Additional file 3: Standard Operating Procedure for *MET* Copy Number Variation (CNV) Detection and Quantification Using Droplet Digital PCR

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Introduction

MET copy number variation (CNV) is a clinically relevant genomic alteration characterized by amplification of the *MET* gene, frequently observed in several cancer types, including non-small cell lung cancer (NSCLC), gastric cancer, and glioblastoma [1]. *MET* amplification leads to aberrant activation of the MET/HGF signalling axis, promoting tumour cell proliferation, survival, migration, and resistance to targeted therapies and immune checkpoint inhibitors [2].

Accurate and quantitative assessment of *MET* CNV is critical for diagnosis, prognosis, and therapeutic management. High *MET* copy number (CN) is associated with poor prognosis and may predict response to MET-targeted therapies such as tyrosine kinase inhibitors (TKIs), including crizotinib and capmatinib [3].

Droplet Digital PCR (ddPCR) enables sensitive, reproducible, and absolute quantification of *MET* gene copies relative to a reference gene, facilitating standardized assessment across diverse sample types. This high level of precision supports better stratification of patients, monitoring of treatment response, and informed clinical decision-making in precision oncology [4,5].

Aims

The aim of this Standard Operating Procedure (SOP) is to provide a reproducible and optimized protocol for the detection and quantification of the *MET* CNV in DNA from various sources (e.g., tissues and cell-free DNA (cfDNA)) using ddPCR.

This protocol ensures high sensitivity, specificity, and accuracy, enabling absolute quantification of the mutation, independent of amplification efficiency. The method is designed to produce standardized and comparable results, which are critical for diagnostic, prognostic, and therapeutic applications. It is particularly useful for detecting low-frequency variants, assessing tumour burden, monitoring Minimal Residual Disease (MRD), and guiding targeted therapy decisions.

Furthermore, this SOP aims to lay the foundation for a new potential Reference Measurement Procedure (RMP), metrologically characterized and suitable for future use in calibrating reference materials. By establishing a method with metrological traceability, this protocol could contribute to improving the reliability of molecular diagnostics, particularly by providing SI-traceable and standardized quantification for Next-Generation Sequencing (NGS) results.

Terminology

In the following SOP, the term “must” is used to denote mandatory steps or requirements within the protocol. When "must" is employed, adherence is non-negotiable, as these actions are essential for the correct execution and reliability of the procedure. Conversely, the term "should" has been employed to highlight recommended practices that, while not mandatory, are strongly advised for achieving optimal results.

List of abbreviations

cfDNA, Cell-Free DNA; CN, Copy Number; CNV, Copy Number Variation; ddPCR, Droplet Digital Polymerase Chain Reaction; HGF, Hepatocyte Growth Factor; MRD, Minimal Residual Disease; NGS, Next-Generation Sequencing; NSCLC, Non-Small Cell Lung Cancer; NTC, No Template Control; RMP, Reference Measurement Procedure; RT, Room Temperature; SI, International System of Units; SOP, Standard Operating Procedure; TKI, Tyrosine Kinase Inhibitor; WT, Wild-Type.

Materials

Equipment

- Droplet Digital PCR System
    - Droplet Generator (QX200 or Automated)
    - Droplet Reader
    - PX1 PCR Plate Sealer
- Thermocycler compatible with ddPCR plates
- Vortex mixer
- Microcentrifuge
- Pipettes
- Fluorometer or spectrophotometer system for DNA quantification
- Benchtop centrifuge for PCR plates (optional)
- PCR Workstation with laminar flow (optional)
- DG8 Cartridge Holder (if using QX200 Droplet Generator)

Consumables

- DG8 Cartridges and Gaskets (QX200) or DG32 Cartridges (Automated)
- ddPCR 96-Well PCR Plates
- Low-Binding microcentrifuge tubes
- Filtered pipette tips
- PCR Plate Heat Seal (foil, pierceable)
- MicroAmp tubes or PCR strips (optional)

Reagents

- Droplet Generation Oil for Probes (QX200) or Automated Droplet Generation Oil for Probes (Automated)
- ddPCR Supermix for Probes (No dUTP)
- Primers/Probes
- Droplet Reader Oil
- Nuclease-Free H_2O

General information

Protective gloves and lab coat should be worn, and all work areas should be thoroughly cleaned before and after testing.

