New candidate reference measurement procedure for NRAS p.Q61R quantification by digital PCR

Methods 254 (2026) 192–201

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

New candidate reference measurement procedure for *NRAS* p.Q61R quantification by digital PCR☆

Jessica Petiti [a,*], Sabrina Caria [a,b,*], Laura Revel [a], Alexandra Bogožalec Košir [c], Giovanna Carrà [d,e], Marika Fava [a], Nathalie Weiss [f], Marcel Kremser [f], Marit Gillmeister [f], Laura Vierbaum [f], Anne Kaufmann-Stoeck [f], Arthur Schmitz [f], Mojca Milavec [c], Carla Divieto [a]

[a] Division of Advanced Materials Metrology and Life Sciences, Istituto Nazionale di Ricerca Metrologica (INRIM), Turin, Italy
[b] Department of Physics, University of Turin, Turin, Italy
[c] Department of Biotechnology and Systems Biology, National Institute of Biology, Ljubljana, Slovenia
[d] San Luigi Gonzaga Hospital, Orbassano, Italy
[e] Department of Translational Medicine (DIMET), University of Eastern Piedmont, Novara, Italy
[f] INSTAND e.V., Society for Promoting Quality Assurance in Medical Laboratories, Duesseldorf, Germany

ARTICLE INFO

Keywords:
NRAS p.Q61R
Digital PCR (dPCR)
Reference Measurement Procedure (RMP)
Precision oncology
Molecular testing

ABSTRACT

Accurate and comparable quantification of somatic mutations is essential for precision oncology, as clinical decision-making increasingly relies on the quantification of molecular biomarkers. Despite major technological advances, inter-laboratory variability and the lack of metrological traceability remain significant barriers to harmonization and confidence in mutation testing results. Reference Measurement Procedures (RMPs) represent a critical framework to address these challenges by anchoring molecular measurements to common quantitative standards.

Here, we describe the development and validation of a candidate RMP for the detection and quantification of the clinically relevant *NRAS* p.Q61R mutation using digital PCR (dPCR). The assay was systematically optimized to maximize specificity and minimize cross-reactivity between wild-type and mutant alleles. Analytical characterization demonstrated excellent linearity across a broad range of variant allele frequencies (vAF), with a limit of detection of 0.1 %. Precision studies performed on commercially available circulating tumour DNA reference materials (RM) showed good repeatability and intermediate precision, while a full measurement uncertainty budget confirmed the robustness of the approach. Comparison with a commercial dPCR assay provided independent support for assay comparability and consistent vAF estimates across the investigated range.

Preliminary inter-laboratory assessment supported transferability of the candidate RMP and comparability of the resulting measurements. Overall, this work establishes a metrologically characterized and transferable dPCR-based RMP for *NRAS* p.Q61R quantification. Its implementation can support the harmonization of molecular measurements, the value assignment of RM, and the alignment of routine and secondary methods, thereby strengthening the reliability of quantitative biomarker assessment in precision oncology.

1. Introduction

Mutations in the *NRAS* gene, particularly the p.Q61R variant (c.182A > G, COSM584), play a crucial role in oncogenesis and are recurrently detected in several solid tumours, including non-small cell lung cancer and colorectal cancer. The *NRAS* gene encodes a small GTPase involved in intracellular signal transduction, primarily through the RAS/MAPK pathway, which regulates cell proliferation, differentiation, and survival. The substitution of a glutamine (Q) with an arginine (R) at codon 61 disrupts the intrinsic GTPase activity of NRAS, resulting in a constitutive pathway activation and a sustained oncogenic signalling [1,2].

Accurate detection and quantification of *NRAS* mutations are critical for clinical decision-making. Specific variants, including p.Q61R, have

☆ This article is part of a special issue entitled: 'Editors Collection - YMETH' published in Methods.

* Corresponding authors at: Division of Advanced Materials Metrology and Life Sciences, Istituto Nazionale di Ricerca Metrologica (INRIM), Turin, Italy (S. Caria).
E-mail addresses: j.petiti@inrim.it (J. Petiti), sabrina.caria@unito.it (S. Caria).

https://doi.org/10.1016/j.ymeth.2026.07.005
Received 21 April 2026; Received in revised form 15 June 2026; Accepted 5 July 2026
Available online 10 July 2026

been associated with resistance to targeted therapies such as cetuximab, dabrafenib, and vemurafenib [3,4]. In this context, quantitative assessment of variant allele frequency (vAF) is increasingly relevant for patient stratification, treatment monitoring, and prognosis. However, conventional molecular methods, including Sanger sequencing and allele-specific PCR, are limited by insufficient sensitivity and specificity, and poor quantitative reproducibility, particularly when dealing with heterogeneous tumour samples or low-frequency variants [5]. While next-generation sequencing (NGS) enables comprehensive mutation profiling and has become a cornerstone of molecular oncology, its quantitative performance at low vAF remains method- and laboratory-dependent, as standardized, SI-traceable quantification is often lacking. This can result in significant inter-laboratory variability, ultimately compromising result comparability and clinical reliability in precision oncology [6,7].

Recent advances in digital PCR (dPCR) have substantially improved the sensitivity and precision of mutation detection. By partitioning DNA samples into thousands of nanolitre-sized reactions, dPCR enables absolute quantification of target molecules and robust estimation of low-frequency variants in complex genomic backgrounds [8–10]. These features make dPCR well suited for applications such as measurable residual disease (MRD) monitoring and assessment of treatment response, where accurate quantification of rare mutant alleles is crucial [11,12].

Despite these analytical advantages, high technical performance alone does not ensure inter-laboratory comparability. In the absence of standardized and SI-traceable measurement frameworks, differences in assay design, calibration strategies, and data analysis can still lead to systematic biases across measurement systems.

In this context, the establishment of a Reference Measurement Procedure (RMP) represents a critical step toward anchoring molecular measurements to common metrological standards. Unlike routine diagnostic assays, RMPs are intended to provide higher-order measurement results with documented metrological traceability and uncertainty evaluation. Their role is not to replace routine methods, but to support calibration hierarchies, value assignment of RM, external quality assessment schemes, and inter-method harmonization, in line with quality-assurance frameworks and regulatory requirements, including EU Regulation 2017/746 on *in vitro* diagnostic medical devices (IVDR) and International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q2(R2): Validation of Analytical Procedures [13,14]. European metrology initiatives, such as the GenomeMET [15] and Bio-SITrace projects [16], have been launched to address these needs by advancing metrological frameworks for clinically relevant cancer-associated biomarkers.

Here, we present the development and validation of a candidate RMP for the detection and quantification of the *NRAS* p.Q61R mutation using dPCR. By providing metrologically traceable measurement results through a transferable measurement procedure, the proposed approach supports inter-laboratory harmonization, value assignment of RM and alignment of dPCR and secondary methods, such as NGS. Ultimately, the availability of robust RMPs for clinically actionable mutations provides an important basis for reliable and reproducible quantitative biomarker assessment in precision oncology.

2. Materials and methods

2.1. Assay design

A competitive duplex dPCR assay was developed for the detection and quantification of the *NRAS* p.Q61R (c.182A > G) mutation. The assay uses a single primer pair and two allele-specific hydrolysis probes to enable precise discrimination between the mutant and wild-type (WT) alleles. The target region encompassing exon 3 of *NRAS* (NC_000001.11) was amplified, with the p.Q61R mutant allele detected using a FAM-labelled MGB probe, and WT allele detected using a HEX-labelled MGB probe.

