GenomeMET project: establishing high-accuracy ddPCR reference methods for cancer genomic profiling

Jessica Petiti[1], Marika Fava[1], Laura Revel[1], Sabrina Caria[1,2], and Carla Divieto[1]

1. Istituto Nazionale di Ricerca Metrologica (INRiM), Turin, Italy 2. University of Turin, Turin, Italy

Background

Cancer is one of the leading causes of death in Europe[1]. Next Generation Sequencing (**NGS**) has transformed cancer diagnostics and treatment by enabling earlier detection, more precise classification, and development of targeted therapies[2]. This approach, known as **precision medicine**, has the potential to significantly improve patient outcomes and make cancer treatment more effective.

However, genomic profiling using NGS still faces major challenges. The quality and accuracy of genetic test results can vary significantly between laboratories due to differences in technology, analytical methods, and data interpretation[3]. This **lack of consistency** makes it difficult to compare results, potentially leading to incorrect diagnoses or inappropriate treatment choices. Currently, **standardized reference systems** and **metrological tools** to ensure the reliability and comparability of genomic data are still in their early stages.

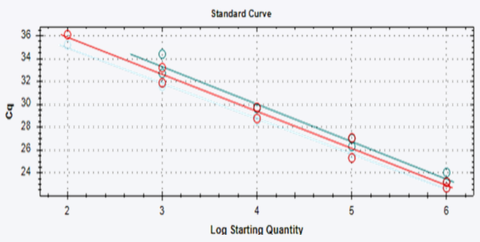
Aims

The **GenomeMET** project **-** *Metrology for genomic profiling to support early cancer detection and precision medicine,* coordinated by INRiM - aims to bridge this gap by developing **robust metrological frameworks** and **reference measurement systems** (RMS) to support high-quality, reproducible genomic testing. By applying metrological principles, the project will contribute to improve cancer diagnostics and support the implementation of precision medicine in clinical practice. Specific aims include: **i)** developing RMS; **ii)** establishing Reference Measurement Procedures (RMP); **iii)** creating SI-traceable Reference Materials (RM); **iv)** developing a robust framework to assess the measurement uncertainty (MU); **v)** facilitating adoption of developed metrological infrastructure and methods by stakeholders.

To achieve these goals, the **Biometrology Lab** at INRIM has developed two novel RMP using droplet digital PCR (ddPCR) for the accurate and SI-traceable quantification of two key cancer biomarkers: **MET copy number variations** (CNV) and **NRAS p.Q61R** mutation.

M&M and Results

1 Primers efficiency

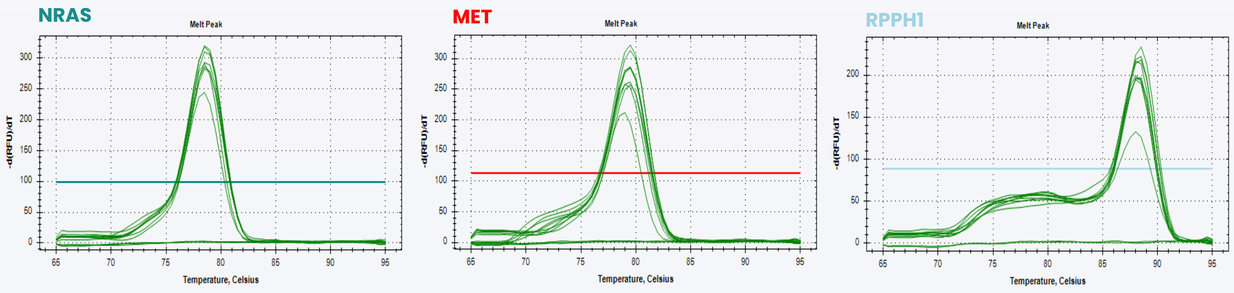


1) Left panel. Primers' efficiency (*E*) was assessed for NRAS, MET and RPPH1 (selected as reference gene for MET CNV) using qPCR on standard curves prepared with scalar dilutions of gDNA from a wild-type (WT) cell line (P-EP/SVTER28-2). The *E* were found to be optimal for all the primer pairs:

