Similarly, tumors with highly unstable genomes may present widespread structural alterations that complicate the interpretation of CNV results. To mitigate these issues, several strategies have been proposed in the literature. For FFPE samples, optimized DNA extraction methods, quality assessment metrics, and inclusion of quality controls can improve assay reliability [32, 33]. For samples with genomic instability, the use of multiple reference genes/loci can help distinguish true biological variation from technical noise [20, 34, 35].

Moreover, although the *RPPH1* gene has been previously reported as a stable diploid reference locus and has been successfully used as a reference gene for CN determination [36–38], including metrology-oriented and interlaboratory studies, its suitability should be further confirmed as CN stability may vary depending on tumor type and genomic context. Nevertheless, the suitability of any reference gene should be verified for the specific biological and clinical context under investigation.

Future efforts should focus on external validation in multi-center settings and on integrating this RMP into quality assessment frameworks to further support clinical implementation. To facilitate method adoption and ensure consistency across laboratories, the complete Standard Operating Procedure (SOP) is provided in Additional File 3, offering detailed instructions for the correct implementation of the candidate RMP for *MET* CNV quantification.

Conclusions

This work presents a rigorously validated candidate RMP for *MET* CNV quantification using ddPCR, offering a metrological approach to provide traceable, precise, and reproducible approach suitable for both research and clinical applications. By addressing the current lack of standardized methodologies for *MET* CNV assessment, the proposed method can serve as a cornerstone for the development of certified reference materials and contribute to inter-laboratory harmonization of CNV testing.

The availability of such procedure is particularly relevant in the context of precision oncology, where accurate *MET* quantification informs diagnostic and therapeutic decisions. Implementing this RMP across clinical laboratories could enhance the reliability of molecular diagnostics, support the comparability of clinical trial outcomes, and ultimately improve patient stratification and care. This study thus represents a significant step toward the standardization and quality assurance of *MET* CNV measurements in cancer genomics.

Abbreviations

AF	allele frequency
CCQM	Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology
cfDNA	cell free DNA
CN	copy number
CNV	copy number variation
CV	coefficient of variation
dPCR	droplet PCR
ddPCR	droplet dPCR
D_f	dilution factor
FISH	fluorescence in situ hybridization
FFPE	Formalin-Fixed Paraffin-Embedded
gDNA	genomic DNA
MU	measurement uncertainty
NAWG	Nucleic Acid Working Group
NGS	next-generation sequencing
qPCR	quantitative PCR
RMP	Reference Measurement Procedure
RM	reference material
SD	standard deviation
SOP	Standard Operating Procedure
T_a	annealing temperature
VAF	variant allele frequency
V_d	droplet volume
WT	wild-type

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12575-026-00325-5.

Additional file 1: Assessment of Measurement Uncertainty (MU) - detailed description of the uncertainty estimation model.

Additional file 2: Evaluation of the optimal input DNA concentration.

Additional file 3: Standard Operating Procedure for MET Copy Number Variation (CNV) Detection and Quantification Using Droplet Digital PCR - Optimized step-by-step protocol for detecting and quantifying the MET CNV in DNA from various sources using droplet digital PCR.

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Authors' Contributions

JP design the study, performed the experiments, analyzed the data, and wrote the manuscript; SC and LR performed the statistical analyses and revised the manuscript; MF revised the manuscript; GC, RA and SG characterized cell lines, TV provided cell lines and revised the manuscript; CD revised the manuscript, supervised the study, and acquired the funding. All authors read and approved the final manuscript.

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Data Availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare no competing interests.

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