Primer and probe sequences were designed using Primer3Plus according to criteria optimized for dPCR applications [17]. In silico specificity was assessed using NCBI Primer-BLAST to exclude off-target amplification within the human genome. Known polymorphisms and restriction sites within the amplicon region were avoided to ensure robust assay performance. All oligonucleotides were synthesized by Eurofins Genomics. Primer and probe sequences are reported in the Standard Operating Procedure (SOP, Supplementary file 1).

2.2. Control materials and test samples

Synthetic double-stranded DNA fragments corresponding to exon 3 of *NRAS* WT and p.Q61R mutant alleles were used as control templates for assay development, optimization and characterization. Both sequences were cloned into two separate plasmid vectors (pEX-A128 backbone), synthesized and sequence-verified by Eurofins Genomics. Upon receipt, plasmids were resuspended according to the manufacturer's instructions, quantified using the Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific), and diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Working solutions (0.1 ng/µL) were aliquoted and stored at −20 °C to avoid repeated freeze–thaw cycles. Plasmid maps and insert sequences are reported in Fig. 1 and Table 1.

Genomic DNA (gDNA) was extracted from four commercial human cell lines with confirmed *NRAS* WT status (P-EP/SVTERT28-2 and P-MSC/TERT308, purchased from Evercyte; MG-63 and THP-1, purchased from ATCC). Cell lines were cultured according to the manufacturer's recommended conditions and harvested upon reaching confluence. gDNA extraction was performed for each cell line using the QIAamp DNA Mini Kit (QIAGEN) following the manufacturer's instructions. DNA concentration and purity were assessed using a NanoQuant Plate (Tecan), and WT gDNA was stored at − 20 °C until use. These samples served as WT controls and as background matrices for spiking experiments, where defined amounts of the *NRAS* p.Q61R plasmid were added to generate samples with controlled vAF.

Repeatability, intermediate precision, and measurement uncertainty (MU) were assessed using commercially available RM with nominal vAF of 0.1 %, 0.5 %, and 5 %, each provided as a separate Seraseq ctDNA Mutation Mix v4 product, along with a corresponding WT control (SeraCare Life Sciences). RM were subdivided into independent aliquots to ensure homogeneity and to minimize freeze–thaw cycles prior to analysis.

2.3. dPCR assay optimization

The dPCR assay was systematically optimized to ensure high specificity, robust cluster separation and minimal cross-reactivity between mutant and WT alleles. Primer amplification efficiency and specificity were initially evaluated by SYBR Green-based quantitative real-time PCR (qPCR) using the CFX Connect Real-Time PCR System (Bio-Rad) and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Reactions were performed using a 5-fold serial dilution of WT gDNA prepared gravimetrically in nuclease-free H_2O, starting from 150 ng DNA per reaction. Each dilution point was analysed in duplicate. Amplification efficiency was calculated from the slope of the standard curve. Amplicon specificity was verified by melting curve analysis.

Annealing temperature optimization was subsequently performed by gradient dPCR using synthetic *NRAS* WT and p.Q61R mutant plasmids (100 % WT and 50 % p.Q61R). A temperature range from 55 °C to 65 °C was evaluated, and the optimal temperature was selected based on separation between positive and negative populations, fluorescence amplitude, and minimal rain.

Given the single-nucleotide difference between the mutant- and WT-specific hydrolysis probes, primer and probe concentrations were empirically optimized to achieve optimal signal discrimination, while maintaining low cross-reactivity and adequate assay sensitivity. Different primer/probe concentration combinations were tested in

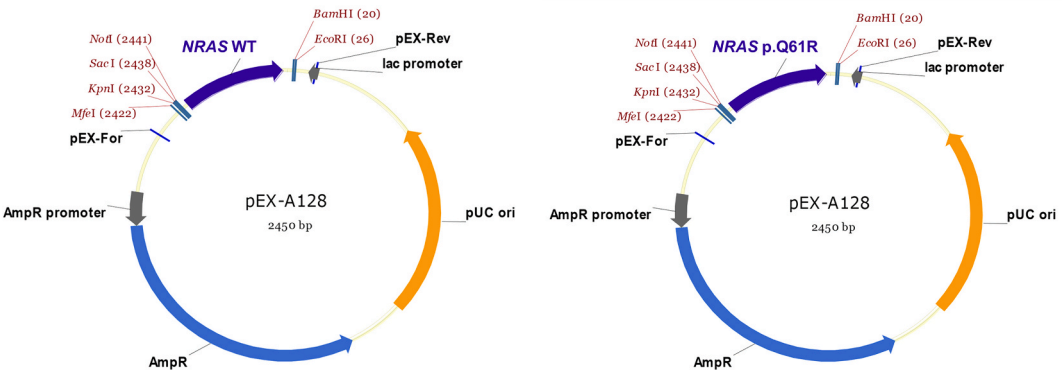

Fig. 1. *NRAS* p.Q61R and WT plasmid maps. The exon 3 *NRAS* inserts (WY and p.Q61R mutated) were cloned into two different pEX-A128 plasmids. The *NRAS* insert region is highlighted in purple. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

duplex using WT gDNA and samples spiked with defined amounts of *NRAS* p.Q61R plasmid (vAF: 0 %, 5 %, 10 %, and 50 %). Optimal discrimination was defined as the condition that provided clear separation between positive and negative populations in both fluorescence channels, minimal "rain", negligible cross-reactivity between mutant and WT signals, and stable vAF quantification across the tested concentrations. Final concentrations were selected based on these criteria and were used for all subsequent experiments.

2.4. Analytical performance assessment

Analytical performance of the duplex dPCR assay was evaluated in terms of linearity and sensitivity. Linearity was assessed using two complementary experimental approaches. In the first approach, synthetic plasmid DNA carrying the *NRAS* p.Q61R mutation was mixed with WT plasmid DNA at defined ratios to generate samples to cover a wide range of expected vAFs, from 0 % to 100 % (0 %, 1 %, 5 %, 10 %, 50 %, and 100 % vAF). In the second approach, independently prepared samples were generated by spiking defined amounts of mutant plasmid DNA into WT gDNA to obtain low and clinically relevant vAF levels (0 %, 0.25 %, 0.50 %, 2.50 %, and 5 %). Each vAF level was analysed in triplicate under optimized assay conditions.

Measured vAF values were plotted against expected values, calculated from the predefined mixing ratios of quantified gDNA and plasmids, and linear regression analysis was performed to evaluate assay linearity, expressed as the coefficient of determination (R^2). The same dilution series were used to estimate the analytical sensitivity of the assay. The limit of blank (LoB), limit of detection (LoD), and limit of quantification (LoQ) were initially derived from regression analysis, in accordance with established analytical validation principles [14,18]. Specifically, the LoB was calculated as the mean blank response plus 1.645 times the standard deviation (SD) of the blank measurements. LoD and LoQ were estimated from the SD of the response and the slope of the calibration curve (S), using the expressions LoD = 3.3SD/S and LoQ = 10SD/S.

The resulting estimates were subsequently experimentally verified using commercially available RM with nominal vAF values close to the calculated thresholds. The LoD was defined as the lowest vAF level showing a statistically significant difference from the blank material [14], whereas the LoQ was defined as the lowest vAF level showing a statistically significant difference from the blank and acceptable precision and accuracy [19] (a CV < 30 % is generally considered acceptable for quantitative molecular assays).

The estimated limits were subsequently experimentally verified using commercially available RM with nominal vAF values close to the calculated thresholds.

2.5. Assessment of the impact of gDNA digestion

To evaluate the impact of gDNA digestion on *NRAS* p.Q61R quantification, WT gDNA spiked with defined amounts of *NRAS* p.Q61R mutant plasmid was analysed. Identical DNA–plasmid mixtures were analysed under two conditions: undigested and enzymatically digested.