Experimental workflow

Sample Preparation

- Extract DNA from biological samples using an appropriate extraction kit.
- Quantify the extracted DNA using a fluorometer or spectrophotometer to assess concentration and quality.
- Dilute the DNA in nuclease-free H_2O or another appropriate buffer (e.g., TE buffer) to a final concentration between 5–20 ng/µL.

Primer and Probe Preparation

- While not mandatory, it is strongly recommended to prepare the assay in a "pre-PCR" area, under a dedicated PCR hood, using dedicated pipettes. This should be done in a designated space to minimize the risk of nucleic acids contamination.
- Prepare the duplex *MET* CNV assay (see Table 1 for primer and probe sequences).

Component	Sequence (5'-3')	Target/Reference gene
MET forward primer	TCGCTACGATGCAAGAGTACACA	Target
MET reverse primer	GGCTTACACTTCGGGCACTTA	
MET probe	[FAM]TCCTCATTTGGATAGGCT[MGBEQ]	
RPPH1 forward primer	ACGTCATCAACCCGCTCCAAG	Reference
RPPH1 reverse primer	CCACTCCCCTGTCCCTCACA	
RPPH1 probe	[HEX]GTGTCACTAGGCGGGAACACC[BQ]	

Table 1. Primer and probe sequences for *MET* CNV assay.

- For convenience, we recommend preparing a 20x assay. If a different concentration is chosen, ensure that the final concentration of each reagent in the PCR reaction mix is as indicated in Table 2.
- Primers and probes must be mixed and diluted in nuclease-free H_2O.

Component	Concentration for 20x assay [µM]	Final concentration in PCR reaction mix [µM]
MET forward primer	18	0.9
MET reverse primer	18	0.9
MET probe	5	0.25
RPPH1 forward primer	18	0.9
RPPH1 reverse primer	18	0.9
RPPH1 probe	5	0.25

Table 2. Primer and probe concentrations for *MET* CNV assay.

- Once prepared, *MET* CNV assay should be stored at -20°C for several months.

PCR Reaction Mix Preparation

- While not mandatory, it is strongly recommended to prepare the assay in a "pre-PCR" area, under a dedicated PCR hood, using dedicated pipettes. This should be done in a designated space to minimize the risk of nucleic acids contamination.
- Thaw all components to room temperature (RT).
- Mix thoroughly by vortexing each tube to ensure homogeneity.
- Centrifuge briefly to collect contents at the bottom of the tubes.
- Prepare the PCR reaction mix for the required number of reactions according to Table 3.
    - It is recommended to prepare an excess volume (10-20% more) to ensure sufficient mix for all samples.
    - Add all the required reagents except for the DNA sample, then vortex and briefly centrifuge the tube.

Component	Volume per reaction [µL]
2x ddPCR Supermix for Probes (No dUTP)	11
20x MET CNV assay	1.1
Nuclease-free H_2O	Variable
DNA Sample (between 5-20 ng)	Variable
Total Volume	22

Table 3. PCR reaction mix composition for *MET* CNV assay.

- Dispense 22 µL of the reaction mix into each reaction well (96-well plates, PCR strips, or microtubes).
- If possible, move to a separate room or area and use a dedicated hood and pipette to add the DNA sample as the final step.
- Mix thoroughly by vortexing the plate/strip/microamp and centrifuge briefly.
- Allow the PCR reaction mix to equilibrate at RT for ~3 minutes.

Droplet Generation

Transfer the reaction mix from the reaction well/microtube to the appropriate ddPCR cartridge as follow:

- For the QX200 Droplet Digital PCR System: load 20 µL of each reaction sample into a sample well of a DG8 Cartridge. Follow the instructions specified in the QX200 Droplet Generator Instruction Manual.
- For the QX200 AutoDG Droplet Digital PCR System: follow the instructions in the Automated Droplet Generator Instruction Manual.