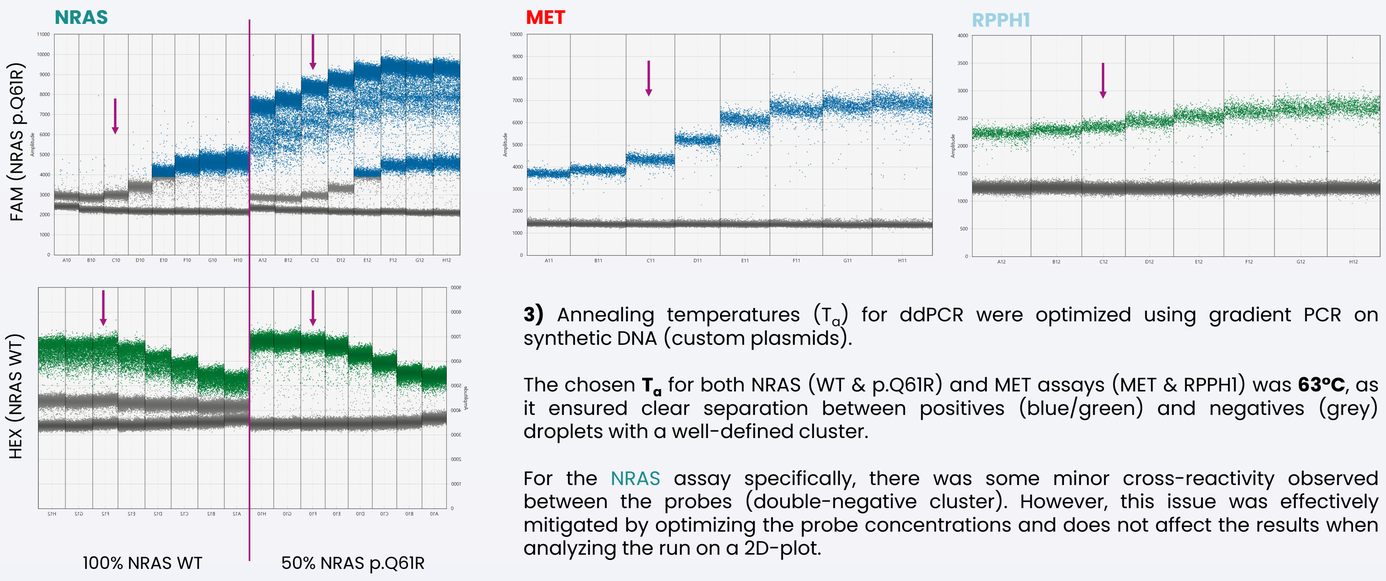
- E_{NRAS} = 101.9% (**Green**)
- E_{MET} = 103.6% (**Red**)
- E_{RPPH1} = 110.6% (**Light Blue**)

2) Right panel. Melting curves were analyzed, confirming the absence of non-specific amplicons

2 Melting curve analysis



3 T_a optimization

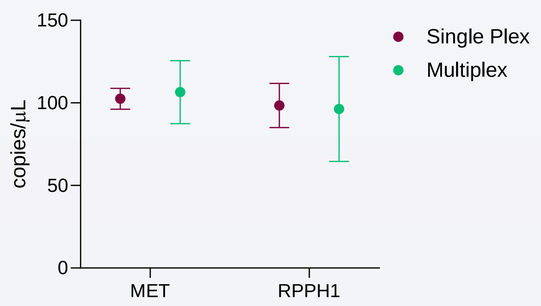


3) Annealing temperatures (T_a) for ddPCR were optimized using gradient PCR on synthetic DNA (custom plasmids).

The chosen **T_a** for both NRAS (WT & p.Q61R) and MET assays (MET & RPPH1) was **63°C**, as it ensured clear separation between positives (blue/green) and negatives (grey) droplets with a well-defined cluster.

For the NRAS assay specifically, there was some minor cross-reactivity observed between the probes (double-negative cluster). However, this issue was effectively mitigated by optimizing the probe concentrations and does not affect the results when analyzing the run on a 2D-plot.

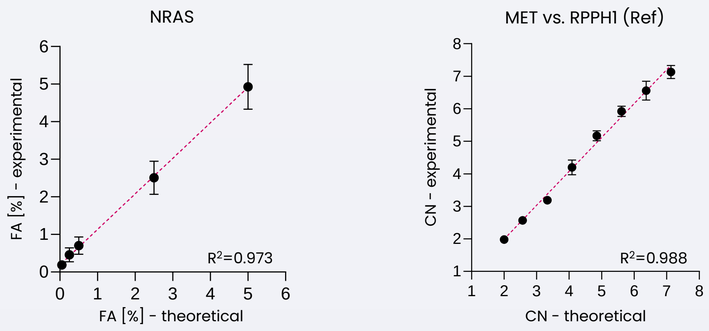
4 MET & RPPH1: single plex vs. multiplex



4) For MET assay, single plex and multiplex approaches were compared using gDNA from a WT epithelial cell line (P-EP/SVTER28-2) to investigate any potential interference between MET and RPPH1 primers/probes.