For the digested condition, gDNA and plasmid were treated with the restriction enzyme EcoRI (Thermo Fisher Scientific) according to the manufacturer's instructions. EcoRI was selected because it does not cut within the NRAS target amplicon, thereby allowing the effects of DNA fragmentation to be evaluated without affecting the target sequence. EcoRI digestion was evaluated to determine whether DNA fragmentation could improve droplet cluster separation or influence vAF estimation. Because cell-free DNA (cfDNA) is naturally fragmented, this assessment was also relevant to the potential application of the procedure to cfDNA samples. Quantitative results obtained from digested and undigested samples were compared EcoRI was selected because it does not cut within the NRAS target amplicon, allowing the effect of DNA fragmentation to be evaluated without altering the target sequence.

2.6. Comparison with commercial assay

Performance of the candidate RMP was compared with a BioRad's commercially available and wet-validated assay (NRAS p.Q61R c.182A > G, Assay ID: dHsaMDV2010071). The comparison was performed as an independent assessment of assay comparability. Specifically, it was designed to evaluate the consistency of vAF estimates obtained with the candidate RMP and a commercially available assay across a broad concentration range, while assessing potential assay-dependent effects related to cluster separation, signal discrimination, and quantitative performance. The analyses performed using the candidate RMP followed the optimized in-house protocol, whereas the commercial assay was carried out according to the manufacturer's instructions.

A dilution series was prepared by mixing synthetic *NRAS* p.Q61R mutant plasmid DNA with a WT gDNA, used as a background to generate samples covering a broad range of expected vAF (0 %, 0.16 %, 0.80 %, 8 %, 20 %, 40 %, 60 % vAF). Each vAF level was analysed in duplicate.

For both assays, fluorescence amplitude data were evaluated to assess cluster separation and potential probe cross-reactivity. Quantitative performance was assessed by comparing measured and expected vAF values through linear regression analysis and by evaluating the agreement between the two assays using correlation analysis.

2.7. Repeatability, intermediate precision, and measurement uncertainty

Repeatability, intermediate precision and MU were evaluated using commercially available RM, including four samples with nominal vAF of

Table 1
NRAS p.Q61R and WT sequences of inserts. The nucleotide affected by the mutation is highlighted in red.

Variant	Sequence (5′-3′)	Length [bp]
NRAS p.Q61R	GATTCTTACAGAAAACAAGTGGTTATAGATGGTGAAACCTGTTTGTTGGACATACTGGATACAGCTGGACGAGAAGAGTACAGTGCCATGAGAGACCAATACATGAGGACAGG CGAAGGCTTCCTCTGTGTATTTGCCATCAATAATAGCAAGTCATTTGCGGATATTAACCTCTACAG	179
NRAS WT	GATTCTTACAGAAAACAAGTGGTTATAGATGGTGAAACCTGTTTGTTGGACATACTGGATACAGCTGGACAAGAAGAGTACAGTGCCATGAGAGACCAATACATGAGGACAG GCGAAGGCTTCCTCTGTGTATTTGCCATCAATAATAGCAAGTCATTTGCGGATATTAACCTCTACAG	

0.1 %, 0.5 %, and 5 %, as well as a WT control (Seraseq ctDNA Mutation Mix v4, SeraCare Life Sciences). These materials were selected to assess assay performance across a clinically relevant dynamic range, including vAFs close to the estimated LoD and LoQ.

Each RM was subdivided into independent aliquots to ensure homogeneity and to minimize freeze–thaw cycles. Measurements were performed over four non-consecutive days by the same operator. In each run, all materials were analysed in triplicate, resulting in a total of twelve replicate measurements per material.

Repeatability (intra-run precision) was estimated by calculating the pooled standard deviation (SD) across replicate measurements within each run. Intermediate precision (inter-run precision) was assessed by calculating the SD of the mean values obtained across independent runs. Repeatability and intermediate precision were expressed as coefficients of variation (CV).

MU was expressed as relative expanded uncertainty (U%) and estimated by combining the main sources of uncertainty, including the dilution factor (D_f), partitioning statistics (N_{neg}/N), and partition volume (V_d). Uncertainty propagation was performed in accordance with established metrological principles and guidance documents[20,21]. A detailed description of the uncertainty model and calculations is provided in the Supplementary file 2.

The resulting dataset was also used to experimentally verify the previously estimated LoB, LoD, and LoQ.

2.8. Inter-laboratory comparison study

Operational transferability and reproducibility of the candidate RMP were assessed through a small inter-laboratory comparison study involving two independent laboratories: the National Metrology Institute of Italy (INRiM, Turin, Italy) and the National Institute of Biology (NIB, Ljubljana, Slovenia).

An external quality assessment (EQA) material based on circulating cfDNA, provided by INSTAND, was used for the comparison (Supplementary file 3). The EQA material consisted of two identical blinded samples distributed to both laboratories. Each partner performed dPCR analyses independently using routine instrumentation and software settings, and strictly adhered to the shared SOP (Supplementary file 1) for assay setup, thermal cycling conditions, and data acquisition. Operators, instruments, and data analysis thresholds were not harmonized across laboratories in order to reflect realistic implementation conditions.

In both laboratories cfDNA was extracted with the QIAamp Circulating Nucleic Acid Kit (QIAGEN) and quantified with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific), according to manufacturer's instructions.

At INRiM each EQA sample was analysed undiluted over three independent experiments performed on three different days, with triplicate measurements in each run (18 replicate measurements in total), using the QX200 Droplet Digital PCR System (Bio-Rad). Value assignment and the associated MU were estimated according to the uncertainty model described in Supplementary file 2, considering the two EQA samples as biological replicates.

At NIB undiluted cfDNA samples were analysed using the QX600 Droplet Digital PCR System (Bio-Rad) in one experiment. One EQA samples was analysed in triplicate and one in duplicate. Target copy number concentrations were calculated as the mean of the five measurements, and MU was estimated according to the model described in Supplementary file 2.

Inter-laboratory agreement was assessed by comparing the *NRAS* p.Q61R vAF values measured at both laboratories.

2.9. Statistical analysis

Statistical analyses and graphical representations were performed using Origin 2022 software (OriginLab Corporation). Linear regression

analysis was used to assess assay linearity and agreement between expected and measured vAF, as well as to compare results obtained with the candidate RMP and the commercial dPCR assay. Correlation between methods was evaluated using Pearson's correlation coefficient.

Repeatability and intermediate precision were expressed as coefficients of variation (CV). Differences between experimental conditions were assessed using one-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple-comparison test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Optimization of the dPCR assay conditions

Key assay parameters were systematically optimized to ensure robust and reproducible quantification of the NRAS p.Q61R mutation. Primer efficiency and specificity were first evaluated by SYBR Green-based qPCR. The primer pair showed an amplification efficiency of 102 % ($R^2 = 0.978$; Fig. 2a), and the melting curve analysis confirmed a single specific amplicon, with no evidence of non-specific products or primer–dimer formation (Fig. 2b).

Annealing temperature optimization was subsequently performed by gradient dPCR. Among the tested temperatures, 63 °C yielded the clearest separation between positive and negative populations (1D-amplitude plot, Fig. 2c) and discrimination of the four expected droplet populations (negative, WT-positive, mutant-positive, and double-positive), with balanced cluster positioning, minimal overlap between populations, and limited rain (2D-amplitude plots, Supplementary Fig. S1). These characteristics supported its selection as the optimal annealing temperature.