Thermocycling

- Carefully transfer the droplets into a clean 96-well plate (for the QX200 Droplet Digital PCR System only)
- Seal the 96-well plate using the PX1 PCR Plate Sealer at 180°C for 5 seconds.
- Proceed with thermal cycling as described in Table 4.

Cycling Step	Temperature [°C]	Time [min]	N. of Cycles	Ramp Rate [°C/sec]
Enzyme activation	95	10	1	2
Denaturation	94	0.5	40	2
Annealing/Extension	63	1		2
Enzyme deactivation	98	10	1	2
Hold	4	30	1	1
Hold	4	∞	1	-

Table 4. Thermal cycling conditions for MET CNV assay.

Droplet Reading and Data Acquisition

- Transfer the PCR plate to the Droplet Reader after thermocycling.
- Refer to the QX200 Droplet Reader Instruction Manual for detailed guidance: QX200 Droplet Reader and QuantaSoft Software or QX200 Droplet Reader and QX Manager Software Standard Edition.
- Set up the experiment in the *Sample Panel*:
    - Enter the sample name or ID.
    - From the *Experiment* dropdown menu, select *CNV – Copy Number Variation*.
- Select *ddPCR Supermix for Probes (no dUTP)* from the *Supermix* dropdown menu.
- Define the targets:
    - *Target 1* (Ch 1, FAM channel) → *MET* (Target gene)
    - *Target 2* (Ch 2, VIC/HEX channel) → *RPPH1* (Reference gene)
- Click *Run* to start the analysis.
- In the *Run Options* window, select *FAM/HEX*.

Data Analysis

- Analyse the data using the ddPCR analysis software. Refer to the software instruction manuals for further details (QuantaSoft Software or QX Manager Software)
- Open the acquired ddPCR data in the ddPCR analysis software.
- Ensure that the total number of accepted droplets per well exceeds 10,000, as a lower count may reduce statistical confidence.
- Set *RPPH1* as *Target type*→Ref and set *Ref Copies*→2
- Evaluate the 2D fluorescence plot to verify proper clustering of droplets:
    - *MET* positive droplets (FAM channel, blue)

- *RPPH1* positive droplets (HEX/VIC channel, green)
- Double-positive droplets (if applicable, orange)
- Negative (empty) droplets (grey)

- Set the fluorescence threshold (manually or automatically): the threshold should be placed above the baseline fluorescence of negative droplets but below the lowest intensity of true positive droplets to avoid misclassification (Fig. 1).
- Use a no-template control (NTC) to establish a baseline and confirm the absence of non-specific signals (Fig. 1).
- Express results as copy number (CN).