No interference was observed between MET and RPPH1 assays; therefore, the **multiplex approach** was used for subsequent experiments.

5 Linearity



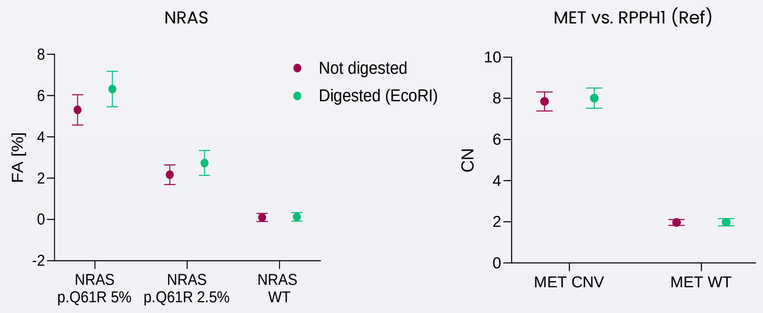
5) Linearity was assessed by generating dilution curves with increasing amounts of NRAS p.Q61R and MET plasmids, respectively, in a pool of gDNA from WT cell lines (P-EP/SVTERT28-2, P-MSC/TERT308, and MG-63 cells).

For the **NRAS** assay, theoretical and experimental fractional abundance (FA%) were evaluated from 0% (WT) to 5%, yielding an **R^2** of **0.973**.

For the **MET** assay, theoretical and experimental copy number (CN) were evaluated from 2 (WT) to 7, with an **R^2** of **0.988**.

Both assays demonstrated **high linearity**.

6 Enzyme digestion

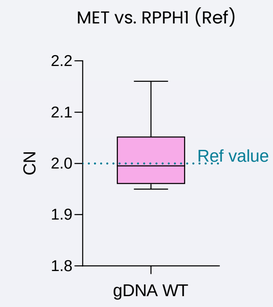


6) The impact of enzyme digestion on ddPCR results was investigated by comparing **digested** (EcoRI) and **non-digested DNA** (NRAS p.Q61R and MET plasmids in a pool of gDNA from WT cell lines and a pool of gDNA from WT cell lines).

No significant differences were observed between the two approaches for either assays, indicating that **both methods can be used**.

FA[%]=fractional abundance
CN=copy number

7 Assay limits



Estimated values for **NRAS assay** as FA[%]

LoB = 0.06%

LoD = 0.10%

LoQ = 0.35%

7) Limit of Blank (LoB), Limit of Detection (LoD), and Limit of Quantification (LoQ) were estimated[4,5] for the **NRAS** assay. The results indicated values consistent with other commercial assays and suitable for diagnostic applications, usually requiring a LoD of 0.1% or higher.

For the **MET** assay, the baseline (WT) value was assessed by analyzing gDNA from different WT cell lines (P-EP/SVTERT28-2, P-MSC/TERT308, and MG-63). Preliminary results were promising, with WT samples showing a value of **2.06 ± 0.21 copies** (Ref. value=2 copies), making it reliable and suitable for MET CNV analysis.

FA[%]=fractional abundance
CN=copy number

Conclusions

This study successfully developed two novel **ddPCR RMP** for the accurate quantification of **NRAS p.Q61R** mutation and **MET CNV**, addressing key challenges in cancer genomic profiling.

The next step involves validation and metrological characterization using reference materials.

These results contribute to the standardization of **SI-traceable genomic reference methods**, supporting precision medicine implementation and ultimately improving cancer diagnostics and patient care.

References

1. Cancer EURO – World Health Organization (WHO). https://www.who.int
2. Satam H et al. *Next-Generation Sequencing Technology: Current Trends and Advancements.* Biology (Basel), 2023. doi: 10.3390/biology13050286.
3. Quy PN et al. *Inter-assay variability of next-generation sequencing-based gene panels.* BMC Med Genomics, 2022. doi.org/10.1186/s12920-022-01230-y
4. Chandran S et al. *Comparison of various international guidelines for analytical method validation.* Pharmazie, 2007.
5. Committee for Medicinal Products for Human Use ICH Guideline Q2(R2) on Validation of Analytical Procedures 2022; ICH Guideline Q2(R2) on Validation of Analytical Procedures; Guideline. Available online: https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-q2r2-validation-analytical-procedures-step-2b_en.pdf.

Contact Info

Jessica Petiti, PhD
Phone: +39 011 3919971
E-mail: j.petiti@inrim.it

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