Given the single-nucleotide difference between the mutant- and WT-specific probes, primer and probe concentrations were further optimized to minimize cross-reactivity while preserving assay sensitivity. The selected concentration combination (0.63 µM primers, 0.175 µM wild-type probe, and 0.125 µM mutant probe) was used throughout the study as it provided the best overall balance among fluorescence amplitude, cluster separation, and stable vAF quantification, resulting in clear discrimination between *NRAS* p.Q61R mutant and WT signals.

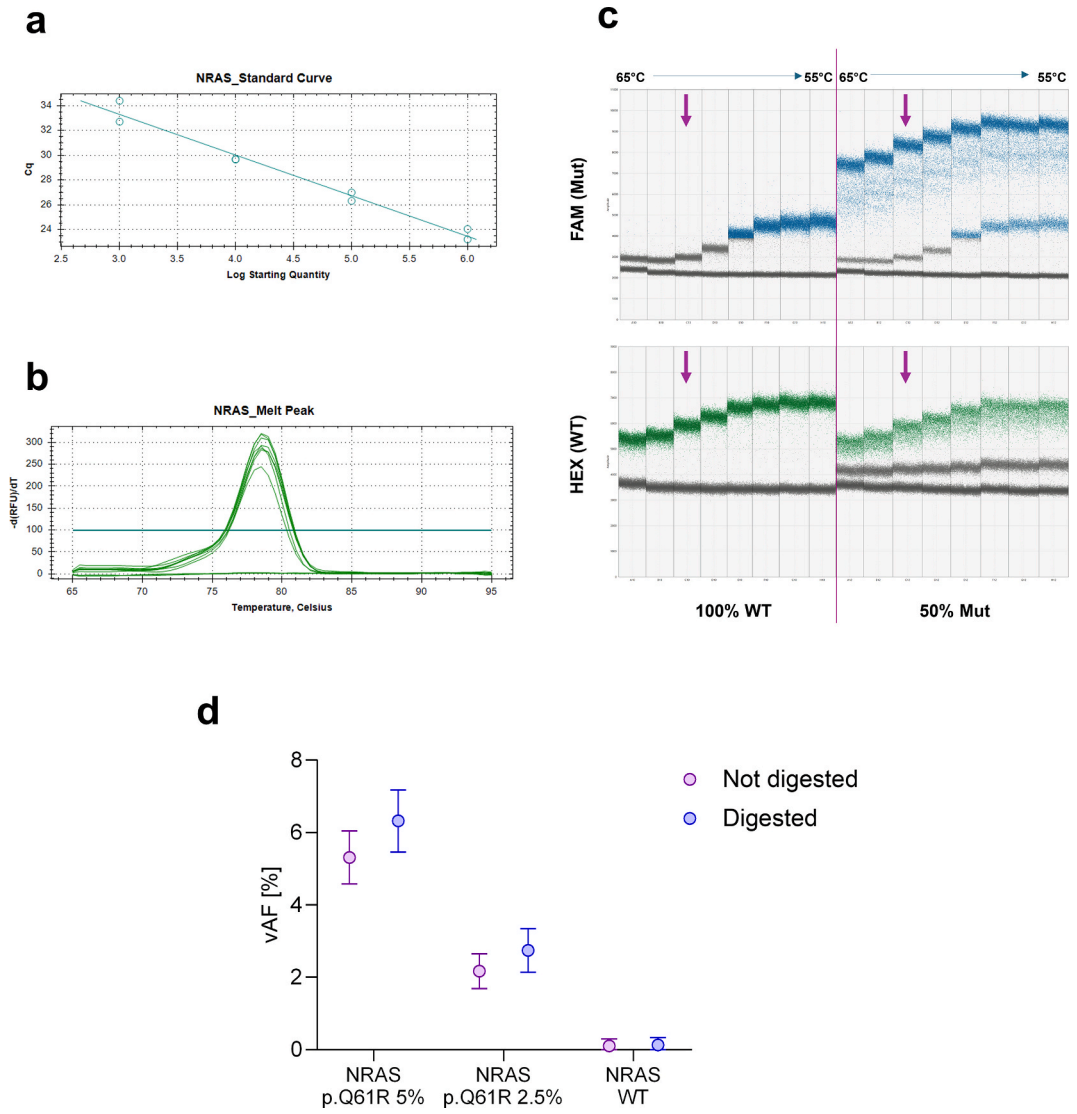

Fig. 2. Optimization of the *NRAS* dPCR assay. a) Primer efficiency curve for *NRAS* (qPCR). b) Melting curve analysis confirming specificity of *NRAS* amplicon (qPCR). c) Annealing temperature (T_a) optimization using a gradient dPCR. The purple arrows indicate the chosen T_a. d) Comparison of assay performance using undigested and EcoRI-digested gDNA and plasmid in terms of *NRAS* vAF (y-axis) in p.Q61R mutated (vAF ~ 5 % and 2.5 %) and WT *NRAS* samples. Error bars indicate SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

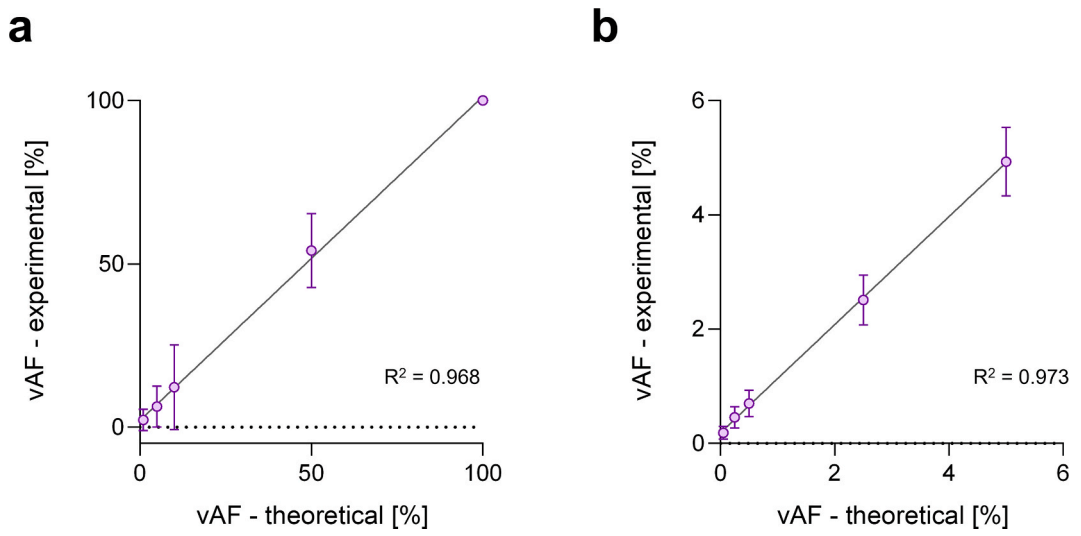

Fig. 3. Linearity assessment of the *NRAS* dPCR assay. Error bars indicate SD. a) Linearity curve obtained by mixing the *NRAS* p.Q61R mutant plasmid with the WT plasmid (vAF range: 0 %-100 %). b) Linearity curve obtained by spiking the mutant plasmid into WT gDNA extracted from NRAS WT cell lines (vAF range: 0 %-5 %).

Finally, the potential impact of gDNA digestion on assay performance was evaluated. No statistically significant differences were observed between digested and undigested samples at any tested vAF level (two-way ANOVA followed by Tukey's multiple-comparison test, all $p > 0.05$), indicating that enzymatic digestion was not required under the proposed RMP and its applicability on cfDNA (Fig. 2d).

3.2. Analytical performance: Linearity and sensitivity

The analytical performance assay was evaluated by assessing linearity and analytical sensitivity using two complementary experimental approaches.