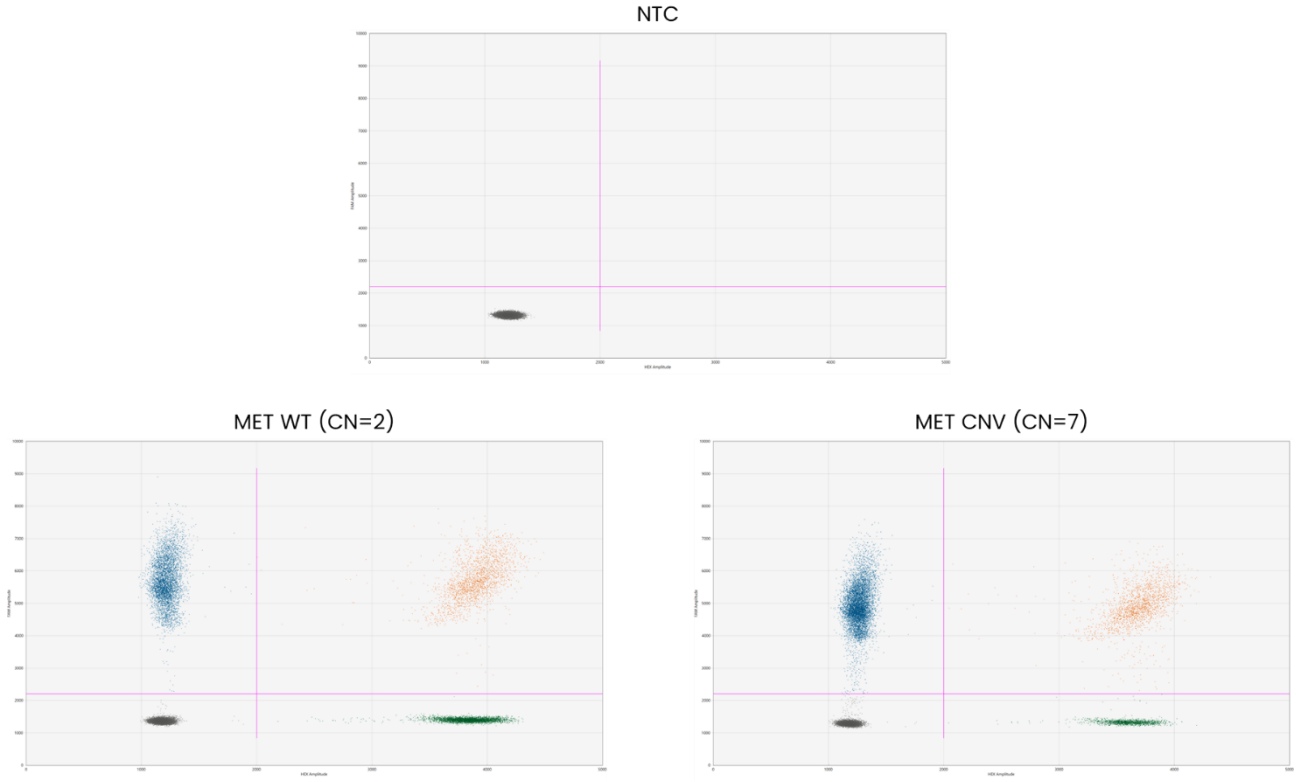

Figure 1. Example of 2D-plots. The FAM channel (*MET*) is indicated on the *y*-axis and the HEX channel (*RPPH1*) on the *x*-axis. NTC: no template control; CN: copy number, WT: wilt-type, CNV: copy number variation.

Quality Control

In addition to the NTC, it is recommended to include:

- One positive control for *MET*
- One positive control for *RPPH1*

Example of control samples are synthetic DNA containing the target amplicon region. Several commercial control or reference materials can be purchased. Alternatively, the following sequences can be used to prepare or purchase control samples:

- *MET* sequence (5'-3'):

ATCTGGGCAGTGAATTAGTTCGCTACGATGCAAGAGTACACACTCCTCATTTGGATAGGCTTG
TAAGTGCCCGAAGTGTAAGCCCAACTACAGAAATGGTTTCAAATGAATCTGTAGACTACCGA
GCTACTTTTCCAGAAG

- *RPPH1* sequence (5'-3'):

TTCCCAAATCCAAAGACATTTCACGTTTATGGTGATTTCCCAGAACACATAGCGACATGCAAA
TATTGCAGGGCGCCACTCCCCTGTCCCTCACAGCCATCTTCCTGCCAGGGCGCACGCGCGCT
GGGTGTTCCCGCCTAGTGACACTGGGCCCGCGATTCCTTGGAGCGGGTTGATGACGTCAGCG
TTCGAATTCCATGGCGGCGCGGCGG

Test parameters

Test parameters	Values
CN wild-type (Ref. CN=2.00)[1]	2.25 ± 0.30
Mean Relative Expanded Uncertainty[2]	23.8%

Table 5. Test parameters associated with the specific protocol outlined in this SOP.

[1] Estimated from results obtained in several samples (cell lines and RM) with no *MET* amplification.

[2] Evaluated using the Seraseq ctDNA Mutation Mix v4 (SeraCare, CNV range: 2-15 CN).

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