In the first approach, synthetic plasmid DNA carrying the *NRAS* p.Q61R mutation was mixed with WT plasmid DNA at defined ratios to generate samples spanning the full range of expected vAF, from 0 % to 100 %. Measured vAF showed a strong linear relationship with expected values ($R^2 = 0.968$, $r = 0.984$), supporting adequate assay linearity across the tested concentration range (Fig. 3a).

In the second approach, defined amounts of mutant plasmid DNA were spiked into WT gDNA extracted from *NRAS* WT cell lines, generating samples with low vAF representative of clinically relevant scenarios (0–5 %). In this more complex genomic background, measured vAF correlated with expected values ($R^2 = 0.973$; Fig. 3b), indicating assay robustness at low mutant allele frequencies. Based on these dilution series, the analytical sensitivity of the assay was estimated. The LoB was determined to be 0.07 % vAF, the LoD was estimated at 0.1 % vAF, while the LoQ was established at 0.5 % vAF.

3.3. Comparison with the commercial dPCR assay

The quantitative performance of the candidate RMP was compared with a commercially available assay using a dilution series spanning a broad range of expected vAF. Inspection of 1D and 2D-fluorescence amplitude plots showed comparable cluster separation and signal discrimination for both assays. In both assays, a minor population of negative droplets with slightly increased fluorescence intensity was observed, although it was more evident in the commercial assay. This is a regular phenomenon occurring with competitive duplex assays, primarily caused by a partition specific competition [22]. This population remained clearly separated from the positive droplet clusters and did not interfere with threshold placement, droplet classification, or vAF quantification (Fig. 4a).

For both assays, measured vAF values showed a strong linear relationship with the expected values across the tested range. Linear regression analysis yielded a slope of 1.032 (95 % CI: 0.991–1.073) and an intercept of 0.895 (95 % CI: −0.282–2.071) for the candidate RMP, and a slope of 1.069 (95 % CI: 1.023–1.115) and an intercept of 1.215 (95 % CI: −0.097–2.527) for the commercial assay. The corresponding coefficients of determination were $R^2 = 0.996$ and $R^2 = 0.995$, respectively (Fig. 4b), indicating a comparable quantitative performance across the investigated vAF range..

Direct comparison of vAF values obtained with the two assays showed a strong correlation (Pearson's $r = 0.99$, $p < 0.0001$, Fig. 4c), indicating highly comparable quantitative estimates across the investigated vAF range.

3.4. Repeatability, intermediate precision, and measurement uncertainty

Repeatability, intermediate precision, and MU were evaluated using commercially available ctDNA RM spanning clinically relevant vAF, including 0.1 %, 0.5 %, and 5 %, as well as a WT control.

At the highest tested vAF (~5 %), the assay demonstrated high precision, with a repeatability of 7.8 % and an intermediate precision of 8.2 %, confirming robust performance in a clinically relevant concentration range (Fig. 5a and b). For the low-frequency materials, experimental results were consistent with the previously estimated analytical sensitivity. The 0.1 % vAF sample, corresponding to the estimated LoD, differed from the WT sample ($p < 0.001$) but showed increased variability, with repeatability and intermediate precision values of 62.8 % and 57.5 %, respectively. In contrast, the 0.5 % vAF sample, corresponding to the estimated LoQ, showed repeatability and intermediate precision values of 32.7 % and 32.4 %, respectively, within the range generally considered acceptable for quantitative molecular assays (~30 %) [23,24].

The WT material was used to experimentally verify the LoB. Measured vAF values ranged from 0 to 0.08 %, with a mean value of 0.03 %, supporting the previously established LoB threshold of 0.07 %.

As expected, MU was inversely related to variant abundance. The sample with the highest vAF (~5.16 %) exhibited the lowest relative expanded uncertainty (17 %), whereas the lowest-frequency sample (~0.12 %) showed the highest (67 %). An intermediate uncertainty value (44 %) was obtained for the 0.5 % vAF sample (Fig. 5c). In addition, the vAF values measured in this study were consistent with the producer's declared values for the RM, showing perfect agreement (Pearson's $r = 1.00$, $p = 0.004$; Fig. 5d).

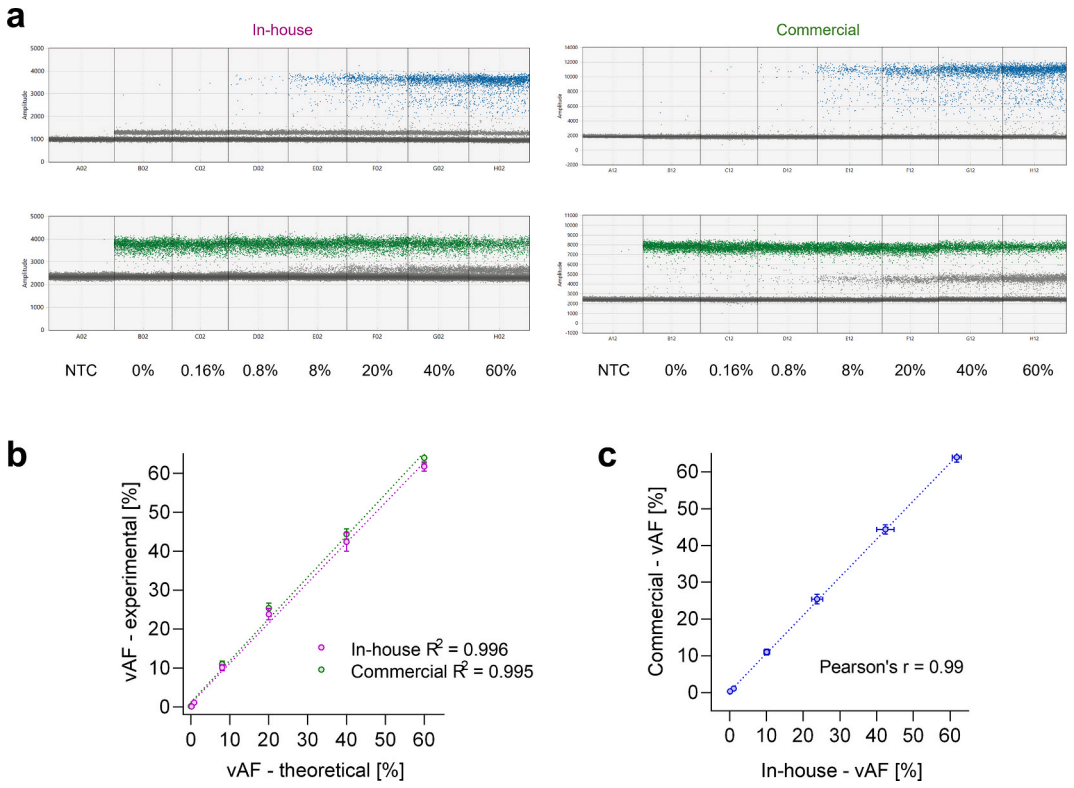

Fig. 4. Comparison between the candidate RMP for *NRAS* p.Q61R and the commercial dPCR assay. A synthetic *NRAS* p.Q61R plasmid was mixed with a pooled WT gDNA background to generate a dilution series simulating different vAF. a) 1D-amplitude plots. FAM channel (mutant) is indicated in blue, while HEX channel (WT) in green. b) Theoretical (*x*-axis) vs. experimental (*y*-axis) vAF for both the assays (In-house: pink; Commercial: green). Error bars indicate SD. c) In-house (*x*-axis) vs. commercial (*y*-axis) assay: correlation between experimental vAF results. Error bars indicate SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. *Measurement comparability and inter-laboratory transferability*

Preliminary assessment of inter-laboratory transferability and measurement comparability of the candidate RMP were evaluated in a small inter-laboratory study using an EQA cfDNA material distributed as two identical blinded samples to two participating laboratories.

At INRiM, the *NRAS* p.Q61R vAF measured in the EQA material was 2.30 %, with a relative standard MU of 18.15 % and U% ($k = 2$) of 36.31 %. At NIB, analysis of the same EQA material showed a vAF of 1.88 %, with a relative standard measurement uncertainty of 20.41 % and U% ($k = 2$) of 40.83 %.

The vAF values obtained by both laboratories were in good agreement and overlapped within their respective expanded MU (Fig. 6). The absolute difference between the measured vAF values ($\Delta = 0.42$ %) was compared with the combined expanded uncertainty, yielding a normalized difference (E_n) = 0.37, where values < 1 indicate measurement compatibility. The use of the E_n allows inter-laboratory compatibility to be assessed while accounting for the associated MU and is therefore particularly suitable for metrological comparisons. Both results were also consistent with the nominal value indicated by the supplier (vAF = 2 %).

4. Discussion

Accurate and comparable quantification of somatic mutations is critical in precision oncology, where clinical decisions increasingly rely not only on mutation presence but also on quantitative parameters [6,7]. Despite major technological advances, including the widespread adoption of NGS and dPCR, the lack of harmonized and metrologically traceable measurement systems remains a barrier to inter-laboratory comparability and clinical reliability [11,13,25]. Here we describe a candidate RMP for the quantification of the clinically relevant *NRAS* p.Q61R mutation using dPCR, designed to establish traceable and comparable measurements across laboratories.

The proposed RMP showed robust analytical performance across all evaluated parameters. Systematic optimization of assay conditions ensured high specificity and minimal cross-reactivity between mutant and WT alleles, a critical requirement given the single-nucleotide difference between the two targets. Analytical characterization showed linearity across a wide dynamic range and reliable quantification of low-frequency variants, with a LoD of 0.1 % vAF and a LoQ of 0.5 % vAF. These performance characteristics are well aligned with the requirements for applications such as MRD monitoring and therapy response assessment, where accurate quantification of rare mutant alleles is essential [8–12,26].

A key methodological aspect of this work is the explicit integration of MU into the quantification of vAF. While uncertainty estimation is a fundamental component of metrology, it is rarely systematically implemented in molecular diagnostics. By incorporating a transparent uncertainty budget, the proposed RMP enables quantitative results to be interpreted in relation to decision thresholds, thereby improving comparability and supporting more informed clinical interpretation. As expected, uncertainty increased at low variant frequencies, highlighting the importance of reporting quantitative results together with their associated MU, particularly near decision thresholds. This approach represents a step toward transforming high-performance molecular assays into standardized measurement systems, essential for informed clinical interpretation and inter-laboratory comparability [13,20,27].

Comparison with a commercial dPCR assay demonstrated highly comparable quantitative performance and consistent vAF estimates across the investigated range. Although such comparison does not constitute evidence of trueness in a metrological sense, it provides independent

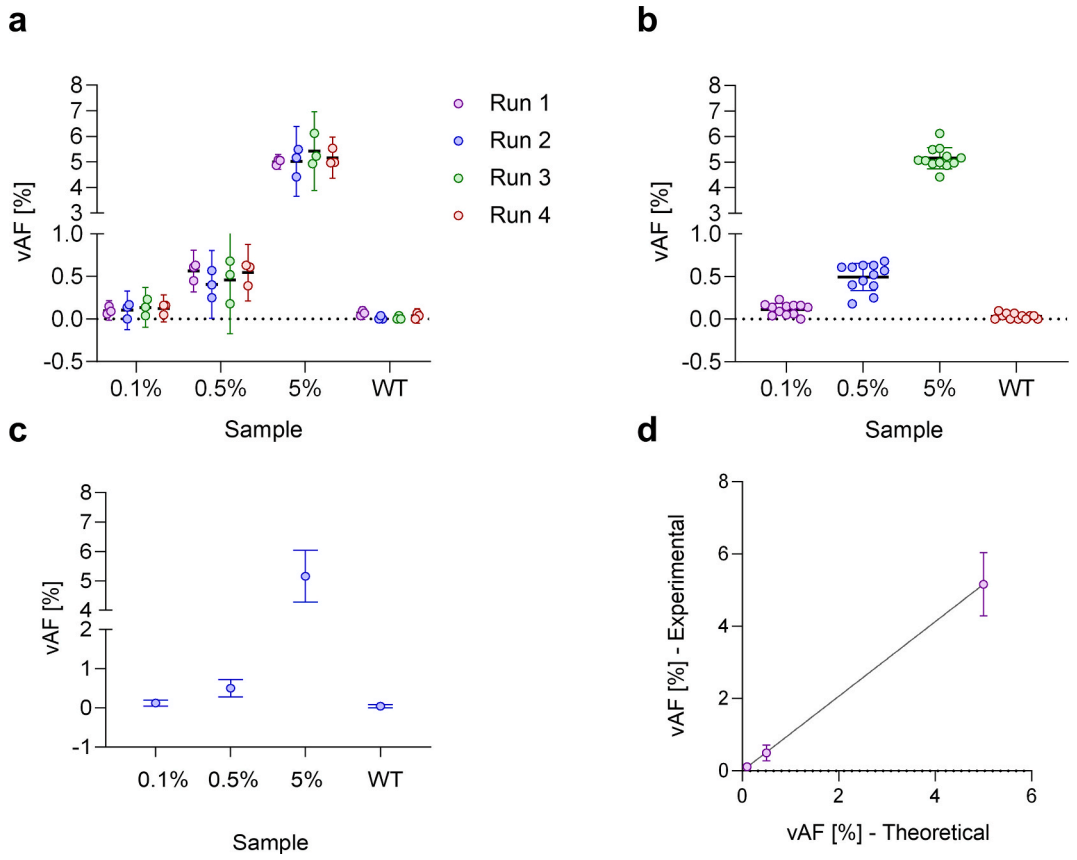

Fig. 5. Repeatability, intermediate precision, and *MU* assessment of the assay. a) Repeatability (intra-run precision). b) Intermediate precision (inter-run precision). c) Samples value assignment. Error bars indicate the absolute expanded uncertainty ($k = 2$). d) Correlation between measured *NRAS* p.Q61R vAF values (*y*-axis) and values stated by the material producer (*x*-axis). Error bars indicate the absolute expanded uncertainty ($k = 2$).

support for assay comparability and suggests the absence of major assay-dependent effects under the tested conditions. The proposed candidate RMP is supported by its metrological characterization, including the implementation of a defined uncertainty budget, traceable value assignment strategy, and demonstrated inter-laboratory applicability. Therefore, the comparison should be interpreted as an assessment of assay comparability, providing independent support for the consistency of vAF measurements obtained with the proposed candidate RMP [6,13,25,28].

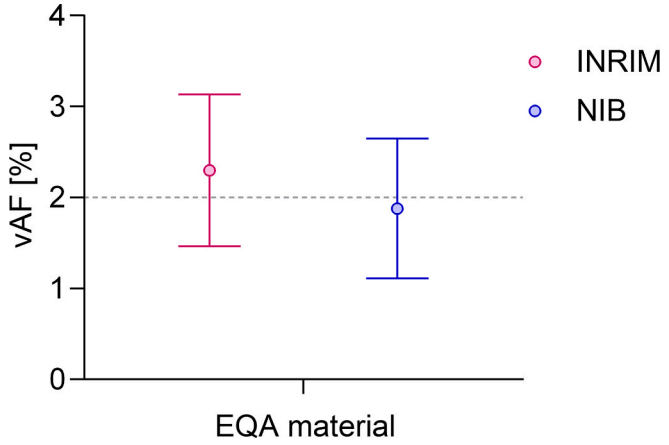

Fig. 6. Inter-laboratory comparison of *NRAS* p.Q61R vAF measured in an EQA cfDNA material at INRiM (red) and NIB (blue). Error bars represent the expanded MU ($k = 2$). The grey dashed line indicates the material nominal value. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The operational transferability and reproducibility of the candidate RMP was further evaluated through a preliminary small inter-laboratory comparison using an EQA cfDNA material. Although limited in scale, this comparison showed that, implementing the shared SOP under routine conditions, independent laboratories achieved comparable results within their combined expanded MU. The normalized difference provided a quantitative assessment of inter-laboratory compatibility, indicating the absence of significant systematic bias. These findings support the applicability of the proposed RMP under realistic laboratory conditions and highlight its potential role in EQA schemes and multi-centre studies [13,25,29].

The added value of the proposed candidate RMP should be considered in the context of metrological infrastructure rather than routine assay implementation. Although commercially available assays already enable reliable detection and quantification of *NRAS* p.Q61R, they are not primarily intended to provide higher-order reference measurements supporting value assignment of RM, uncertainty evaluation, calibration hierarchies, and harmonization across measurement systems. The proposed candidate RMP therefore complements rather than replaces existing diagnostic workflows. By providing a metrologically characterized framework for quantitative mutation assessment, it can support EQA schemes, improve comparability among laboratories, and facilitate the alignment of routine molecular testing methods with reference measurement systems.

From a regulatory and quality-assurance perspective, the availability of metrologically characterized RMPs is increasingly relevant in the context of the EU Regulation 2017/746 on IVDR medical devices. RMPs provide a reference point for method validation, calibration hierarchies, and EQA schemes. In doing so, they support harmonization and reduce

variability in molecular diagnostics, in line with international validation and quality-assurance guidance [14,18,25,30].

Although this study was conducted using one dPCR platform, the assay design and the proposed candidate RMP are not inherently platform-dependent. The SOP was developed and validated on BioRad QX200 system; however, the underlying measurement principles, assay design, and uncertainty framework could in principle be implemented on other dPCR platforms following appropriate verification and optimization [31,32]. Broader inter-platform validation, including evaluation on array-based dPCR systems, will be required to further establish transferability and support wider adoption of the proposed candidate RMP.

Beyond the specific application to *NRAS* p.Q61R, the methodological framework presented here is inherently generalizable. The integration of assay optimization, SI-traceable quantification, and uncertainty estimation can be extended to other clinically relevant biomarkers and analytical platforms. As such, this work provides a blueprint for the development of RMP in molecular diagnostics, contributing to the broader goal of harmonizing quantitative measurements across laboratories and technologies.

Nevertheless, this study has some limitations. Firstly, larger inter-laboratory studies and extended EQA schemes will be required to further consolidate the role of the proposed RMP in multicentre clinical and quality-assurance settings. Second, the study relied on synthetic constructs, well-characterized cell lines, RM and EQA material, which may not fully capture the complexity of clinical specimens. In addition, the potential impact of different DNA extraction workflows on assay performance was not evaluated. Although standardized extraction procedures were used throughout the study, future investigations should assess extraction-related variability, particularly in the context of low-frequency variant detection and routine clinical implementation. Future work should evaluate the performance of the proposed RMP in real-world clinical samples and across multiple platforms to further strengthen its applicability [6,7,28].

In conclusion, this study establishes a robust, metrologically traceable, and transferable dPCR-based candidate RMP for the quantification of *NRAS* p.Q61R. By providing absolute quantification with a well-defined uncertainty budget and demonstrated inter-laboratory comparability, the proposed RMP provides an important basis for harmonizing molecular measurements in precision oncology and strengthening the reliability of quantitative biomarker assessment in clinical practice. The complete SOP is provided in Supplementary file 1 to facilitate method adoption and promote consistency across laboratories.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This project has received funding from the European Partnership on Metrology, co-financed from the European Union's Horizon Europe Research and Innovation Programme and by the Participating States (Grant number: 22HLT06 GenomeMET). Funder: European Partnership on Metrology (ID: 10.13039/100019599).

This work was also supported by the European Metrology Network for Traceability in Laboratory Medicine (EMN-TraceLabMed) through scientific collaboration and networking activities. GC is supported by the Ricerca Finalizzata Giovani Ricercatori (GR-2021-12374957) and by AIRC under MFAG 2025 (ID: 31911). ABK and MM also acknowledge Slovenian Research Agency (ARIS) for supporting research infrastructure used in this work through grant IO-0004.

The funder played no role in study design, data collection, analysis and interpretation of data, or the writing of this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2026.07.005.

Data availability

The data supporting the findings of this study are available within the article and its Supplementary files. Additional data are available from the corresponding author upon reasonable request.

References

[1] I.A. Prior, P.D. Lewis, C. Mattos, A comprehensive survey of ras mutations in cancer, Cancer Res. 72 (2012) 2457–2467, https://doi.org/10.1158/0008-5472.CAN-11-2612.
[2] M. Malumbres, M. Barbacid, RAS oncogenes: the first 30 years, Nat. Rev. Cancer 3 (2003) 459–465, https://doi.org/10.1038/nrc1097.
[3] R. Nazarian, H. Shi, Q. Wang, X. Kong, R.C. Koya, H. Lee, Z. Chen, M.-K. Lee, N. Attar, H. Sazegar, T. Chodon, S.F. Nelson, G. McArthur, J.A. Sosman, A. Ribas, R.S. Lo, Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation, Nature 468 (2010) 973–977, https://doi.org/10.1038/nature09626.
[4] W. De Roock, B. Claes, D. Bernasconi, J. De Schutter, B. Biesmans, G. Fountzilas, K. T. Kalogeras, V. Kotoula, D. Papamichael, P. Laurent-Puig, F. Penault-Llorca, P. Rougier, B. Vincenzi, D. Santini, G. Tonini, F. Cappuzzo, M. Frattini, F. Molinari, P. Saletti, S. De Dosso, M. Martini, A. Bardelli, S. Siena, A. Sartore-Bianchi, J. Tabernero, T. Macarulla, F. Di Fiore, A.O. Gangloff, F. Ciardiello, P. Pfeiffer, C. Qvortrup, T.P. Hansen, E. Van Cutsem, H. Piessevaux, D. Lambrechts, M. Delorenzi, S. Tejpar, Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis, Lancet Oncol. 11 (2010) 753–762, https://doi.org/10.1016/S1470-2045(10)70130-3.
[5] H. Do, A. Dobrovic, Sequence artifacts in DNA from formalin-fixed tissues: causes and strategies for minimization, Clin. Chem. 61 (2015) 64–71, https://doi.org/10.1373/clinchem.2014.223040.
[6] L.J. Jennings, M.E. Arcila, C. Corless, S. Kamel-Reid, I.M. Lubin, J. Pfeifer, R. L. Temple-Smolkin, K.V. Voelkerding, M.N. Nikiforova, Guidelines for validation of next-generation sequencing–based oncology panels, J. Mol. Diagn. 19 (2017) 341–365, https://doi.org/10.1016/j.jmoldx.2017.01.011.
[7] I. Kinde, J. Wu, N. Papadopoulos, K.W. Kinzler, B. Vogelstein, Detection and quantification of rare mutations with massively parallel sequencing, PNAS 108 (2011) 9530–9535, https://doi.org/10.1073/pnas.1105422108.
[8] C.M. Hindson, J.R. Chevillet, H.A. Briggs, E.N. Gallichotte, I.K. Ruf, B.J. Hindson, R.L. Vessella, M. Tewari, Absolute quantification by droplet digital PCR versus analog real-time PCR, Nat. Methods 10 (2013) 1003–1005, https://doi.org/10.1038/nmeth.2633.
[9] L.B. Pinheiro, V.A. Coleman, C.M. Hindson, J. Herrmann, B.J. Hindson, S. Bhat, K. R. Emslie, Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification, Anal. Chem. 84 (2012) 1003–1011, https://doi.org/10.1021/ac202578x.
[10] A.S. Whale, J.F. Huggett, S. Cowen, V. Speirs, J. Shaw, S. Ellison, C.A. Foy, D. J. Scott, Comparison of microfluidic digital PCR and conventional quantitative PCR for measuring copy number variation, Nucleic Acids Res. 40 (2012) e82–e, https://doi.org/10.1093/nar/gks203.
[11] S.C. Taylor, G. Laperriere, H. Germain, Droplet Digital PCR versus qPCR for gene expression analysis with low abundant targets: from variable nonsense to publication quality data, Sci. Rep. 7 (2017) 2409, https://doi.org/10.1038/s41598-017-02217-x.
[12] V. Taly, D. Pekin, L. Benhaim, S.K. Kotsopoulos, D. Le Corre, X. Li, I. Atochin, D. R. Link, A.D. Griffiths, K. Pallier, H. Blons, O. Bouché, B. Landi, J.B. Hutchison, P. Laurent-Puig, Multiplex picodroplet digital PCR to detect KRAS mutations in circulating DNA from the plasma of colorectal cancer patients, Clin. Chem. 59 (2013) 1722–1731, https://doi.org/10.1373/clinchem.2013.206359.
[13] M.M. McGovern, Quality assurance in molecular genetic testing laboratories, J. Am. Med. Assoc. 281 (1999) 835, https://doi.org/10.1001/jama.281.9.835.
[14] Committee for Medicinal Products for Human Use, ICH guideline Q2(R2) on validation of analytical procedures, (2023). https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q2r2-guideline-validation-analytical-procedures-step-5-revision-2_en.pdf.
[15] Metrology for genomic profiling to support early cancer detection and precision medicine, (n.d.). https://www.euramet.org/research-innovation/search-research-projects/details?tx_eurametctcp_project%5Bproject%5D=1868&cHash=ca3877b0eccad38268a612ea8a72f10d.
[16] Traceability for biologically relevant molecules and entities, (n.d.). https://www.euramet.org/research-innovation/search-research-projects/details/project/traceability-for-biologically-relevant-molecules-and-entities.
[17] Droplet Digital PCR Applications Guide, (n.d.). https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf.
[18] S. Chandran, R.S.P. Singh, Comparison of various international guidelines for analytical method validation, Pharmazie 62 (2007) 4–14.

[19] I.D. Hay, M.F. Bayer, M.M. Kaplan, G.G. Klee, P.R. Larsen, C.A. Spencer, American Thyroid Association assessment of current free thyroid hormone and thyrotropin measurements and guidelines for future clinical assays. The Committee on Nomenclature of the American Thyroid Association, Clin. Chem. 37 (1991) 2002–2008.
[20] Working Group 1 of the Joint, Committee for Guides in Metrology (JCGM/WG 1)., Evaluation of measurement data — Guide to the expression of uncertainty in measurement (GUM), (n.d.). https://www.bipm.org/documents/20126/2071204/JCGM_100_2008_E.pdf.
[21] Joint Committee for Guides in Metrology, Evaluation of measurement data, Supplement 2 to the "Guide to the expression of uncertainty in measurement" – Extension to any number of output quantities, (n.d.). https://www.bipm.org/en/doi/10.59161/JCGM102-2011.
[22] A.S. Whale, J.F. Huggett, S. Tzonev, Fundamentals of multiplexing with digital PCR, Biomol. Detect. Quantif. 10 (2016) 15–23, https://doi.org/10.1016/j.bdq.2016.05.002.
[23] C. Dide-Agossou, K. Rossmassler, J. Reid, J. Purohit, R.M. Savic, P. Nahid, P.P. J. Phillips, C.M. Moore, N.D. Walter, MOVER approximated CV: a tool for quantifying precision in ratiometric droplet digital PCR assays, J. Pharm. Biomed. Anal. 212 (2022) 114664, https://doi.org/10.1016/j.jpba.2022.114664.
[24] D. Gleerup, M. Vynck, L. Gysens, C. De Baere, J. Vandesompele, O. Thas, A. Martens, M. Haspeslagh, W. De Spiegelaere, Cross-platform digital PCR evaluation of bovine papilloma virus quantification: introducing PCR-ValiPal for Standardized guided assay validation, (2025). https://doi.org/10.1101/2025.09.04.674204.
[25] UNI, ISO 15189:2022 Medical laboratories — Requirements for quality and competence, (2022).
[26] J.C.M. Wan, C. Massie, J. Garcia-Corbacho, F. Mouliere, J.D. Brenton, C. Caldas, S. Pacey, R. Baird, N. Rosenfeld, Liquid biopsies come of age: towards implementation of circulating tumour DNA, Nat. Rev. Cancer 17 (2017) 223–238, https://doi.org/10.1038/nrc.2017.7.
[27] Ellison SLR, Williams A, EURACHEM/CITAC - Guide Quantifying Uncertainty in Analytical Measurement, (2012). https://www.eurachem.org/images/stories/Guides/pdf/QUAM2012_P1.pdf.
[28] S. Roy, C. Coldren, A. Karunamurthy, N.S. Kip, E.W. Klee, S.E. Lincoln, A. Leon, M. Pullambhatla, R.L. Temple-Smolkin, K.V. Voelkerding, C. Wang, A.B. Carter, Standards and guidelines for validating next-generation sequencing bioinformatics pipelines: a joint recommendation of the association for molecular pathology and the college of american pathologists, J. Mol. Diagn. 20 (2018) 4–27, https://doi.org/10.1016/j.jmoldx.2017.11.003.
[29] Harmonization of Clinical Laboratory Test Results, EJIFCC 27 (2016) 5–14. https://pmc.ncbi.nlm.nih.gov/articles/PMC4975212/ (accessed January 26, 2026).
[30] CLSI., Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures, 2nd Edition., (2012).
[31] P. Corbisier, L. Pinheiro, S. Mazoua, A.-M. Kortekaas, P.Y.J. Chung, T. Gerganova, G. Roebben, H. Emons, K. Emslie, DNA copy number concentration measured by digital and droplet digital quantitative PCR using certified reference materials, Anal. Bioanal. Chem. 407 (2015) 1831–1840, https://doi.org/10.1007/s00216-015-8458-z.
[32] C. Fava, S. Bernardi, E.M. Gottardi, R. Lorenzatti, L. Galeotti, F. Ceccherini, F. Cordoni, F. Daraio, E. Giugliano, A. Jovanovski, J. Petiti, M. Varotto, D. Barberio, G. Rege-Cambrin, P. Berchialla, V. Sciannameo, M. Malagola, G. Saglio, D. Russo, Alignment of Qx100/Qx200 droplet digital (Bio-Rad) and QUANTSTUDIO 3D (Thermofisher) digital PCR for quantification of BCR-ABL1 in Ph+ chronic myeloid leukemia, Diseases 9 (2021) 35, https://doi.org/10.3390/diseases9020035.